MOLECULAR STUDIES ON THE HYPOXANTHINE PHOSPHORIBOSYLTRANSFERASE OF PLASMODIUM FALCIPARUM

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I hereby declare that I alone have composed this thesis, and that except where stated, the work presented within is my own.

Md. Shahabuddin

March, 1990

ABSTRACT

Hypoxanthine phosphoribosyltransferase (EC 2.7.2.8) of *P. falciparum* has been studied for its biological properties and cellular location. The enzyme plays an important role in the parasite's life, and therefore is a putative target for chemotherapy against malaria.

Due to the difficulty in obtaining large amounts of the enzyme from the parasite, it was over-expressed in *E. coli*, first as a fusion protein with *E. coli* -β-galactosidase. This facilitated the one step purification of the protein, using β-galactosidase substrate affinity chromatography, for making antibodies against the enzyme.

The antibodies thus made was used to investigate the cellular location, a prerequisite for a successful drug design against the parasite enzyme. Immunofluorescent microscopy (IFA) and immunogold electron microscopy revealed that;

1. the enzyme is expressed at all the stages of the parasite's life,
2. the enzyme is concentrated in some vesicular bodies of unkown origin,
3. in the sporozoite, it may be released into the intrapellicular spaces.

Subsequently, the enzyme was over-expressed directly in *E coli*, as a non-fusion protein and retained its enzymatic activity. This opened the way to study the enzyme for its biochemical properties and structure-activity relationship.

The active *E. coli*-expressed *P. falciparum* HPRT compensated a *S. typhimurium hpt* mutation. The strain, named *S. typhimurium* SH4 can now be used to screen large number of putative antimalarial drugs which might act against the parasite HPRT. This will be both simple and inexpensive.

The cell free extract of induced SH4 was used to study some biochemical properties of the enzyme. Such a study confimed the finding that unlike any

other *Plasmodia* studied so far, *P. falciparum* HPRT can use xanthine as its substrate in addition to hypoxanthine and guanine. However, competitive inhibition studies revealed that hypoxanthine is the most favourable substrate. The possible biological significance of such properties is discussed.

This thesis is dedicated to my mother
and father.

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Chapter 1

Introduction

Malaria is a parasitic disease of man, caused by the species of genus *Plasmodium* primarily characterised by periodic fever and transmitted by the bite of infected mosquitoes.

Clinical symptoms of malaria generally develop within 8 to 30 days following the inoculation of the parasite in sporozoite form by the bite of an infected *Anopheline* mosquito. The symptoms involve mainly periodic chill, fever and sweating, but, in severe cases, accompanied by anemia, enlargement of the spleen and pigmentation of tissues. In cerebral malaria, changes of behaviour may occur in the early stages, excitement, mania, coma and epileptiform attack may follow.

Only four species of the parasite of genus *Plasmodium* infect humans naturally. Among these *P. falciparum* is the most deadly and causes malignant form of malaria. *P. vivax* is most widely distributed and common. *P. vivax* and *P. ovale* are characterized by the periodic appearance of the symptoms after a latent period of up to 5 years. *P. malariae* is also widely distributed but less than *P. vivax* and *P. falciparum*.

One of the main reasons of continuing research on the disease and its worldwide concern is that malaria is still a major health problem of most of the modern world, specially in tropical and subtropical countries. At present about 102 countries remains endemic and this is placing over half of the world population in risk (TDR report, WHO 1989). The situation in third world countries is more serious, where none or very few antimalarial measures are undertaken. It is estimated that there are at least 100 million cases of malaria reported annually and it contribute at about a million deaths per year (TDR report, WHO,1989). Despite the intensive efforts made against malaria during the last four decades, the original levels of endemicity in Asia, Africa and Amazonian region of Latin America have hardly changed. Malaria can also

cause risks to even people living in nonendemic areas through importing by travellers and immigrants from endemic areas.

P. falciparum the most dangerous species of *Plasmodia*, for which Africa is hyperendemic, causes death due to the cerebral malaria. cerebral malaria has a mortality rate of 20% even with good medical care. In nonimmune children, it causes anemia and growth retardation in mild cases. It is also responsible for the maternal morbidity, foetal dysmaturity and anemia in pregnant mothers.

Apart from the deadly effect on human health, malaria can also affect the economy of a country indirectly. It was shown that during an epidemic of malaria in Paraguay, agricultural development was markedly reduced (Conly, 1976)

1.1 History of malaria.

Malaria is one of man's oldest diseases. Hippocrates in 5th century first described the fever (a symptom of malaria) and noted the observations of the relationship between intermittent fever and the marshy land. From early times the disease was known in France and in Italy. The Italians used to practice the drainage of swamp as a measure of control of malaria during the early 17th century. The belief was that air from these wet areas carried the disease. It was not until the 18th century that the fever took the Italian name mal'aria (bad air), which recognize the relationship of the disease with the bad air of the marshy land.

Although there was a lot of description of the disease, the causative agent was not known at that time. It was in 1847 when the German pathologist Meckel first realised the relationship between the black pigment he saw in the blood, spleen and liver of dead bodies of malaria patients and the disease.

About 30 years later the French pathologist Kelsch saw the pigment in the blood of malaria patients and noted that the pigments are most noticeable just before the onset of fever. Another contemporary French pathologist, Laveran, who was working in an Algerian hospital, claimed to see parasites in red blood cells. He described the presence of clear bodies which contained pigment and noted the oval and crescent shape of the clear bodies. He confirmed his observation in 1880, when he saw that the blood of a young soldier was enriched with the crescent form. These are now known as the **gametocytes** (the sexual stage) of *P. falciparum*. Marchiafava and Celli, in 1885, saw the multiplicative form of the parasite in red blood cell of patients. At about the same time, Golgi saw these parasites and described their synchronous release from the red cell just before the onset of fever. This form is now known as the merozoite of the *P. falciparum*. In Russia, Danilewsky discovered avian and amphibian malaria parasites.

But it was still a big mystery how the parasite infected its hosts. It was long suspected, but not proved in parts of India and China that mosquitoes and malaria were related. In the late 1870s, Patrick Manson working in China showed the mosquito was a possible vector of filariasis. In 1893, Smith and Kilbourne showed that a tick could transmit a cattle red blood cell parasite, Babesia.

On the basis of these results, Ronald Ross, in 1894, speculated the possible role of mosquitoes in the spread of malaria. He started examining mosquitoes for the presence of the malaria parasite. But it was only after a long search that he saw black pigment, an indication of the presence of malaria parasites, in the midgut of a particular type of mosquito, *Anopheles*. To investigate the further role of the mosquito, Ross started working on an avian malarial parasite (*P. relictum*). He discovered that the infective form of the

parasite can be found in the mosquito salivary glands and he successfully infected sparrows using these mosquitoes. Hence the role of mosquito in the spread of malaria discovered. The life cycle starts when the mosquito feeds on the bird. The parasitized blood sucked in the mosquito midgut, multiplies and becomes a form infective to the bird and migrate to the mosquito's salivary gland. An uninfected bird becomes infected when it eats the infected mosquito or when the infected mosquito takes its meal on the bird. Soon after this discovery , the presence of a similar cycle for human malarial parasites was confirmed. In 1897, MacCallum in Canada described the maturation of the sexual form and fertilization of *Haemoproteus columbiae* in mosquito midgut. The exoerythrocytic stage of plasmodium was first proposed by Grassi in 1902 and finally demonstrated by Raffaele in 1934. The liver stage of the mammalian Plasmodia was demonstrated by Short and Garnham in 1948.

1.2 The life cycle and developmental biology of malaria parasite.

Figure 1.1 shows the detailed life cycle of *P. falciparum* as described by Garnham (1966). The life cycle of the parasite starts, in the mammalian host, when an infected female *Anopheles* mosquito bites a human host. The **sporozoites** from salivary glands along with saliva are injected into the capillaries of the host's peripheral circulatory system. These sporozoites are motile and are about 11 µm in length and 1 µm in diameter. porozoites enter into the main blood stream though the capillaries in the skin and within 30 to 40 minutes they accumulate in liver and disappear from the blood stream. Studies with rodent parasites indicated that before invading the hepatocyte the parasite may passage through the Kuffer cell in the liver sinusoid (Meis *et al*, 1983). Nothing about the nutrition and metabolic activity of the sporozoite during this

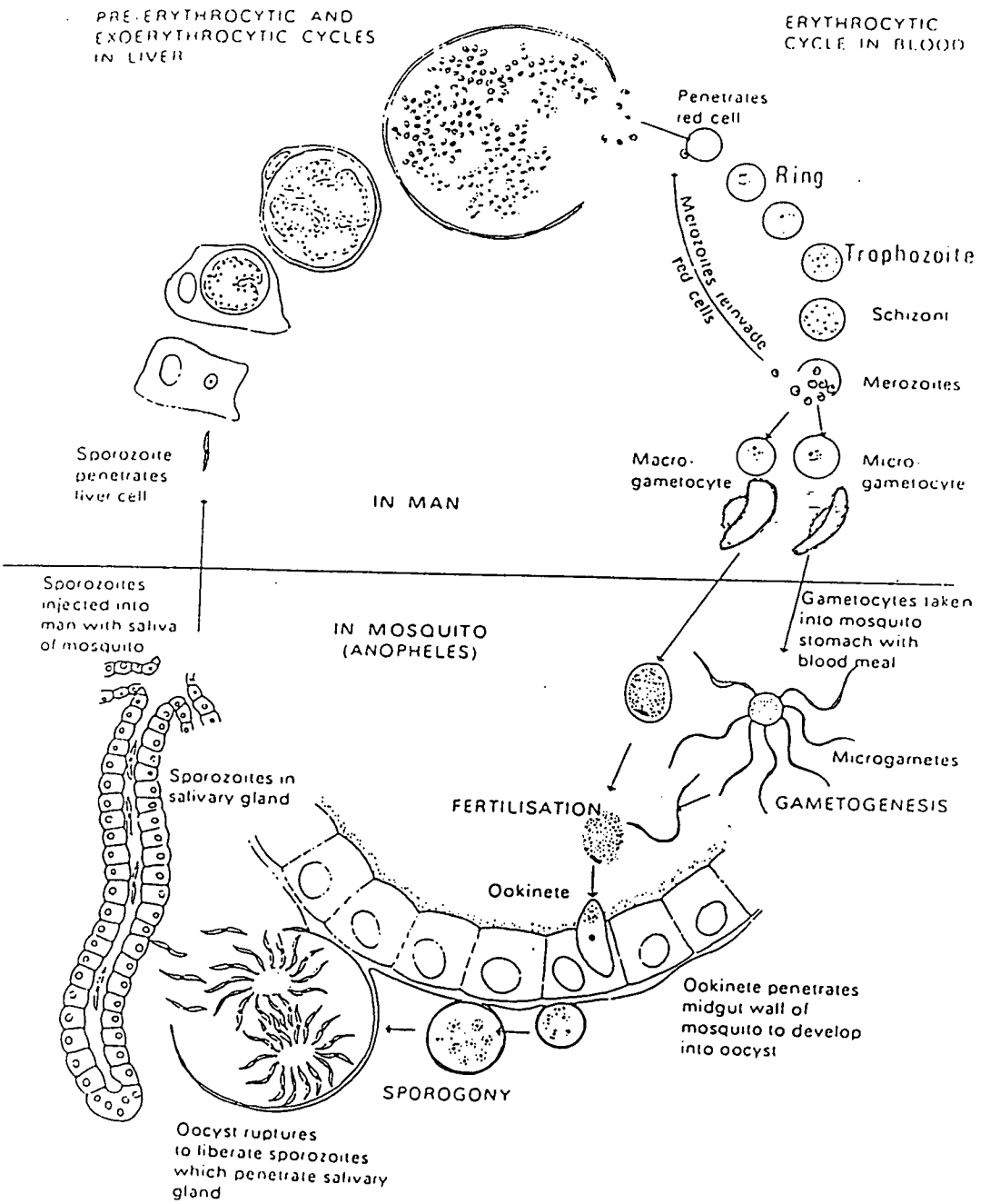


Figure 1.1 Life cycle of *P. falciparum*

brief blood stage is known. Since sporozoites do not undergo any multiplication, presumably the biosynthetic rate during this period is very low. And perhaps most of the energy is spent in the mechanical movement of the parasite in transit to the liver cell. Within the hepatocyte the parasite remains in a membrane bound structure, called the **parasitophorous vacuole**.

In hepatocytes the parasite undergoes a rapid transformation. It grows bigger in size, The nucleus replicates several times and cellular components for several thousand new parasite are synthesized. Then within the parasitophorous vacuole the parasite divides, the hepatocyte bursts and about 40,000 merozoites are released into the blood stream. After release from the hepatocyte *P. falciparum* merozoites invade red blood cells. The mechanism of merozoite release is not clear. This stage is called the **preerythrocytic** stage. The **merozoites** of *P. falciparum* can not reinvade hepatocytes, however there is evidence that other human malarial parasites can reinvade hepatocytes (Krotoski *et al.*, 1980; 1982).

The merozoite is pear shaped and about 1.5 µm long. The apex consists of a polar ring, two rhoptries, which contain electron dense material, micronemes and dense granules. The content and the origin of dense granules is not clear (Torii *et al.*, 1989). The merozoite is surrounded by outer, inner and a plasma membranes. On the surface there are rough bristle like structures. Invasion only occurs when the merozoite is attached to the erythrocyte at its epical end.

The mechanism of attachment and invasion has been studied extensively (Ladda, 1969; Dvorak *et al*, 1975; Aikawa *et al*, 1978; Aikawa and Seed, 1980). Most of these results are on the basis of electron microscopy, and the molecular basis of the mechanism is not clearly known. After proper orientation, the merozoite binds possibly to glycophorin A on the surface of

the erythrocyte membrane. After attachment the content of the rhoptries are released into the junction. This causes a rapid invagination of the red cell membrane, which allows the merozoite to invade. The invasion is a very rapid process, taking no more than a minute.

Once the merozoite is in, it remains in the parasitophorous vacuole, surrounded by the parasitophorous vacuole membrane. This membrane was believed to be a part of the red cell membrane, but there is evidence that most of this vacuolear membrane structure is of parasitic origin. During invasion the merozoite sheds its surface coat. Inside the red cell the merozoite transforms into a feeding form, rapidly grows bigger and in a Giemsa stained slide under a light microscope looks like a signet ring. This is called the **ring stage**.

The nucleus undergoes a second round of mitotic division about three to five times and the vacuole grows bigger. This is the **trophozoite stage** of the parasite. The parasite then grows much bigger in size till it occupies most of the red cell. Following this rhoptries, micronemes and apical ring form, the cytoplasm divides and buds out with one nucleus in each. This is the **schizont stage**. The schizont then bursts and 8 to 24 new merozoites are released which reinvade new red cells. This is called the **erythrocytic cycle**. It takes 48 hours in case of *P. falciparum* and longer in the case of other human parasites. The erythrocytic cycle is the stage which is responsible for the clinical symptoms of malaria.

During parasite maturation inside, massive changes occur on the surface of the red blood cell (reviewed by Howard R. J., 1988). Although very little is known about the biochemistry and genetics of the development there is evidence that novel proteins are expressed during the developmental stages (Vermeulen, *et al.*, 1985). Some of these proteins have been studied in detail for their apparent medical importance.

At the later stage of infection some of the maturing parasites inside the red cell differentiate into sexual form by an unknown mechanism (Bruce-Chwatt, 1980). The process is called the **gametocytogenesis**. There is evidence that the extent of parasitemia may trigger this process but external metabolites may also participate (Pietro Alano, personal communication). In the course of differentiation the parasite is transformed into crescent shaped male gametocyte, called **microgametocyte**, or female gametocytes, called **macrogametocytes**. This stage of the parasite is infective for the mosquito and can survive in the mosquito gut. The fate of these gametocytes in human blood is not clear , however it is believed that they are eliminated from the blood stream if not taken into the mosquito gut.

The mosquito stage of the parasite starts when a mosquito takes its blood meal from an infected human. The gametocytes are taken into the midgut of the mosquito where the process of **gametogenesis** occurs. The microgametocytes lose the red cell membrane and differentiate into a flagellated round shape. Within 10 minutes the nucleus divides mitotically into 8 daughters which migrate into the flagella. These nucleated flagella extrude and detach. They are the male gametes. Meanwhile, the macrogametocytes after loosing the red cell membrane, differentiate into macrogametes ready for fertilisation.

Within a few minutes fertilisation occurs, the two nuclei fuse to form **zygote**. The round zygote transforms into a leaf shaped **ookinete** within next 12 to 18 hours. These ookinetes then cross the mid gut wall, usually intracellularly, and eventually reach the basal lamina of the mosquito mid gut where they differentiate into **oocysts**. Over the next 10 to 12 days the oocysts grow bigger in diameter and a capsular wall is formed. Very little is known about the biochemistry of oocysts. They do not have any cytostome to feed

themselves. presumably, they obtain their food through the wall.

On the outer surface of the stomach wall, there may be several hundreds of oocysts in one stomach. Inside the oocyst the genome undergoes several cycles of replication and the parasite divides to form slender sporozoites. Several thousands of sporozoites are released into the haemocoel, from where they enter into the salivary glands ready to be inoculated into a new human host at next blood meal (Pringle, 1966). The overall duration of the cycle in the mosquito depends on the environmental temperature. At 28°C it is about 8 to 10 days, whereas at 20°C about 16 days. Under 15°C the cycle can not be completed.

1.3 Methods of controlling malaria.

To complete the life cycle, it is very important for the malaria parasite to passage through the two hosts, mosquito and human. So, for the transmission of the disease in a community, successful contact between this two hosts and sound development of the parasite within them is very important indeed. Hence, control of malaria may involve: i) The reduction of the numbers of the vector in the community, ii) Interruption of the transmission cycle and iii) Blocking the normal growth of the parasite in either one or both of the hosts. In the following sections I will discuss these methods and pinpoint some particular steps where molecular biology can play a major role in the control of malaria.

1.3.1 Vector control.

Reducing the number of mosquitoes in nature could be the easiest and preferable choice of malaria control. Because it does not require the treatment of humans, it is ethically more acceptable for most countries. Hence, it may be the perfect tool for a global eradication programme for malaria.

Indeed, in the 1950s the WHO malaria eradication campaign was based predominantly on the spraying of insecticide. This was successful in some countries. Reduction of the number of mosquitoes can be achieved in several ways:

Preventing the mosquito from feeding on man.

The transmission cycle can be interrupted by preventing mosquitoes from feeding on man. Use of bed nets, Mosquito nets on windows, insect repellent and protective clothing are the most common measures that can be used for this purpose. However, in developing countries where malaria is hyperendemic, these could be very expensive for individual families.

Number of breeding site reduction.

Anopheles mosquito lays their eggs on standing fresh water, like wells, paddy fields and swamps or sometimes even on very slowly running water. The eggs hatch and spend their larval and pupal stages in the same place. So, reducing breeding sites could be another way of reducing number of mosquitoes. This method of controlling malaria has long history. Ross himself suggested this method in India. However, in tropical countries, where monsoon rain is heavy, it is very common that rain water collects in foot prints, cart-tracks, leaf axils. Besides, in agricultural areas, making irrigation channels and water reservoirs is common.

Use of antilarval agents.

After hatching, the larvae feeds on the decomposing biomaterials in the waters and usually lie parallel to the surface for breathing. Use of larvicide, like Purple green (Copper aceticarsenite) and use of a film of oil on the water surface which interrupts the breathing of larvæ can be used to reduce the number of mosquitoes. Larvivorous fish, such as guppy and minnows have also been successfully used to control malaria.

Use of insecticide.

Since the introduction of DDT (dichloro diphenyl trichloroethane) and dieldrin in the mid forties, direct attack on the adult mosquito was the main focus.

The rationale was after taking blood meal at night a mosquito is at rest in a dark corner of a house. If the corner was previously sprayed with residual insecticides, the mosquito would die before transmitting malaria. There are several advantages of this method. Firstly it is easy to organize spraying of houses. Also it is easy to train personnel to carry out the procedures and is more easily effected on a countrywide basis than antilarval measures. Indeed, it was the major tool of 1955 WHO malaria eradication programme. During the early stages the house spraying was a great success. In 1957, it seemed that malaria eradication from the world was possible. However, the insecticides and other organochlorines which were introduced afterwards have fallen out of favour to some degree. The first reason was that these insecticides were found to be toxic at high concentrations for plants and animals. Second but more serious, resistant mosquito vectors were beginning to emerge rapidly. By 1976, 3 species were resistant to DDT only, 21 species to both DDT and dieldrin and 42 species to dieldrin only.

1.3.2 Vaccination.

Vaccination of humans against the malaria parasite is possibly the best way to control the disease. Indeed, a cheap, effective and easily administered vaccine would be of great value in the eradication of malaria from the world. Hence, it is not surprising that a major goal of current malaria research is vaccine development. Although global eradication was successfully achieved with the smallpox immunisation campaign, despite the worldwide search, it has not yet been possible to develop a successful vaccine for preventing malaria, or for any other human parasitic diseases such as Trypanosomiasis, Leishmaniasis, Filariasis, Leprosy and Schistosomiasis. However, there are successful vaccines against some veterinary parasitic diseases. For example, dog hookworm a (*A. caninum*), cattle and sheep lung

worm (*D. viviparus, D. filaria*) (Miller, 1971; Urquhart, 1962). These vaccines are mostly attenuated parasites and the procedure is not possible for application on man, mainly for ethical reasons and also for the danger of possible immunopathogenicity.

One of the major difficulties in developing vaccines against malaria is its complexity as an infectious agent and its unique ability for adaptation and the evasion mechanism which the parasite has evolved to its vertebrate hosts immune system (Sher 1988). Another great difficulty which concerns this work to a certain extent, is that until now there is no practical way of obtaining sufficiently large quantity of antigen to immunise a large population. The introduction of the *in vitro* culture method for erythrocytic stage of *P. falciparum* (Trager & Jensen, 1976) opened a new era for research in the field of vaccine development. However, this method is not economically feasible for the production of a vaccine antigen to a commercial level.

1.4 Immunity against malaria and vaccine design.

It is important for a good immune response that the parasite and the host immune system should come into close contact. An infection can take one of three different courses. Firstly, the parasite can multiply very fast, faster than the hosts immune response and kill the host. Secondly, the immune resistance to the parasite can supersede the parasite multiplication rate and the host can recover from the disease. Both of these courses are unfavourable for transmission. Finally, the infection can be kept to sub-clinical levels by the immune mechanism, but it can never cure it. This gives the parasites enough time to go through the several developmental stages and get ready for transmission.

To do this the malaria parasite has evolved several evasion mechanisms,

which allows it to adapt to the host's immune system such as antigen variation, sequestration of parasitized red blood cells in capillaries etc. These evasion mechanisms, common in other pathogenic parasites as well, are some of the major obstacles in the way of vaccine development. Hence, the use of classical immunology and methods of vaccine development are not suitable for parasite vaccine development. These difficulties actually led to the development of the new subjects, like parasite immunology and molecular parasitology.

With regard to the life cycle stages as vaccine targets, designing of antimalarial vaccines fall into several classes (Figure 1.2).

1.4.1 Sporozoite vaccines.

Immunisation against the sporozoite stage is aimed to destroy the parasite immediately after entry into the human body. This will stop subsequent development of the parasite to the pathogenic stage i.e. the erythrocyte stage. Within a few minutes after introduction into the human body the sporozoites disappear from the blood stream. In contrast, a period of several days are required for immune response to develop in a naive host. Hence the immunological selection pressure which would favour the development of an evasion mechanism against the sporozoite stage is small. Thus, it has been proposed that at least theoretically, vaccination against the sporozoite stage is the most logical approach (Nussenzweig & Nussenzweig, 1988).

Although the sporozoite stage in the vertebrate host is very short, protective immunity has been achieved in rodent, monkeys and humans with γ-irradiated mosquitoes (Cochrane *et al.*, 1980; Nussenzweig and Nussenzweig, 1986). The immunity was fully stage and species specific. The animal was fully protected against the sporozoite stage and susceptible to infection by erythrocytic stage parasites. This suggests that separate vaccine components

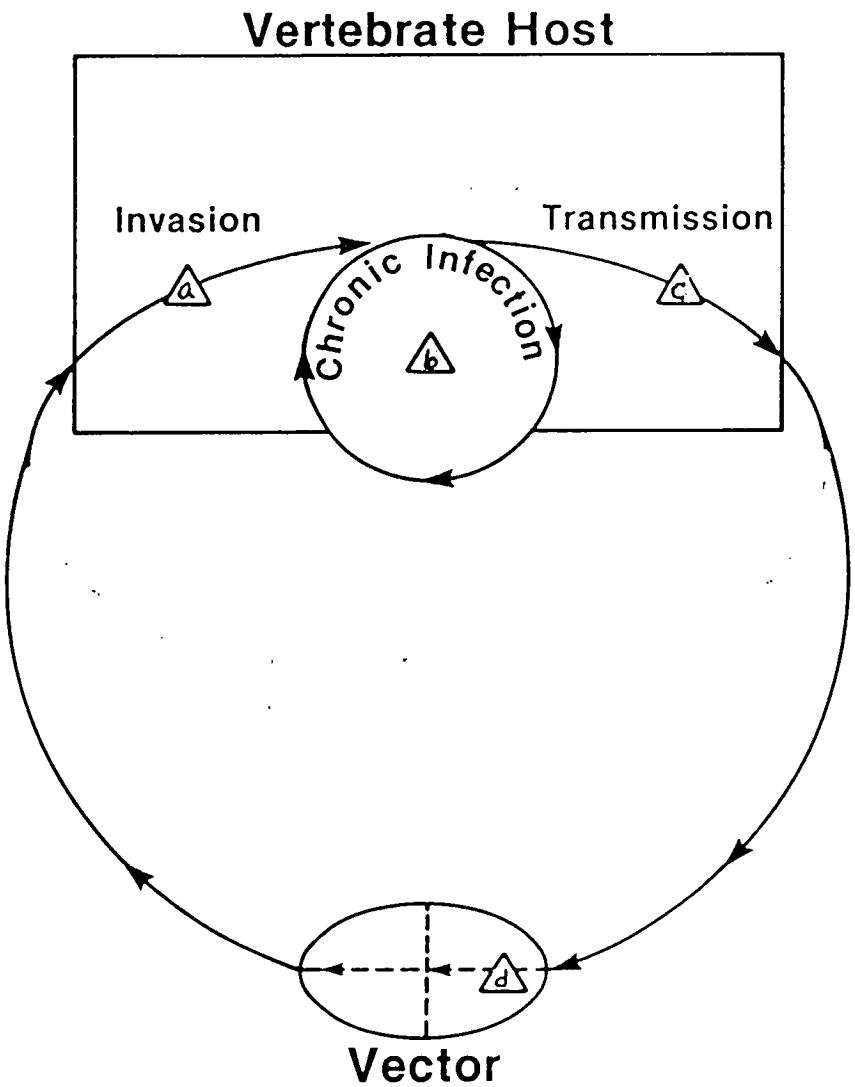


Figure 1.2 Stage specificity of antimalarial vaccine. Malaria vaccines are directed against: a) Sporozoites in vertebrate host; b) asexual or erythrocytic stage; c) gametocyte of sexual stage; or d) gametes in mosquitoes. Antibodies against gametes can be produced in vertebrate host and act on the parasite in the mosquito gut when ingested with the blood meal.

will be needed against the various human malaria parasites. Moreover, the immunity was very short lived - about three months, although it could be boosted (Clyde *et al.*, 1973, 1975; Rieckmann *et al.*, 1974).

Search for the protective antigen revealed that the immune response against sporozoites is essentially directed against a single protein molecule, the circumsporozoite protein (CSP). The protein constitutes the surface coat of the sporozoite. The gene for the protein has been cloned from different species of *Plasmodia* including *P. falciparum* and the deduced primary structure has shown that the protein has an unusually long repeat of the tetrapeptide, which in *P. falciparum* is NANP (Dame *et al.*, 1984; Enea *et al.*, 1984). These repeat regions of the protein have been shown to be the most immunodominant part of the protein (Ballou *et al.*, 1985; Zavala *et al.*, 1985). These and other studies put the repetitive epitope of the CSP in the focus of antisporozoite vaccine development.

The results of immunisation with two different vaccines both based on these repeats were not conclusive (Ballou *et al.*, 1987; Herrington *et al.*, 1987). The immune response was poor and the subsequent challenge with *P. falciparum* showed very insignificant protection. However, one person in each case was fully protected and some acquired a certain degree of protection. Moreover, the immune response was found to vary with age and also higher immune response did not necessarily correlate with the higher degree of protection (Hoffman *et al.*, 1986). Furthermore, the mechanism of the immune response against the sporozoite seems very complex. Recent studies have shown that response may be T-cell mediated, which may act against the hepatocytic stage of the parasite by releasing γ-interferon (Schofield *et al.*, 1987). These results indicate that vaccine development against the sporozoite stage needs further study of the CSP immunology and the mechanism of the

immune response.

1.4.2 Asexual erythrocytic stage vaccines.

The second and most important stage of parasite development to which the vaccine development is aimed, is the asexual blood stage. Compared to the sporozoite stage, antibody responses to the asexual blood stages of *P. falciparum* and other malaria parasites are very polyspecific. This and other advantages such as the introduction of an *in vitro* cultivation of *P. falciparum* which facilitated the preparation of large amount of antigen has made this stage most popular to the vaccine developers.

A number of different approaches have been used to identify the asexual blood stage antigens and a wealth of data has been accumulated in this field (Brown *et al.*, 1982; Freeman *et al.*, 1980; Hall *et al.*, 1983). Although not all of these antigens have proven to be good candidates for vaccines, some of them by their location on either the merozoite surface or in some secretory organelles of merozoites are strong candidates for malaria vaccine. Table 1.1 shows some of these important candidate antigens. Merozoite surface antigens are interesting because these antigens are easily accessible to immune attack. Moreover, there is evidence that the host protective immune response can be directed against these antigens (Miller *et al.*, 1975). Preclinical testing on Aotus and Saimiri monkey has been done with purified asexual stage antigens (Hope *et al.*, 1984, Ridley *et al.*, personal communication). Although the results of these tests were not conclusive, however encouraging.

One of the major problems of immunisation of a large human population with such vaccines is to prepare them in large amounts, free from the contamination of the erythrocyte membrane material. Contaminants could induce complex immunopathogenicity and cause fatal haemolytic disease in the recipient. Development of synthetic vaccines or preparation of the vaccine antigen in

Table 1.1 Cloned genes of *Plasmodium falciparum* (TDR/WHO 9th program report, 1989)

	Estimated relative molecular mass, M, (K=000)	*Year gene cloned*	*Sequence complete*	*Expression*[a]	*Protein location*	*Potential application*[b]
Acidic basic repeat antigen (ABRA, Pf p 101)	102 K	1986	√	E	schizonts	
Actin (Pf actin-I, II)		1988	√			T
Aldolase	41 K	1988	√	E		V
Asparagine-rich protein (ARP)	160–220 K	1986		E	all stages	
Clustered asparagine-rich protein (CARP)	15–36 K	1986		E		
Circumsporozoite protein (CSP)	40–60 K	1984	√	E, Y, V, P	sporozoite surface	V, D
Circumsporozoite-protein-related antigen (CRA, 5.1, *exp*-1)	23 K	1985	√	E	trophozoite/schizont vacuoles	
Dihydrofolate reductase (DHFR)/thymidylate synthase (TS)		1987	√			T
Falciparum interspersed repeat antigen (FIRA)	>300 K	1985		E	infected erythrocytes	D
Glycophorin-binding protein (GBP130)	96–130 K	1985	√	E	secreted; cytoplasm and merozoite surface	V
Heat-shock-related proteins (Pf hsp 70)	71–75 K	1986	√	E	all stages	V
Pf HRP-I (knob-associated histidine-rich protein, KAHRP)	85–108 K	1986	√	E	knobs; infected cell cytoskeleton	
Pf HRP-II	60–72 K	1986	√		secreted; cytoplasm	
Pf HRP-III (small histidine-alanine-rich protein, SHARP)	28–33 K	1985	√	E	schizonts	
Hypoxanthine-guanine phosphoribosyl transferase (HGPRT)		1987	√			T
Lactate dehydrogenase (LDH)	36 K	1985				
Liver-stage-specific antigen (LSA-1)		1987		E	exoerythrocytic schizonts	V
Mature erythrocyte surface antigens (MESA, Pf EMP2)	>250 K	1986		E	cytoskeleton of mature infected cell	
Merozoite surface antigen-2 (MSA-2)	45–51 K	1988	√	E, P	merozoite surface	V
Precursor to major merozoite surface antigens (PMMSA, P190, Pf195, PSA, gp195)	185–210 K	1985	√	E, Y, V, P	schizont/merozoite surface	V
Rhoptry proteins	55 K	1987		E	rhoptries/host membrane	V
	105 K	1988		E		
Ring-infected erythrocyte surface antigen (RESA/Pf155)	155 K	1985	√	E, V, P	micronemes of merozoites; ring cytoskeleton	V
S-antigen	140–230 K	1985	√	E, V, P	parasitophorous vacuole	
Thrombospondin-related anonymous protein (TRAP)		1988	√			
Pfp 126	113–140 K	1987	√	E	schizonts; parasitophorous vacuole	
Pf 11-1	260–350 K	1984		E	mature infected cell membrane	
Pfs 25	25 K	1988	√	E	ookinete surface	

[a] E = *Escherichia* coli; Y = Yeast; V = Vaccina virus; P = Synthetic peptides.
[b] D = Diagnostics; V = Vaccines; T = Drug therapy. (Note that all proteins defined as antigens by the human immune response may have potential in vaccine development.)

genetically engineered bacteria may be a solution. The latter is possibly economically more feasible.

1.4.3 Sexual stage vaccines.

As it was mentioned in the life cycle, some of the infected red cells transform into macro and microgametocytes, thus initiating the sexual stages of the cycle. During this and the following steps upuntil the invasion of the ookinete of the mosquito midgut wall parasites spend more than 24 hours in contact with the vertebrate blood. During this time the sexual stage parasites are vulnerable to the vertebrate immune attack, both in the vertebrate body and mosquito midgut. This is, therefore, the third point of attack against parasite development. In comparison to the sporozoite and asexual blood stage, very little work has been done to assess the potential of the sexual stage antigens as candidates for vaccines. The main reason for this is that the sexual stages are nonpathogenic and do not directly contribute to the disease. But from an epidemiological point of view, the prevalence of malarial infection depends on the availability of the gametocyte to the mosquito and successful zygote formation in the mosquito midgut. Therefore the relevance of immunity to these stages can not be ignored.

Mendis and her colleagues (1987) have recently shown that human populations in endemic areas possess high titres of antibody to sexual stages. They suggested that these may play an important role in the control of malaria epidemics. Immunity against sexual stages is commonly called "malaria transmission blocking immunity". Induction of such immunity has been demonstrated in birds, rodents and monkeys. In most of these cases the immunogens were the sexual stages of the parasite. Immunity against all the sexual stages has been shown; against intracellular gametocytes in the circulation (Harte *et al.*, 1985), extracellular gametes in the mosquito midgut

(Mendis *et al*, 1979), fertilized zygote in the mosquito midgut (Kaushal *et al*., 1983) and ookinetes in the mosquito midgut (Vermeulen *et al*., 1985).

Searching for the target antigen for such immune response resulted in the identification of three sets of proteins in *P. gallinaceum* and *P. falciparum*. These are 230 kD (Grotendorst *et al*., 1984; Rener *et al*., 1983), 48/45 kD and 31/25 kD (Grotendorst *et al*., 1984; Vermeulen *et al*., 1985) proteins. The 230 kD and the 48/45 kD proteins are the surface proteins of the gametes and are also found on the surface of the gametocytes. Antibodies against these proteins were found in the individuals exposed to *P. falciparum*. The 31/25 kD protein is the ookinete surface protein. Neither the immunology nor the biochemical properties of these proteins have been elucidated to date. Further study may reveal the importance of these antigens.

1.5 Chemotherapy.

Despite the tremendous efforts towards designing a vaccine against malaria, it seems that we still have to go a long way to fulfill the dream. What remains for the immediate cure of malaria is to use chemoprophylactic agents. Successful chemotherapy of malaria was carried out long before the cause of the disease was known. About 300 years ago powder of the barks of the cinchona tree was used to treat malaria fever in Peru. This treatment was then transported to Spain and then spread to other parts of Europe. Later the active ingredient quinine was isolated and used against malaria. Since then a large number of other antimalarial drugs have been introduced for malaria treatment. The rationale of selecting a drug against the parasite is to find a chemotherapeutic agent which is toxic against malaria parasites at a certain concentration but would be completely harmless to humans at the same concentration.

The choice of drug depends on whether it will be used for preventive purposes or for curative purposes and also depends on the species of the parasite being treated. The dose determination is also important. A high dose may cure the disease by rapidly eliminating the parasite from the blood stream but this would reduce the extent to which the parasite is exposed to the host immune system and subsequently a very low immune response will result which will not be good for a long lasting immune response. On the other hand an exact dose would reduce the clinical malaria but allow a longer exposure of the parasite to the immune system.

Structures of some of the important antimalarial drugs are shown in Figure 1.3. Not all antimalarials are active against all the stages of the parasite. According to the stage against which they are active, the antimalarial drugs are classified into four catagories. None of the antimalarials are active against the sporozoite stage, but some are taken up by the mosquito in a blood meal and inhibit development of the oocyst, thus preventing the production of sporozoites. These drugs are called the **sporontocides**. Members of such class are primaquine and pamaquine (chemically both are 8-aminoquinolines) and also proguanil and pyrimethamine (chemically diaminopyrimidine). Drugs like proguanil and pyrimethamine which also destroy the exoerythrocytic stages are called the **tissue-schizontocides**. The drugs acting on the asexual blood stages are called the **blood-schizontocides**. These include quinine. mepacrine, chloroquine, pyrimethamine and dapsone. Some antimalarials affect gametocytes. These are the **gametocytocides** and includes chloroquine, amodiaquine and quinine etc.

Now, if there are drugs against all the stages of malaria parasites, why do we need any more drugs or vaccines? The main reason is the horrific emergence of resistant parasite strains against existing antimalarials.

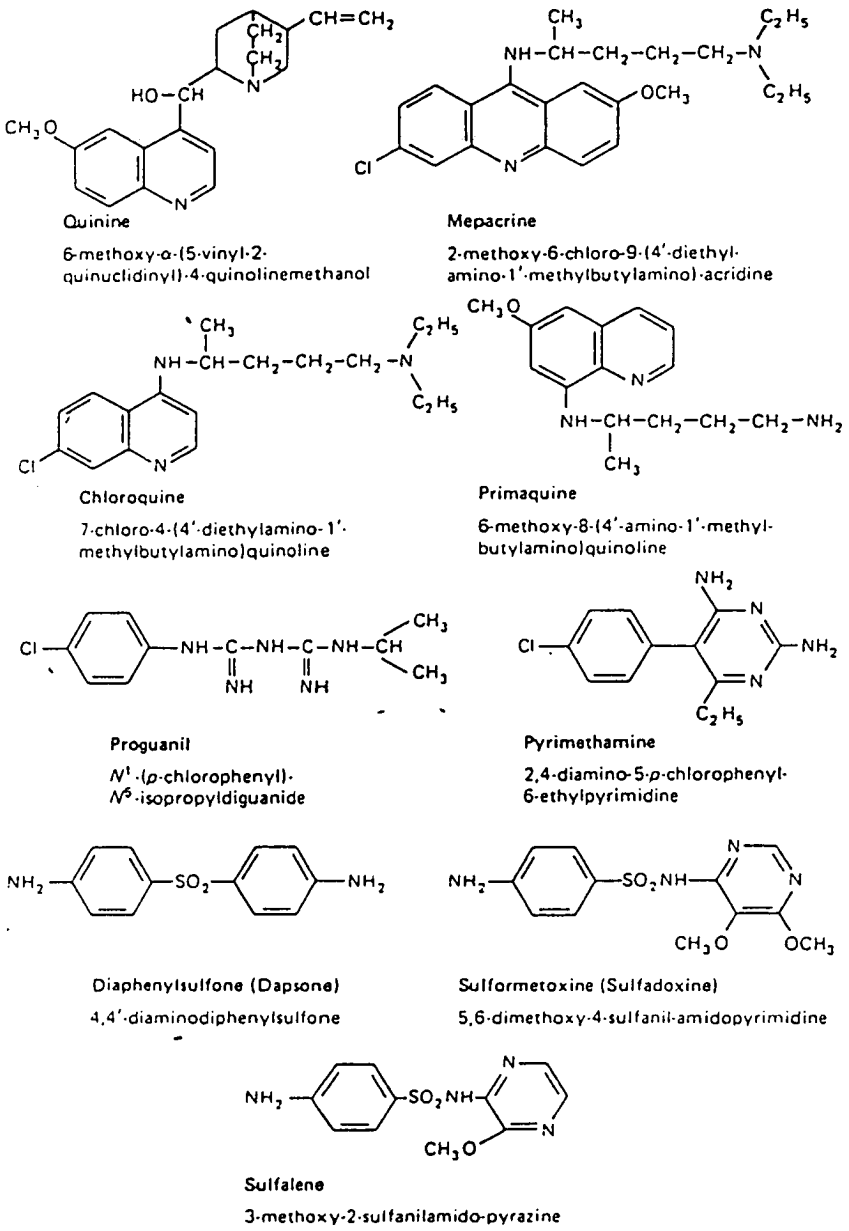


Figure 1.3 The molecular structure of some antimalarial drugs.

Resistance to causal prophylactics, like pyrimethamine and proguanil has been known for about 30 years, especially in south-east Asia and Africa. Such resistant strains are found in all four species of human malaria. These types of resistant stains are not too dangerous, because the drugs to which they are resistant are not used for the treatment of the acute malaria. In contrast, resistance against the drug of choice for acute malaria, i.e. chloroquine, has been described for the most dangerous species *P. falciparum*. Such resistant strains were first described in Columbia, subsequently found in Brazil and in many parts of south-eastern Asia, and recent studies shows that chloroquine resistant strains of *P. falciparum* are present in eastern Africa. The alarming emergence of chloroquine resistance is doubled by the fact that chloroquine resistant strains are sometimes also resistant to amodiaquine and mepacrine and that some have a lower sensitivity to quinine. Quinine is normally used to treat resistant *P. falciparum* malaria. If such multidrug resistant strains of *P. falciparum* become widespread, particularly in the malaria endemic countries, the disease may become completely uncontrollable.

1.6 Mechanism of action of some antimalarial drugs.

A large amount of work has been done on the mechanisms of drug action during the last half century. This has been reviewed by Peters and Howells (1978) and Elslager (1974). Recent knowledge about the action of some drugs has been described here.

Sulfonamides, sulfones and drugs of this group show their selective toxicity by competing with paraaminobenzoic acid (PABA) for the active site of the enzyme dihydropteroate synthase, in the pathway of folic acid metabolism (Figure 1.4). The enzyme catalyses the condensation of PABA with phosphorylated pteridine to form dihydropteroate. This in turn converts into

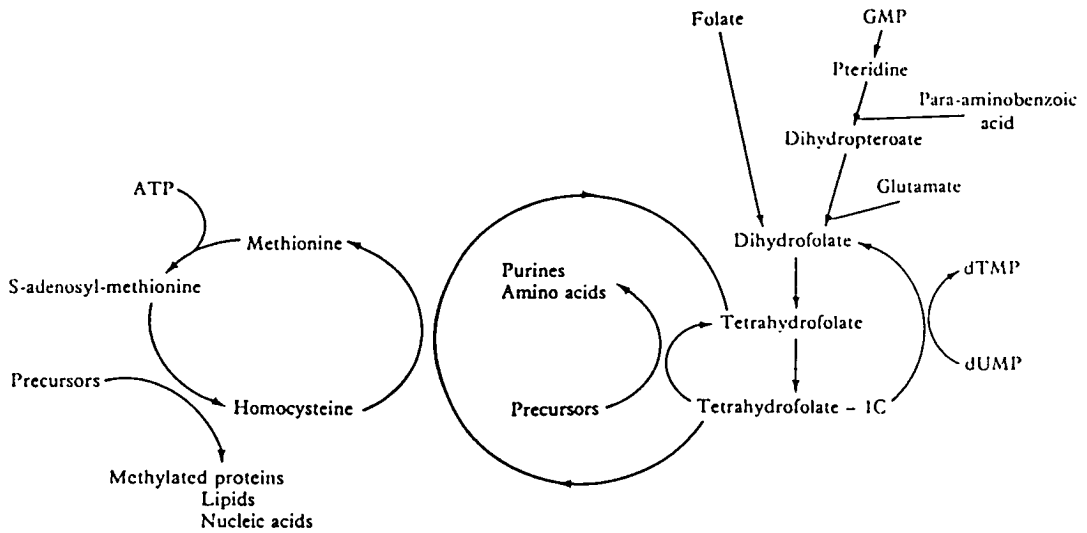


Figure 1.4 Folate metabolism in *Plasmodia*

dihydrofolate, a cofactor in purine and pyrimidine precursor biosynthesis. Humans utilise preformed folic acid for their requirement of dihydrofolates. The above mechanism was accepted for long time but recent study shows that folic acids interfere with the sulfonamide drug activity, indicating that the action of these drugs may be on other pathways as well (McCormick *et al.*, 1972).

Little is known about the mechanism of the resistance against the sulfonamide group of drugs. Resistant strains can be obtained by slow increase of the drug concentration in the culture medium. It appeared that the resistant parasites survive by bypassing the PABA utilisation steps. Instead they use the host cell folates. The resistance against sulfonamides is usually associated with less sensitivity to dihydrofolate reductase inhibitors. This type of resistance is stable and is carried through all stages and subsequent generations (Peters., 1971)

The selective action of 2,4-diaminopyrimidines, like pyrimethamine and trimethoprim and triazines like cycloguanil resides in their greater affinity to the parasite enzyme dihydrofolate reductase (DHFR) than to the host enzyme. DHFR is a key enzyme in folate metabolism since it converts the pteridine ring to the tetrahydro reduction state required forthe reaction in which the folate cofactors are synthesized. it has been shown that this drug binds 100 to 1000 times more tightly to the Plasmodial enzyme than to that of the host (McCormick *et al.*, 1971).

Perhaps the mechanism of resistance of DHFR inhibitors is most thoroughly studied. Recent comparison of amino acid sequence of sensitive and resistant PfDHFR, showed that one amino acid change in the active site due to a point mutation in the gene conferred the change in substrate binding of the enzyme (Peterson et al 1988).But there may be other mechanisms involved as

well, such as; alteration of the transport of drug across the cell membrane, gene amplification which may result in larger amount of the enzyme production in resistant cells (Schimke., 1980), and involvement of a deletion in 5′ flanking region of the gene on the drug resistance has also been hypothesized (Shiu wan Chan and John Scaife, personal comm.).

The mechanism of action of blood schizontocides such as chloroquine, quinine, mefloquine and amodiaquine is not clearly understood. These drugs can be divided into two groups on the basis of their structure and effect on the morphology of the parasite. In the first group, exemplified by chloroquine, there are two easily protonable highly electronegative nitrogen atoms present in the molecule and the most noticeable morphological effect is swelling and fusion of adjacent digestive vacuoles, followed by their sequestration in autophagic vacuoles, i.e. pigment clumping (Warhurst and Hockley, 1967).

The second group, exemplified by quinine do not cause clumping of the pigment but the digestive vacuole may swell and the pigment in them may become less electron dense (Peters *et al.*,1977). This group of drugs has only one electronegative nitrogen atom. The exact effect on the biochemistry of the parasite by these drugs is not clear. However, it has been suggested that these drugs may complex with the ferriprotoporphyrin IX, the degradation product of haemoglobin, and interact with the subcellular membrane resulting in swelling of the vesicles and subsequent lysis (Chou and Fitch, 1981). Resistance to this group of drugs does not emerge as often as to others, and the primary mechanism of this resistance may be due to the change in the transport system of the drugs.

Naphthoquinones and hydronaphthoquinones are found to be active against both primary tissue and blood forms of the parasites. It has been suggested that these compounds exert their antimalarial effect by blocking

electron transfer at a cytochrome mediated step in the energy producing respiratory chain (Roberts *et al.*, 1978). Later it was found that these compounds act on dihydroorotate dehydrogenase, an enzyme involved in pyrimidine biosynthesis which also coupled the pyrimidine synthesis with oxygen utilisation (Gutteridge *et al.*, 1979).

1.7 Parasite biochemistry as the target for chemotherapy.

The above discussion on mechanism of degradation and drug resistance emphasized the fact that antiparasitic drugs exert their effect by inhibiting or disturbing major biochemical pathways which may be unique to the parasite. So, it may be imagined that the complete and exhaustive knowledge of biochemical pathways is the prime requirement for success in antimalarial chemotherapy. To date, knowledge of the malaria parasite's biochemistry is far from complete, but the above discussion indicated that there exist in the parasite unique biochemical determinants and that these could provide a basis for the successful development of chemotherapeutic agents.

Attempts have been made to rationalise drug design. Although folate metabolism has been a fruitful target for drug development success has been very limited in other areas. This is due to several technical reasons. One major factor has been the lack of appropriate test systems. For example, the target of naphthoquinones was first believed to be ubiquinone 8, a counterpart of mammalian ubiquinone 10 when the early inhibition assays were carried out with beef heart mitochondria, although correlation of the inhibition with the activity data was very poor (Sweeney *et al.*, 1983). As stated in section 1.6 the target is now known to be dehydroorotate dehydrogenase.

The development of techniques of in vitro culture of *P. falciparum* and of isolation and fractionation of parasite material have removed this constraint in

part. But obtaining enough material for structural study of enzymes is still not possible by these techniques. Moreover, maintaining cultures and screening drugs is expensive and needs trained personnel.

Recent development of genetic engineering and monoclonal antibody techniques for manipulating parasite DNA and protein could play an important role in understanding the parasite biochemistry and in the identification of potential chemotherapeutic targets. The difficulties of the production of sufficient quantities of the enzyme from cultured parasites could be overcome by cloning in a suitable vector, the Plasmodial genes which code for the particular enzyme. Simmons *et al* (1985), showed the feasibility of such an approach. They identified the antibody to *P. falciparum* lactate dehydrogenase (LDH)(EC 1.1.1.27) and subsequently cloned the gene for this enzyme in *E. coli*. These studies indicated that the parasite-specific LDH was structurally distinct from the host cell enzyme.

In the following sections I will be discussing some possible areas of metabolic pathways in *P. falciparum* as potential chemotherapeutic targets.

1.7.1 Energy metabolism.

The glucose metabolism route is fundamentally similar in all organisms. So, it seems unlikely that these pathway could be a target for malaria chemotherapy. However, there is evidence that Plasmodial enzymes involved in this pathways can be quite distinct from those of the host. Sherman (1966) reported the heterogeneity of lactate dehydrogenase in *P. lophurae*. He also showed that the active site of the parasite enzyme is distinct from that of the host. This knowledge was later extended to *P. falciparum* by Simmons *et al* (1985). To date, there has been no report on the biochemical investigation of any other purified glycolytic enzymes. This is mainly because of the problems of growing the parasite in large quantities for conventional biochemical

analyses and also due to the excessive lability of Plasmodial enzymes. Detailed characterisation may provide some parasite-specific receptors and this may now be possible using molecular biological approaches.

1.7.2 Protein synthesis.

It is now generally agreed that erythrocytic stage *Plasmodia* get amino acids for protein synthesis from three different routes ; CO_2 fixation, free amino acid pools of plasma and red cells, and red cell haemoglobin (Sherman 1979).

CO_2 fixation is a minor source for parasite amino acid requirement, as has been shown by the fact that the parasite mostly depends on the exogenous sources. Despite this there is no detailed description of the mechanism by which the parasite performs nitrogen fixation. For examples one of the important CO_2 fixation enzyme, phosphoenolpyruvate carboxylase has reported only in *P. berghei* (Siu 1967). Likewise, the mechanism of uptake of CO_2 and free amino acids have not been described either. However, if this involved some parasite-specific carrier, it may be possible to inhibit protein synthesis by blocking such transport proteins. Kutner *et al* (1983) have described a new pathway for anion transport in human red cells infected with *P. falciparum*.

It is believed that haemoglobin is the major source of parasite amino acids. Parasite-specific proteases have been identified in different malaria parasites (Gyang *et al.*, 1982; Sherman and Tonigoshi 1983). These aminopeptidases therefore could be specific targets for the design of antimalarial drugs. Although the mechanism of protein synthesis in the malaria parasite appears to be typically eukaryotic, nothing is known about the specific details for this synthesis.

1.7.3 Lipid biosynthesis.

It has been shown that the malaria parasite obtains most of its phospholipids, lysophospholipids, cholesterols and fatty acids and phospholipids

by exchanging the components with plasma during the turnover of erythrocytic lipids (Sherman 1979). Moreover, both membrane lipids of the host cell as well as the parasite become available for Plasmodial lipid biosynthesis by endocytosis. It seems therefore unlikely that lipid biosynthetic pathways could be targets for antimalarial drug design. However, parasite specific enzymes of lipid biosynthesis such as phosphatidylserine decarboxylase (EC 4.1.1.65) and phosphatidylethanolamine methyltransferase (EC 2.1.1.17) (Vial *et al.*, 1982; 1985), have been identified and so this approach cannot be disregarded.

1.7.4 Folate metabolism.

Malaria parasites can synthesize folic acid by the *de novo* pathway. Sulfonamide, a sulfone, interferes with the biosynthesis of dihydrofolate and pyrimethamine, an inhibitor of dihydrofolate reductase block the formation of tetrahydrofolate. It is known that the malaria parasite, in contrast to host can use PABA and not folic acid for synthesis of folates. But more recent studies have revealed that in *P. falciparum* both folic acid and PABA interact with salfadoxin (McCormick *et al.*, 1972). This may indicate the existence of another metabolic pathway by which the parasite is capable of utilising red blood cell folic acid, in the absence of plasma folate or PABA. Further investigation of this pathway may reveal some parasite-specific receptor which could be a target for an antimalarial drug.

1.7.5 Nucleic acid metabolism.

During intraerythrocytic schizogony, malaria parasites multiply rapidly to produce merozoites. Sinden (1978) suggested that during intraerythrocytic development from an invading merozoite to a fully mature schizont the amount of DNA increases 10 to 20 fold. The parasite doubling time is approximately 6 h (Sherman 1979), and so this needs large amount of nucleic acid synthesis in certain stages of parasite's life. Studies have shown that the DNA of

P. falciparum has a unique base composition of approximately 17-19% G+C (Goman *et al.*, 1982). Similarly RNA from malaria parasites is typical of a protozoan having a G+C content of 35% in contrast to that of 65% in human (Sherman 1979). These features suggest that if such a unique DNA and RNA composition is important for the parasite's life, then this could be a suitable target for drug design.

Malaria parasites can synthesize pyrimidine by the *de novo* pathway. The pathway is shown in Figure 1.5. The enzymes for thymidylate synthesis have been identified in several plasmodia species. Inhibition of one of these, dihydrofolate reductase (DHFR) has been shown to be the basis of the action of pyrimethamines. DHFR and thymidylate synthase (TS) in *Plasmodium* exist as a bifunctional enzyme complex whereas the mammalian enzymes exist separately (Ferone and Roland 1980). It is not known yet if this is an exploitable difference.

Some of the enzymes of pyrimidine biosynthesis have been identified in *P. falciparum* (Gero *et al.*, 1981; Rathod and Reyes 1983). The same enzymes have also been detected in *P. berghei* (Hill *et al.*, 1981). Their studies showed that the first three enzymes in the pathway, carbamoylphosphate synthase, aspartate transcarbamylase and dihydroorotase seem to exist in a complex similar to eukaryotes. The fourth enzyme, dihydroorotate dehydrogenase is of considerable interest, since it is inhibited by cyanide and antimycin A. It has been suggested that during oxidation of dihydroorotate, electrons are fed into the cytochrome chain at the ubiquinone level. The prime purpose of this electron transport chain is its involvement in the orotate biosynthesis. The cytochrome is the only cytochrome known in *Plasmodia* and the inhibition of the cytochrome oxidase could therefore, be one of the chemotherapeutic targets (see Fig. 1.5).

In contrast to the *de novo* biosynthesis of pyrimidines *Plasmodia*, like

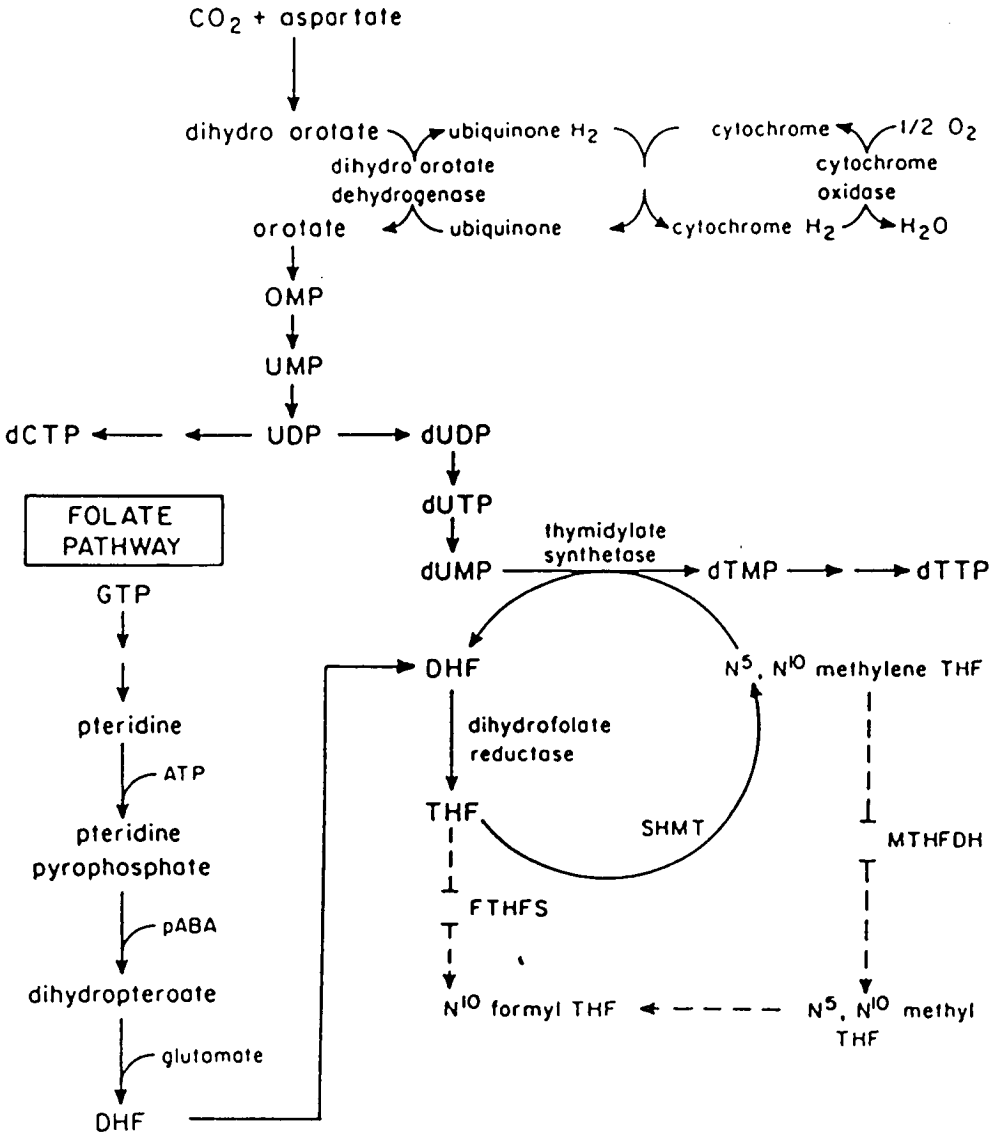


Figure 1.5 The *de novo* pyrimidine biosynthesis in *Plasmodium*. The relationship with the folate pathway.

other parasites cannot synthesize purine rings *de novo*, and these are obtained preformed, preferably as hypoxanthine (Konigk 1977), by the purine salvage pathway. All the major enzymes of purine salvage pathway have been identified in *P. falciparum* (Sherman 1979). These include adenosine deaminase, purine nucleoside phosphorylase, hypoxanthine-guanine phosphoribosyl transferase (HGPRT) and adenosine kinase. Based on the kinetic parameters, Reyes *et al* (1982) suggested that HGPRT and adenosine deaminase are structurally distinct from the host enzymes. Another enzyme of interest in purine salvage pathway is adenylosuccinate synthase. This enzyme catalyses the synthesis of adenylosuccinate, an intermediate in the conversion of inosine monophosphate to adenosine monophosphate. One inhibitor of this enzyme, halacin has been studied for its ability to inhibit synthesis of adenylate. Webster *et al* (1984) showed that halacin blocks synthesis of adenylates from hypoxanthine.

1.8 Rationale of chemotherapy.

In the above discussion it has been demonstrated that parasite-specific enzymes exist which could be potential targets for drug design against malaria. As discussed above, the major problem of studying these enzymes is difficulty in getting enough purified enzyme from *in vitro* cultured parasites. Further problems arise due to the excessive lability of plasmodial enzymes for some unknown reason. Development of genetic engineering technologies could contribute considerably in overcoming at least some of these problems. For instance, if the genes specifying these enzymes can be cloned and sequenced, structural information can be readily obtained. Expression of these genes in well-studied prokaryotes could give enough protein for crystallography. Structural data could then be compared with that of the host protein and key

differences could then be pin pointed and putative drugs could be designed by computer aided designing (CAD) (Silverton 1984). Such drugs may only act on the parasite enzyme but not on the host's enzyme.

Another advantage of such a structural study is that, it could give information about any functional change in the protein which causes resistance against a drug. One excellent example of this is pyrimethamine resistance. Pyrimethamine is a specific inhibitor of DHFR. Resistance to pyrimethamine has been shown to be due to a point mutation in the active site of the enzyme (Peterson et al 1988). This has been identified by cloning the gene from both sensitive and resistant strains.

Direct and functional expression of enzyme-encoding genes in a bacterium may present an extra advantage. Expressing such a gene in a bacterium with a mutation in the homologous gene could result in complementation of the bacterial mutation. Such a system could be excellent for screening putative drugs against the parasite enzyme. This would be both inexpensive and simple to perform.

As mentioned before, *P. falciparum*, cannot synthesize purine nucleus *de novo* and exclusively relies on the activity of the salvage pathway for its purine requirements. This could be a potential target for drug action. The main rationale of applying such an approach for understanding the salvage pathway is that the key enzyme HPRT has been studied extensively in several organisms. The cDNA of the *P. falciparum* HPRT has been cloned and sequenced (King and Melton 1987). Comparison of the predicted amino acid sequence with that of the host revealed several dissimilarities between them.

In the present work described in this thesis, hypoxanthine guanine phosphoribosyl transferase of *P. falciparum*, denoted PfHPRT has been chosen for study at the molecular level. To place the PfHPRT in context, I will review

the essential features of the nucleic acid metabolism in *Plasmodia* in detail, concentrating on *P. falciparum*. This will in turn lead to a brief review of purine metabolism in plasmodia and then PfHPRT. I will also discuss purine metabolism in both man and mosquito, hosts of human malaria parasites.

1.9 Nucleic acid metabolism in Plasmodia.

DNA

Each intraerythrocytic merozoite in a schizont is haploid, contains about 0.1 pg DNA and has a chromosome number of 14 (Wellems, 1988). Kinetic study of *P. falciparum* DNA synthesis, where the erythrocytic cycle is 48 hours, suggested that in the asexual intraerythrocytic stage DNA synthesis starts at 29.5 hours, at a time when the parasite is in the early trophozoite stage. DNA synthesis increases logarithmically until 44 to 48 hours, the end of the schizogony (Inselberg & Banyal 1984). Waki *et al* (1985) also suggested similar timing. Similar stage specificity for DNA synthesis was reported by Newbold *et al* (1982) for *P. chabaudi*, where the cycle is only 24 hours. These authors observed DNA synthesis at the early trophozoite stage and very little synthesis was noted during the first half of the cycle with the maximum rate of synthesis at late trophozoite and schizont stages. In *P. falciparum* it is reported that during this period the DNA content increases about 10 to 20 times (Sinden 1978). This tremendous amount of DNA synthesis at certain time points in the parasite's life suggest that a huge amount of DNA precursor molecules are needed and raises the question about the source of such precursors.

The genome size of *P. falciparum* has been estimated to be 3.8×10^8 base pairs and 10% of the genome to contain repetitive sequences (Hough-evans and Howard 1982). Dore *et al* (1980), reported the *P. berghei* genome size of 2×10^7 bp with a variable repetitive frequency between 3 to 18%. By comparison,

the human genome consists of 3 x 10^9 base pairs with 25% repetitive sequence (Gaubatz *et al.*, 1976) and that of *E. coli* is 4 x 10^6 base pairs.

One unique character of the Plasmodia genome is its very high A+T content. The G+C content of *P. falciparum* genome as estimated by Goman *et al* (1982) is 18–20%. Similar numbers have also been reported for *P. lophurae* (Walsh and Sherman, 1968b). The G+C content varies somewhat between different species of *Plasmodia*: for instance, in simian malaria parasites, the the G+C content is found to be 37% (Sherman, 1979), whereas in rodent malaria it is 24%.

RNA

In comparison with DNA content of the intraerythrocytic malaria parasite, the total RNA content is 5 times greater. Most of this is ribosomal RNA present in the metabolically highly active cytoplasm (Gutteridge and Trigg 1970). The sedimentation value of plasmodial rRNA was found to be about 25S and about 17S, typical for prokaryotes (Sherman *et al.*, 1975; Tokuyasu *et al.*, 1969). For comparison the values for other eukaryotes are 28S and 18S (Sherman and Jones 1977). Nucleotide composition of rRNA also supported this result. The G+C content of Plasmodial rRNA is about 35% (Sherman 1979) whereas that of eukaryotes is about 65%.

The organisation and copy number of plasmodial ribosomal genes are also novel among eukaryotes. Plasmodial rRNA genes are small in number and dispersed in the genome. In contrast the genes in most other eukaryotes are high in number (100 or more) and tandemly repeated in the genome. Langsley *et al* (1984) cloned most of the genes of both subunits and showed that two genomic clones both contained all three ribosomal genes, but from two different transcriptional units. The ribosomal RNA genes are organised in a typical eukaryotic pattern of 18S–5.8S–28S. Copy number analysis of *P. falciparum* rRNA genes revealed that each of the 2 ribosomal transcriptional

units is present four times in the genome i.e. there are only 8 rRNA genes in the genome (Langsley 1984). They also suggested that the large rRNA subunit gene contained one intron. The pattern of introns in ribosomal genes are another interesting feature of the malaria parasite ribosomal DNA structure. The 25S ribosomal gene has two introns and the 17S gene has one (Unnasch and Wirth 1983). Presence ofthe intron in the 17S gene is unique to Plasmodia, no other eukaryote or prokaryote is known to contain an intron in its 17S rRNA gene.

P. falciparum mRNA has been extracted from blood stage *in vitro* cultured parasites and translated (Hyde *et al* 1984, Wallach *et al.*, 1982a and 1982b). The translation efficiency of *P. falciparum* mRNA in *in vitro* with commercial reticulocyte lysate has been assessed. Wallach *et al* (1982a) observed that the efficiency is markedly increased if supplemented with homologous tRNA. They were able to translate proteins up to Mr 200000 Da. However other workers have successfully translated *P. falciparum* mRNA without the above modification (Hyde *et al.*, 1984; Coppel 1984). Franco da Silvera and Mercereau-Puijalon (1983) isolated mRNA from different stages of erythrocytic parasite. By *in vitro* translation of this mRNA they demonstrated a stage-specific pattern of protein expression as also seen *in vivo*. Hyde *et al* (1984) also produced similar results and found proteins ranging from 16 to 230 kDa proteins which were synthesized consistently, whereas some high molecular mass proteins were only expressed in the schizont stages. These authors also measured the amount of mRNA present in different stages of intraerythrocytic development of *P. falciparum*. They reported about 4-6% of total RNA is mRNA, and 82% rRNA and about 14% is tRNA. The average size of poly A^+ RNA was 1.2 kb with a modal value of about 3.5 kb. The average size of the poly A^+ tail was estimated at approximately 120 to 200 bp.

1.10 Nucleic acid precursor metabolism.

As mentioned above, the intraerythrocytic stages of *Plasmodia* possess high rates of DNA and RNA synthesis. For these purposes they require a vast amount of precursor molecules. The host erythrocytes are capable of providing enough essential precursor molecules to allow the successful growth and multiplication of the parasite within the cell. Mammalian erythrocytes can synthesize nucleotides, although they do not synthesize any nucleic acids. However, the synthesis of purine rings does not take place in erythrocytes, rather they are obtained in the plasma from the liver or other organs and subsequentlyutilised via the salvage pathway.

It was first shown in 1967 that *P. berghei*-infected erythrocytes do not incorporate tritiated uridine and thymidine into parasite nucleic acid (Sherman 1979). Similar attempts to incorporate uridine into plasmodial RNA also failed (Conklin *et al.*, 1973). Later, it was discovered that the parasite can incorporate radioactive bicarbonate and furthermore that they contain several enzymes of pyrimidine biosynthesis (Walsh & Sherman 1968b). Since the discovery of *de novo* pyrimidine biosynthesis, all six enzymes of the *de novo* pathway have been detected in several *Plasmodia* species, which catalyse the conversion of carbamyl phosphate to thymidylate (Hill *et al.*, 1981; O'Sullivan & Ketley 1980). Figure 1.5 shows the *de novo* pyrimidine biosynthesis pathway in *Plasmodia*.

1.10.1 Metabolism of purine nucleotides.

Biosynthesis.

All living organisms which can synthesise purine nucleotides *de novo* follow the same basic pathway (Lehninger, 1982). The detail of the pathway is shown in Figure 1.6. The synthesis begins with the donation of an amino group by glutamine to position one of ribose 5'-phosphate and the purine is then built on it, step by step. The ring closure occurs to yield the

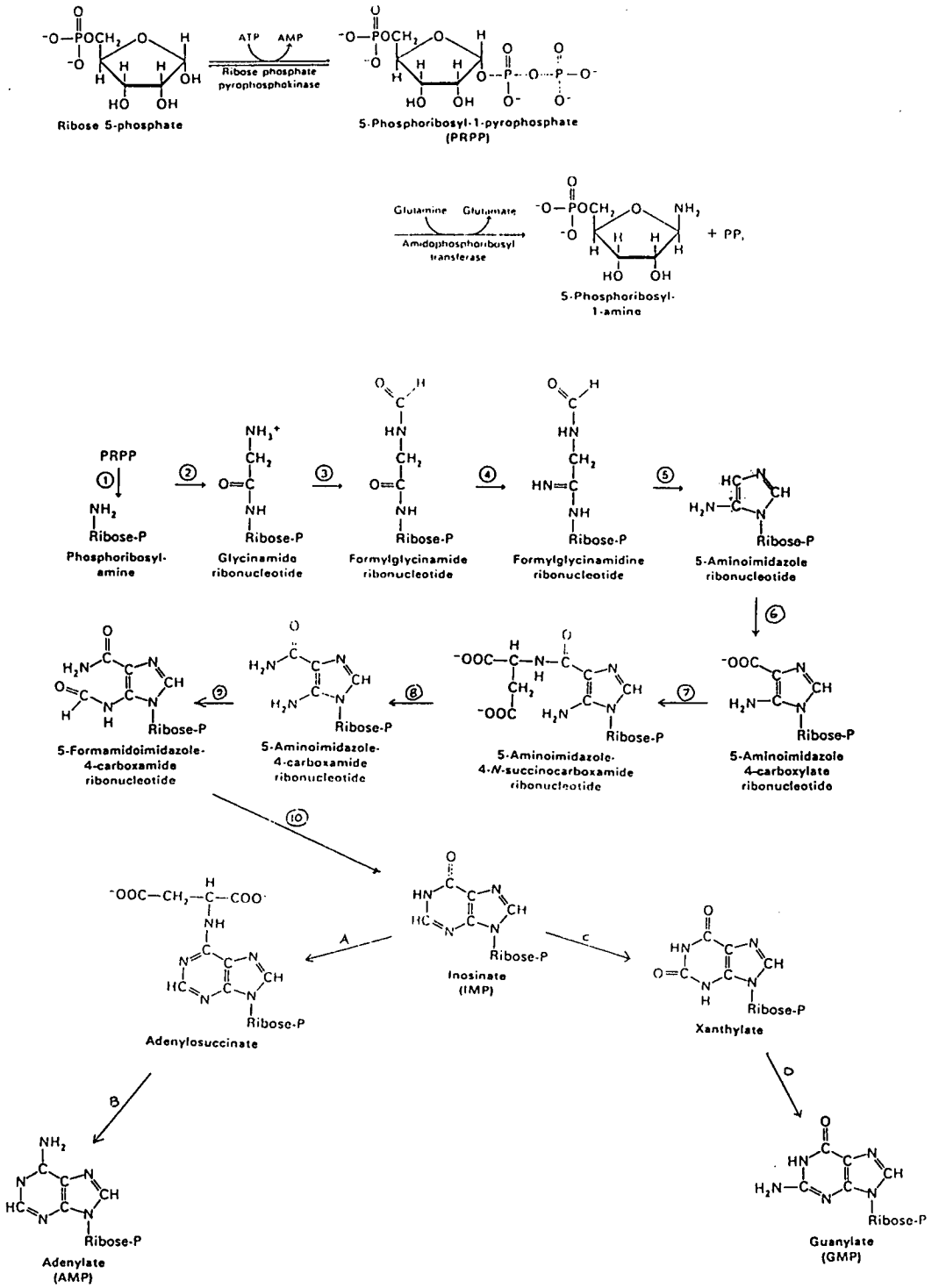


Figure 1.6 The *de novo* purine biosynthesis pathway. (1) displacement of PPi by the side chain amino group of glutamine, (2) addition of glycine, (3) formylation by N^{10}-formyl tetrahydrofolate, (4) transfer of a nitrogen atom from glutamine, (5) dehydration and ring closure, (6) carboxylation, (7) addition of aspartate, (8) elimination of fumarate, (9) formylation by N^{10}-formyltetrahydrofolate, (10) dehydration and ring closure, (A) Adenylosuccinate synthase, (B) Adenylosuccinate lyase, (C) IMP dehydrogenase and (D) GMP synthase.

five-membered imidazole ring. Aspartate donates its amino group to the imidazole ring and a second ring closure causes the fusion of the two rings of the purine nucleus. Thus the first intermediate of the purine nucleotide, IMP is formed. AMP and GMP are synthesised from IMP via separate branches of the pathway, which in turn convert to ATP and GTP by phosphorylation. AMP is formed by two step addition of an amino group, donated by aspartate, catalysed by adenylosuccinate synthase and adenylosuccinate lyase. In *E. coli* these two enzymes are coded by *purA* and *purB* genes. Similarly formation of GMP from IMP is catalysed by IMP dehydrogenase and GMP synthase which are coded by *guaA* and *guaB* respectively in *E. coli*.

Catabolism.

Similar to the biosynthesis, the degradative pathway of purine is also basically the same in all living organisms. The pathway is shown in Figure 1.7. Degradation starts by the action of 5'-nucleotidase on the nulceotides. AMP yields adenosine, which is then deaminated to inosine by adenosine deaminase. Inosine is then hydrolysed into hypoxanthine by nucleosidase and then xanthine and uric acid by one enzyme, xanthine oxidase. GMP is first converted into guanosine by nucleosidase and then to xanthine by guanosine deaminase, which in turn produces uric acid. In primates uric acid is the excretory end product of purine catabolism. But in some animals they further degrade into allantoin, allantoic acid and urea.

Purine salvage pathway.

In all living systems free purine bases are constantly formed in cells during the metabolic degradation of nucleotides by the pathway described above. However, most of these free purine bases are salvaged and used over again to remake nucleotides and only a small fraction are catabolised and excreted. For instance, the human body excretes only 0.6 g uric acid per day (Lehninger 1982).

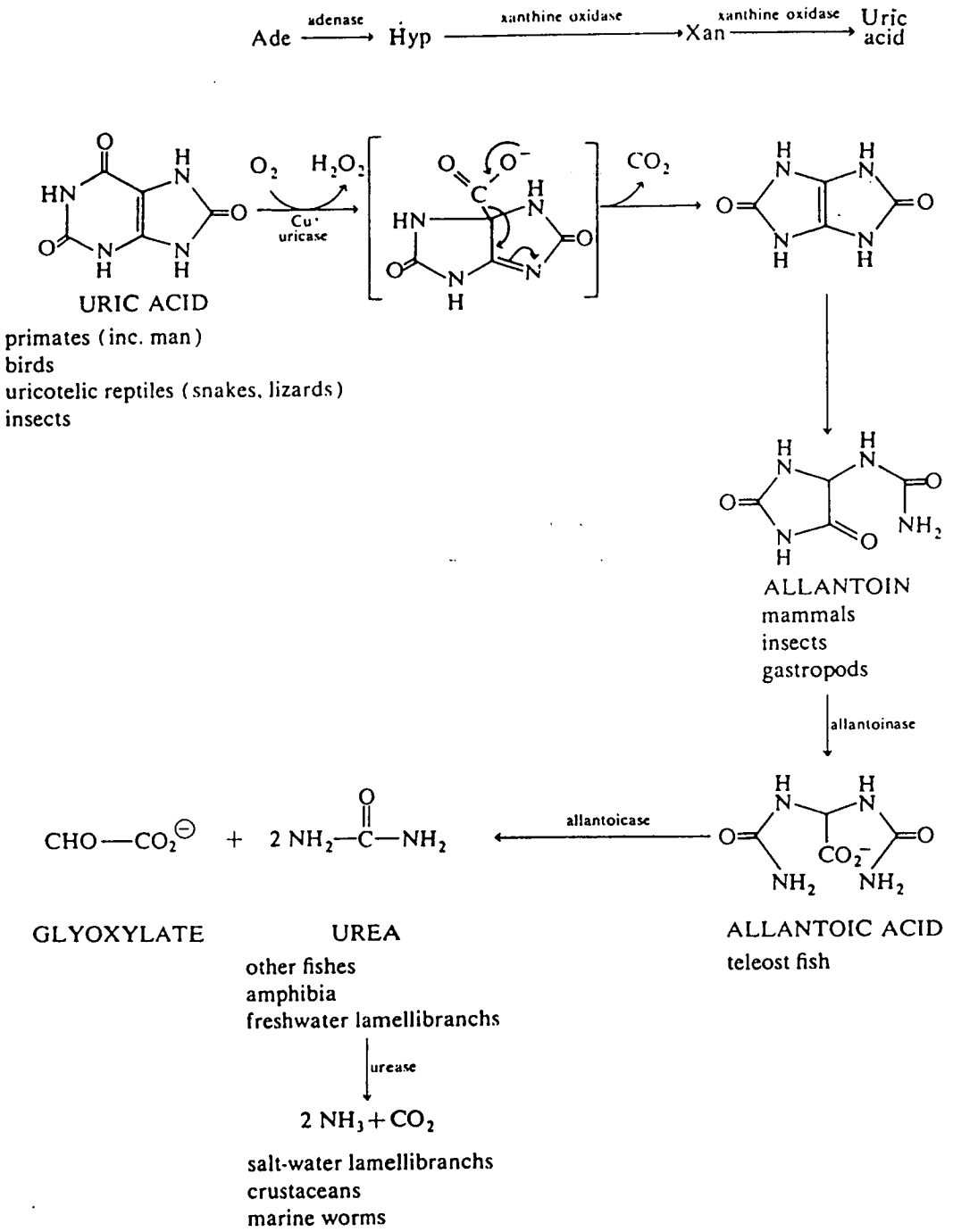


Figure 1.7 Purine degradation pathway. Those organisms excreting each of the various catabolites as end products are indicated.

Compared to the complex biosynthesis of purine by the *de novo* pathway, the salvage pathway is simple. Basically, only one reaction is involved. AMP is produced from adenine by enzyme adenine phosphoribosyl transferase and GMP from guanine by guanine phosphoribosyltransferase (GPRT). In several organisms, including human, GPRT also catalyses the conversion of hypoxanthine to inosine, hence called hypoxanthine-guanine phosphoribosyl transferase (HGPRT). Besides this, most living cells can also catalyse the interconversion of purine bases via IMP, as the common nucleotide. Figure 1.8 shows the details of purine salvage and the interconversion of the free bases and nucleosides and nucleotides.

1.11 Purine metabolism in Plasmodia.

1.11.1 De novo synthesis.

Unlike the thymidine and uridine, when tritiated hypoxanthine or adenosine were injected into the *P. berghei*-infected mice or rats they were readily incorporated into the parasite nucleic acids (Sherman 1979). Similar results were obtained with *P. knowlesi* infected erythrocytes (Gutteridge and Trigg 1970). Tracy and Sherman (1972) showed with *P. lophurae* that the uptake and accumulation of adenosine, inosine and hypoxanthine is directly related to the growth stage of the parasite . The explanation for these results became clear when Walsh & Sherman (1968) showed that the parasite can not incorporate labeled glycine and formate into DNA or RNA, suggesting that *Plasmodia* are incapable of synthesizing the purine ring *de novo*. It was therefore, concluded that *Plasmodia* obtain purine nucleotides necessary for their nucleic acid synthesis or for other metabolic purposes, in intact form through salvage pathways.

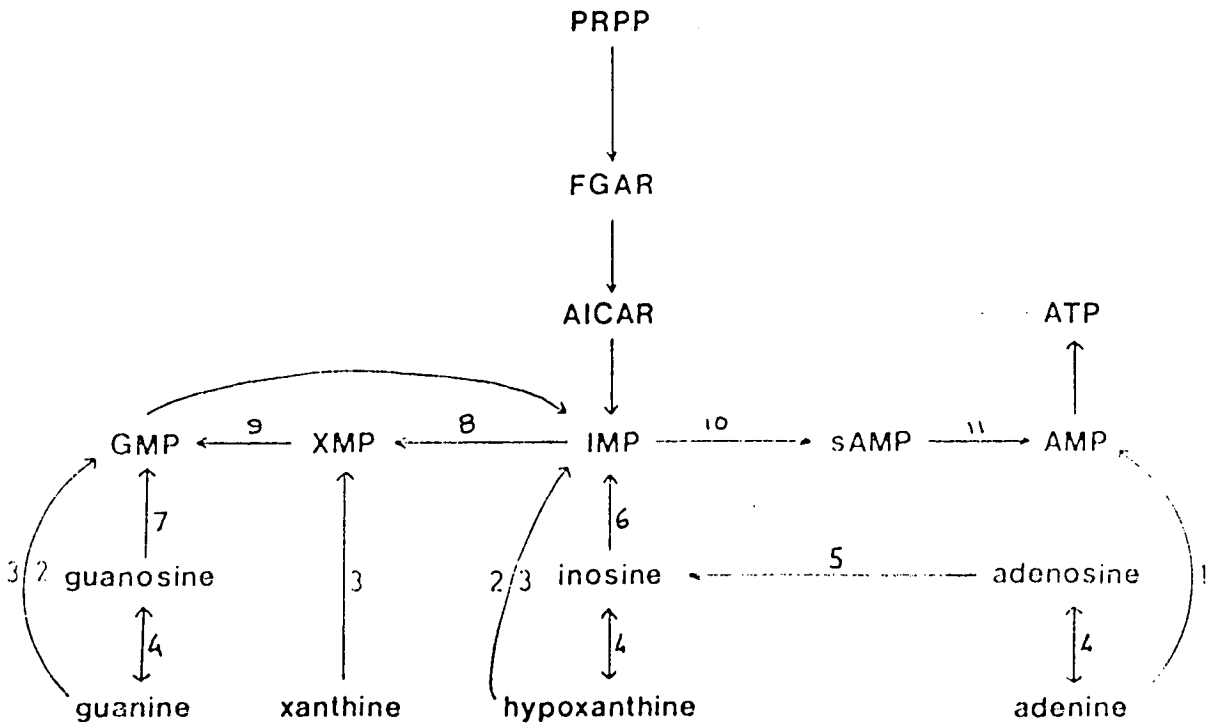


Figure 1.8 Purine salvage pathway. (1) Adenine phosphoribosyl transferase (APRT), (2) hypoxanthine phosphoribosyl transferase (HPRT), (3) guanine phosphoribosyl transferase (GPRT), (4) purine nucleoside phosphorylase (PNP), (5) adenosine deaminase (ADA), (6) inosine kinase, (7) guanosine kinase, (8) IMP dehydrogenase (IMPD), (9) GMP synthetase, (10) adenylosuccinate synthase (ASS) and (11) adenylosuccinate lyase.

1.11.2 Salvage pathway.

Now, if *Plasmodia* completely rely on the salvage of preformed purines, what is the favoured purine which is utilised *in vivo* by the parasite ? Approximately 80% of the erythrocytic purine is in the form of ATP, and probably this ATP serves as the source of purines for the parasite. Trager (1950) reported that addition of ATP enhances the survival of extracellular *P. lophurae*. Later, it was shown that purines from radioactive ATP or AMP are incorporated into saponin freed *P. berghei* (Van Dykes *et al.*, 1969). These authors concluded adenosine was the substrate of choice for Plasmodial purine requirement.

These studies were further elaborated by Manandhar *et al.*, (1975), who suggested that adenosine itself does not enter into the salvage pathway. he proposed that adenosine is first converted to hypoxanthine in the intracellular medium and this hypoxanthine serves as the initial entry compound to the salvage pathway. This suggestion was supported by the fact that Patterson's compound 555, an adenosine transport blocker did not have any effect on the utilisation of adenosine. On the other hand, both uptake and phosphoribosylation of hypoxanthine was completely blocked by purine 6-sulfonic acid-3N-oxide. Tracy and Sherman (1972) also speculated that the added adenosine is probably deaminated to inosine during uptake.

A similar prime role of hypoxanthine in *Plasmodium* purine salvage was confirmed not only for *P. berghei*, but also for *P. chabaudi* and *P. lophurae* (Sherman 1979; Hansen *et al.*, 1980) and for *Leishmania donovani* and *Leishmania braziliensis* (Marr *et al.*, 1978) and *Crithidia fasciculata* (Kidder et al., 1979)

The present knowledge of purine metabolism in *Plasmodia* is primarily based on the enzymatic data as described for the cell free extracts of different

species of this parasite (Konigk, 1977). Reyes *et al.*, (1982) described the presence of purine salvage enzymes in *P. falciparum*. In the following sections, the basic characteristics of the purine salvage pathway in this parasite is discussed in detail.

Adenine, adenosine and AMP reutilisation.

Studies with *P. chabaudi* revealed that the adenine and adenosine can be used by three different routes as shown in Figure 1.9 (Walter & Konigk, 1974). Conversion of adenine to AMP is catalysed by the adenine phosphoribosyl transferase (APRT)(Fig. 1.8). This enzyme has a very low specific activity in *P. falciparum* (Reyes *et al.*, 1982). Such a low level of APRT activity is also reported in *P. chabaudi* (Walter and Konigk 1974). Similarly the specific activity of the enzyme adenosine kinase which converts adenosine to AMP was also found to be remarkably low in *P. falciparum* (Reyes *et al.*, 1982). The authors concluded that these two pathways of adenine nucleotide utilisation are of minor importance in the parasite's life.

In contrast, the activity of adenosine deaminase (ADA), which catalyses the conversion of adenosine to inosine was found to be very high. The specific activity of ADA in *P. falciparum* cell free extract was about 4000 times higher than that of adenosine kinase and about 1000 times higher than APRT (Reyes *et al.*, 1982). So, it was suggested that the enzyme ADA may play an important role in plasmodial purine metabolism.

The small effect of Patterson's compound 555, an inhibitor of adenosine transport blocker, on incorporation of adenine nucleotides into the parasite nucleic acid, indicated that most of the adenosine may be converted into inosine before entry into the parasites, perhaps by the host cell enzyme (Sherman 1979). Human red blood cells have been shown to contain comparatively high amounts of ADA (Reyes *et al.*, 1982). This made the enzyme less suitable as a target for an antimalarial. Moreover, comparison of biochemical parameters,

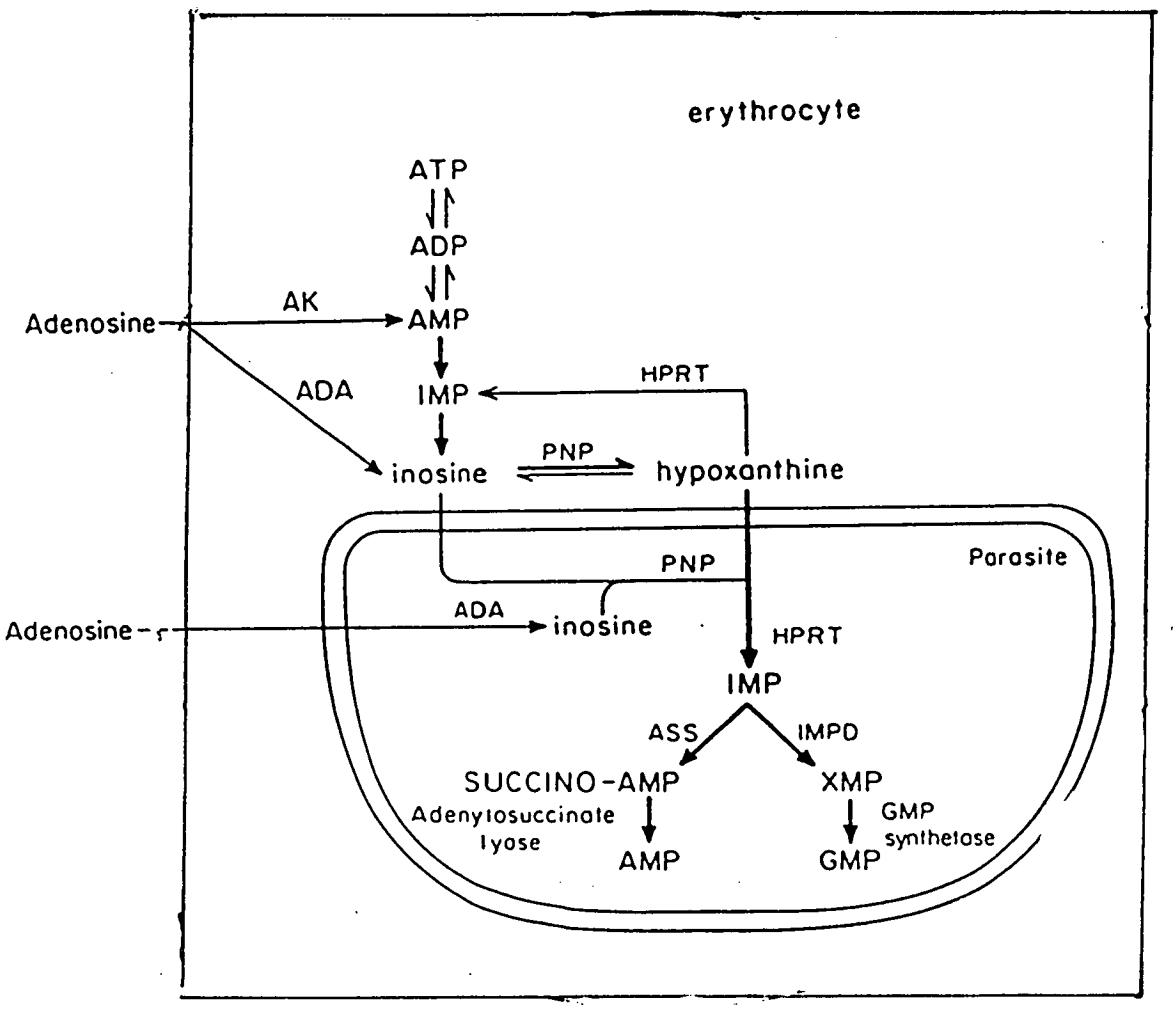


Figure 1.9 The purine salvage pathway in the intraerythrocytic asexual stage parasite. For enzyme abbreviations see Figure 1.8.

substrate specificities and sensitivities to different inhibitors of purified adenine deaminase from different plasmodial and human sources have suggested that the enzymes from the parasite and human are indistinguishable for all the purine analogues surveyed. For example, Schimandle & Sherman (1983), studied purified enzyme from *P. lophurae* and showed that most of its physical properties are similar to the red cell enzyme. However, they reported that the parasite enzyme is unique in its lack of sensitivity to the inhibitor, EHNA (Erythro-9-2-hydroxy-3-nonyl adenine). A similar pattern of insensitivity to EHNA was also reported for *P. falciparum* ADA (Daddona *et al.*, 1984).

AMP can be converted into inosine via two routes. Firstly, by AMP phosphorylase, AMP can be transformed into adenosine, which in turn forms inosine, catalysed by ADA. On the other hand, AMP can directly form IMPcatalysed by AMP-deaminase, which is then dephosphorylated to inosine. Enzymes for both routes are present in *Plasmodia* (Scheibel & Sherman 1988).

Utilisation of inosine and guanosine.

Both *P. falciparum* and human red blood cells contain purine nucleoside phosphorylase (PNP) for inosine and guanosine utilisation (Reyes, et al., 1982). The enzyme catalyses the conversion of nucleosides into corresponding nucleotides; for example, inosine into hypoxanthine. The enzyme is also described in *P. chabaudi* (Konigk 1977). High activities of both ADA and PNP in host red cells and in the parasite suggested that adenosine is readily converted into inosine (both in red cell and parasite cytoplasm). This inosine is in turn converted into hypoxanthine, which is the primary substrate of the purine salvage pathway.

Similarly guanosine is converted into guanine. Since guanosine is not readily taken up by the parasite (Tracy & Sherman, 1972), this route of purine utilisation may be of less importance. That no activity for adenosine and xanthine phosphorylase were detected in *P. falciparum* led to the suggestion

that these steps may not play any important role in the parasite's purine metabolism (Reyes *et al.*, 1982).

PNP from *P. lophurae* has been purified by Schimandle *et al.*, (1985). Their studies in comparing the human enzyme with that of the parasite, revealed that the parasite enzyme is unique in several aspects.

Utilisation of Hypoxanthine and guanine.

Purine nucleotide uptake studies with 'saponin-freed' *P. lophurae* showed that only hypoxanthine, adenosine, inosine and guanine are readily taken up by the parasite. In contrast guanosine, xanthine, adenine, AMP, ATP and IMP uptake was very low (Tracy and Sherman, 1972). Since the guanine concentration in red cell and plasma was found to be very low and adenine and inosine readily converts into hypoxanthine, it has been suggested that hypoxanthine is the most favourable purine for use by *Plasmodia*. Indeed, the hypoxanthine utilising enzyme hypoxanthine phosphorybosyl transferase (HPRT) is shown to be present in *P. falciparum* in high activity. HPRT catalyses the phosphoribosyl group transfer between hypoxanthine and phosphoribosyl pyrophosphate (PRPP), producing inosine monophosphate (IMP) from hypoxanthine.

HPRT activity in *P. falciparum* extract was some 160 times more than that of the APRT (Reyes *et al.*, 1982). HPRT has been purified from *P. lophurae* (Schimandle *et al.*, 1987), *P. chabaudi* (Walter and Konigk 1974) and *P. falciparum* (Queen *et al.*, 1988) and shown to be unique in several aspect from the host enzyme (see below). Through these differences, the enzyme could be a potential target for drug design against malaria. Since the enzyme plays a key role inthe parasite's life and the primary aim of this thesis is to study the *P. falciparum* HPRT (denoted PfHPRT) at the molecular level, I will discuss the properties of this enzyme in detail, later.

IMP is converted to both AMP and GMP by the *de novo* pathway. By one

route, IMP is first converted into adenylosuccinate by adenylosuccinate synthetase which is converted to AMP by adenylosuccinate lyase. Similarly, the other route produces GMP from IMP via XMP in an enzyme catalysed-reaction. The enzyme IMP dehydrogenase, catalysing XMP synthesis has been identified in *P. lophurae*. It is predicted that both adenylosuccinate lyase and GMP sythetase may be also present in the parasite (Scheibel and Sherman, 1988). Both the routes are shown in figure 1.6.

1.12 Purine metabolism in the parasite's host.

Since the malaria parasite depends entirely on both the vertebrate and insect hosts for preformed purine nucleotides the story will remain incomplete without discussing the purine metabolism in the hosts. Any alteration of purine metabolism in these hosts may interfere with the parasite metabolism. For example, addition of allopurinol, an inhibitor of xanthine oxidase in the drinking water of rat mice enhanced the severity of the malarial infection (Sherman, 1979). It was suggested that may be the inhibition of xanthine oxidase increased the concentration of hypoxanthine, the most favoured purine of the parasite.

Mammals including man can satisfy their requirement for purine nucleotides by both *de novo* biosynthesis and salvage pathway as described above.

However, in addition to its usual role purine metabolism serves quite an unusual purpose in terrestrial insects, including mosquito. The work of McEnroe and Forgash (1958), revealed that insects can synthesize purines by *de novo* biosynthesis. They showed that labelled formate is readily incorporated into purines. These purines are formed by a long series of reactions which involve the addition of ammonia, glycine, formate and aspartate to a

phosphorylated derivative of ribose. The pathway is possibly the same as in other animals as shown above.

The absence of functional HGPRT in insects (reviewed in Swerdel & Fallon 1987), suggested that the salvage pathway is not the primary pathway by which these organisms obtain their purines. However, the enzymes XPRT and APRT (Backer, 1974) were detected in insect cells.

The main function of purine metabolism in insects appears to be nitrogen excretion. Excretion of nitrogen in the form of uric acid is characteristic in most terestrial insects and purine biosynthesis in these organisms is very high (Gilmour 1965). In most animals however,the end product of nitrogen excretion is urea. It is suggested that since the solubility of uric acid is low, It can be excreted in solid form, thus, reducing the loss of water from the insect body. Several enzymes which catalyse the interconversion of purines have been detected in insects. These include deaminases, which convert adenine to hypoxanthine and guanine to xanthine (Figure 1.10) and also the enzyme xanthine oxidase, which oxidises hypoxanthine to xanthine and xanthine to uric acids. It appears therefore, that the purines formed are readily converted into uric acid. Xanthine oxidase is probably the crucial enzyme in the formation of uric acids.

1.13 Hypoxanthine phosphoribosyltranferase (HPRT, EC 2.7.2.8)

There is wealth of information on the study of HPRT from a wide range of organisms. However, most studies focus on the human enzyme. The reason is that deficiency in this enzyme causes varying degrees of clinical manifestation in humans. A partial deficiency in HPRT activity causes excessive accumulation of uric acid in the body which normally concentrates in the joints and is

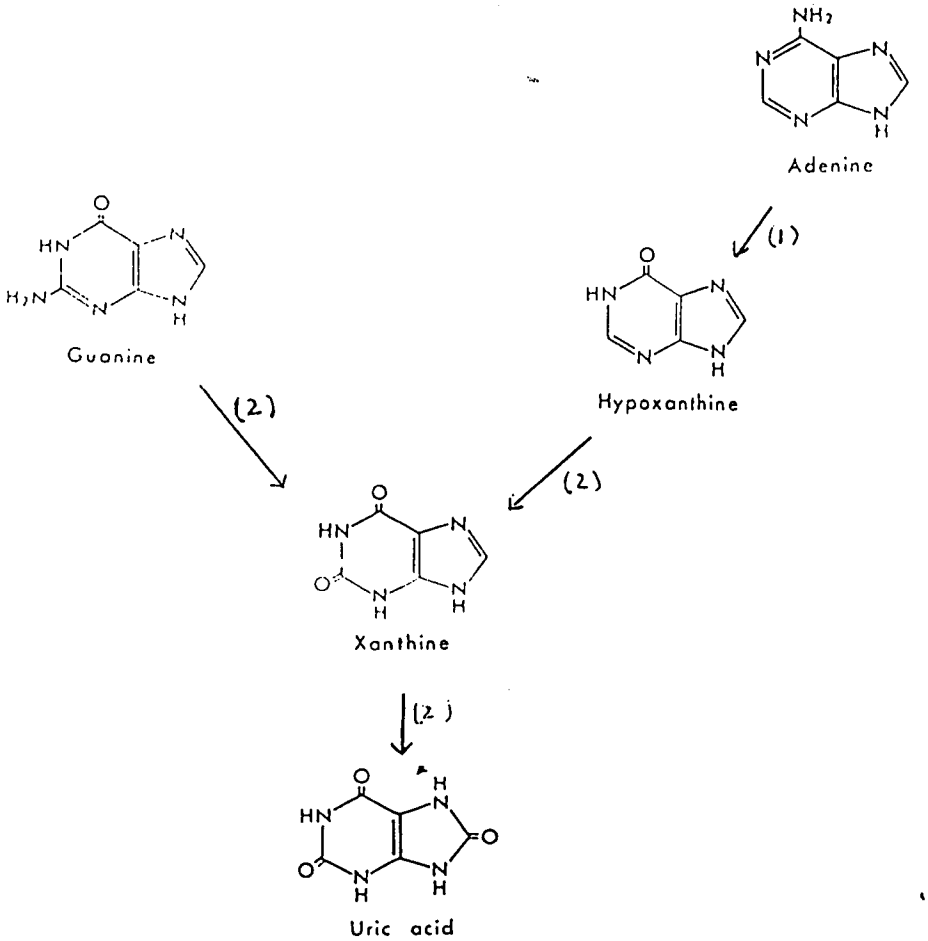
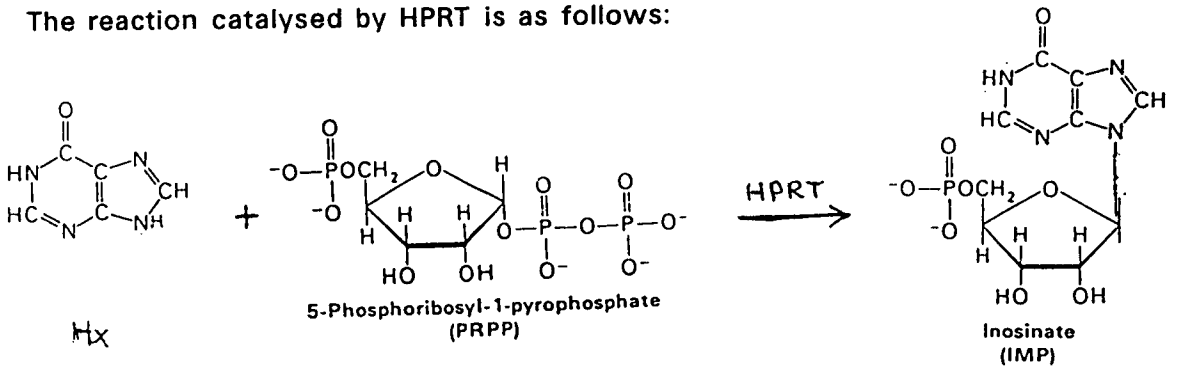


Figure 1.10 Purine metabolism in insect. (1) adenase, (2) xanthine oxidase.

responsible for severe gout. Complete loss of activity causes severe central nervous system disorder, named Lesch-Nyhan syndrome. Moreover, extensive study made this enzyme a widely used marker for gene transfer in the mammalian system and in the study of somatic cell genetics (Melton 1987). The reaction catalysed by HPRT is as follows:



Hx

The primary substrate of the enzyme is hypoxanthine. However, the enzyme can use other purines with different specificities as its substrates. Table 1.2 shows the Km values of HPRTs from different sources for different purine substrates. Krenitsky *et al.*, (1969) showed that the human HPRT can use both hypoxanthine and guanine with similar specificities, whereas the affinity towards adenine and xanthine is about 2500 to 2000 times less, suggesting that these are not the substrate for the enzyme. This is emphasized by the fact that human cells contain a separate enzyme for adenine, named APRT. Human HPRT can also use 6-mercaptopurine with a similar efficiency to hypoxanthine and guanine. HPRTs from parasitic protozoa like *P. lophurae* (Schimandle *et al.*, 1987), *P. chabaudi* (Walter and Konigk, 1974) and *Leishmania donovani* (Tuttle and krenitsky, 1980) also show similar substrate specificity. However, some parasitic HPRTs can use xanthine with higher affinities. For example *P. falciparum* (Queen *et al.*, 1988), *Eimeria tenella* (Wang and Simashkevich, 1981) and *Giardia lamblia* (Aldritt and Wang, 1986). *P. falciparum* HPRT is unique in *Plasmodium* species investigated so far in that it can use xanthine. On the other hand *Crithidia fasciculata*, a kinetoplastid flagellate, does not have

Source	Purine	Km	Reference
P. falciparum	Hx G X	0.46 0.30 29.0	Queen et al. 1988
Human erythrocyte	Hx G X	2-25 2-4 ---	Queen et al 1988
P. chabaudi	Hx G X	2.5 2.0	Walter & Konigk, 1974
P. lophurae	Hx G X	3.8 2.4 ---	Schimandle et al., 1987
L. donovani	Hx G X	2.0 1.0 ---	Allen et al 1989
E. tenella	Hx G X	0.25 0.37 15.0	Wang et al. 1981
G. lamblia	Hx	514.0 30.0 543.0	Aldritt et al.,1986

Table 1.2 Km values of different purines to HPRT of different organisms. Hx = hypoxanthine, G = guanine and X = xanthine.

any xanthine-utilising activity and has two separate enzymes for hypoxanthine and guanine (Kidder & Nolan 1979). *Leishmania donovani* has a separate enzyme for xanthine utilisation (Tuttle & Krenitsky, 1980). In contrast to the eukaryotic HPRTs, most prokaryote enzymes can only use hypoxanthine. *E. coli* has separate enzymes: GXPRT which can use guanine and xanthine and HPRT which predominantly use hypoxanthine as substrate (krenitsky *et al.*, 1970). However, gram positive bacterium *Lactobacillus casei* HPRT can use hypoxanthine and guanine, as observed in eukaryotes and has separate phosphoribosyl tranferases for xanthine and adenine (Krenitsky *et al.*, 1970).

HPRT requires Phosphoribosyl pyrophosphate (PRPP) as its second substrate. The phosphoribosyl moiety of the PRPP is transferred into the 9 position of hypoxanthine. The affinity of HPRT towards PRPP also varies according to its source and also slightly with the purine used (Krenitsky *et al.*, 1970; Queen *et al.*, 1988). The Km for *P. falciparum* HPRT in presence of hypoxanthine is reported to be about 20.7 µM (Queen *et al.*, 1988).

The enzyme also needs magnesium ions for its activity. Krenitsky *et al.*, (1969) studied the effect of the metal ion on the activity of purified human enzyme. From kinetic studies they found that the dimagnesium salt of PRPP is a better substrate for the enzyme than the monomagnesium salt or the free acid. They suggested that magnesium may effect the initial rate of the reaction by forming a complex with the substrate. Addition of extra salt was found to be stimulatory up to a certain extent. However beyond, the optimum concentration the magnesium ion inhibited the reaction drastically. The optimum $MgSO_4$ concentration they reported was in the range of 5 to 20 mM, when the Mg_2PRPP concentrations were between 1mM and 5 mM.

HPRT has been purified from several organisms. The protein in human erythrocytes naturally exists as tetramer with a relative molecular mass (Mr), as

estimated by sedimentation equilibrium centrifugation, of 81-83 kD (Olsen & Milman 1977), and a predicted subunit size of 24.5 kD (King & Melton 1987). Strauss *et al.*, (1978) showed the enzyme can also exist as dimer or trimer depending on the buffer and PRPP concentration. In contrast *P. falciparum* enzyme is independent of the PRPP concentration and present as a dimer, with an apparent Mr of 56 kD (Queen *et al.*, 1988). The predicted Mr of the *P. falciparum* enzyme is 26.4 kD (King & Melton 1987). The estimated size of HPRT in different organisms appears to be different. For example, the HPRT proteins in *P. chabaudi* and *P. lophurae* are 71 and 79 kD respectively (Walter & Konigk, 1974; Schimandle *et al.*, 1987) and that of the *Giardia lablia* is 63 kD (Aldritt and Wang 1986). The protein in *E. coli* and *L. casei* is reported to be 75 kD and 44kD respectively, as estimated by gel exclusion chromatography (Krenitsky *et al.*, 1970).

The HPRTs of different sources also differ in pI values which are indicative of the total charge content of the protein. Whereas human red blood cell HPRT shows three different peaks at pI values of at 5.6, 5.7 and 5.9 (Olsen & Milman 1977), *P. falciparum* HPRT showed only one peak at about 6.2. The pI value of *P. lophurae* (Schimandle *et al.*, 1987), *P. chabaudi* (Walter & Konigk 1974) and *Giardia lamblia* (Aldritt & Wang 1986) are 5.3, 7.6 and 6.8 respectively.

P. falciparum HPRT is most sensitive to heat at 50°C. At this temparature about 60% of the initial activity was lost within 30 seconds. However, the activity could be stabilised if preincubated with PRPP (Queen *et al.*, 1988). Under these conditions more than 90% of the initial activity remained after 30 minutes incubation. A similar stabilisation of activity was also observed with human (Olsen & Milman 1974) and rat liver (Natsumeda *et al.*, 1977) HPRTs. Krenitsky *et al.*, (1970) reported that HPRT from *Lactobacillus casei* is much less sensitive to heat inactivation. They showed that the enzyme retained about

70% activity when heated at 60°C for 10 minutes. In contrast, the same authors reported that the *E. coli* enzyme is comparatively more sensitive; it retains only 20% of the initial activity at the same condition.

A study of the effect of chemical modification of human HPRT in order to identify essential amino acid(s) present in the active site, was reported by Krenitsky [illegible] (1969). The authors showed that the activity was not affected by the serine modifier chemical, diisopropyl fluorophosphate, but was efficiently inhibited by the sulfhydryl reactants p-chloromercuribenzoate and N-ethylmaleimide. This inhibition does not occur when the enzyme is preincubated with PRPP. Moreover, when the enzyme was incubated with sulfhydryl reagents like 2-mercaptoethanol and dithiothreitol the activity was found to be increased. Queen *et al.*, (1988) reported the result of similar experiments with the *P. falciparum* enzyme. They reported a similar efficient inhibition of the *P. falciparum* HPRT with p-chloromercuribenzoate. Wilson *et al* ., (1982) sequenced the human HPRT and showed that the N-terminal methionine is removed by processing and the existing N-terminal alanine is acylated. No such information is known about *Plasmodial* HPRT.

Finally, HPRT is a cytosolic soluble enzyme. The enzyme from human red blood cells is found in the cytoplasm as a soluble protein (Melton, 1987). Reyes *et al.*, (1982) reported that the *P. falciparum* enzyme is present in the soluble fraction of the sonicated supernatant. In contrast, recent studies revealed that the enzyme in *Trypanosoma brucei* and *Trypanosoma cruzi* is present in some membrane bound vesicles, termed glycosomes (Opperdoes, 1987). Glycosomes are specialised vesicles, related to eukaryotic peroxysomes and glyoxysomes and contain most of the glycolytic enzymes (Opperdoes, 1987). A similar location has also been reported for the HPRT of *Leishmania mexicana* (Hassan *et al.*, 1985).

1.14 The project proposal.

The above discussions and literature survey have reflected *P. falciparum* as one of the most dangerous killer parasitic disease in the world. The failure to develop a successful vaccine against the disease and the rapid emergence of resistant parasite strains against the existing chemoprophylactic drugs emphasized the importance of the continuing work on new drug development.

Study on the mechanisms of action of existing drugs and the mechanism of resistance have revealed particular biochemical pathways as the possible targets for effective drug design. One such pathway is purine metabolism in the parasite because the parasite cannot synthesise purine rings by the *de novo* pathway and solely depends on the host's supply of preformed purines. These preformed purines are utilised by the so-called purine salvage pathway. The enzyme HPRT is one of the key enzymes in the pathway, and so plays an important role in the parasite's life. Comparison of HPRT of *P. falciparum* with other organisms, particularly with that of the human host, showed that the parasite enzyme is different in several biochemical aspects. This makes the enzyme a putative target for antimalarial drugs.

However, the problem of isolating large amounts of the native parasite protein from the cultured parasite by conventional methods, and the unusual instability of the parasite enzyme made it difficult to study the parasite enzyme at the molecular level, which is the prime requirement for drug designing.

Recent developments in genetic engineering and molecular biology have made it possible to study eukaryotic genes and manipulate them to overexpress in well-characterised expression systems. This facilitates the obtaining of large amounts of the protein of interest for structural studies. Thus far, no full-length *P. falciparum* enzyme has been expressed in bacteria in a functionally active form.

This thesis is thus proposing the overexpression of *P. falciparum* HPRT in *E. coli*. Such overexpression may provide the following information:

1) Whether *E. coli* can express *P. falciparum* HPRT in an active form.

2) Direct and functional expression of the full-length PfHPRT in *E. coli* will provide enough parasite enzyme for detailed molecular studies.

3) If such a functional recombinant parasite enzyme can complement a mutation in a bacterial homologous gene, the strain may provide an easy way to screen putative anti-PfHPRT drugs.

4) Antibodies against the recombinant PfHPRT could be a probe for PfHPRT *in vivo*, to study the native enzyme processing and location in the parasite cytoplasm.

1.15 Gene expression in E. coli.

As described in the project proposal, it has been mentioned that to obtain a usable quantity of PfHPRT protein, the gene will be overexpressed in *E. coli*. This depends on the development of a suitable host-vector system capable of expressing *P. falciparum* protein in large amounts. Expression of biologically active eukaryotic protein in bacteria has already proven useful in biotechnology (Harris 1983). To place the subject in context, I will first discuss *E. coli* gene expression briely and then review eukaryotic gene expression in the bacterial system.

One of the main reasons for using *E. coli* as the host for a eukaryotic gene is that the control of gene expression in this organism is very well understood. In *E. coli* structural genes form operons with a control region located at begining of the gene. These control elements are the operator and promoter (Miller & Reznikoff, 1980). Upon transcription the operon produces mRNA, which in turn translates into protein. The extent of the expression of

any gene in a cell depends on the type of the promoter and operator of the operon. Usually these control elements are used to express cloned genes in bacteria (Harris, 1983).

In *E. coli*, the transcription of a gene starts when RNA polymerase binds to the promoter of the operon. Study of several operons revealed that *E. coli* promoters have two consensus sequences, the -35 and -10 regions (Rosenberg and Court, 1979). It has been suggested that this region binds closely to the sigma factor of RNA polymerase and ensures the orientation of the enzyme so that transcription will only proceed in the correct direction. The transcription has been stopped by a GC rich palindromic sequence present in the downstream of the gene. Several other factors may also be involved in the regulation of transcriptional termination (Greenblatt, 1981).

The extent at which mRNA is translated into protein depends on the efficiency of the binding of the ribosome to the translation initiation region. This ribosome binding site (rbs) or translation initiation region (TIR) consist of an AUG codon and a small nucleotide sequence 3-9 bases long (Shine-Delgarno (SD) sequence) lying within 3-12 bases upstream of the initiation codon (Shine and Delgarno, 1975). There is no consensus sequence described for this region. Thus, it is possible that the sequence variation in this region may regulate the extent of translation of mRNA at least in part. Other possible regulation may be exerted through the secondary structure of the 5' region of the mRNA. Iserentant and Fiers (1980) showed that translation efficiency is increased if the SD sequence and AUG codon is freely accessible to the ribosome. Translation stops when one of the three stop codons is encountered by the ribosome.

1.16 Expression of eukaryotic genes in E. coli.

There are several potential problems involved in the expression of eukaryotic genes in *E. coli*. The majority of eukaryotic genes contain intervening sequences in the coding region, which are spliced out from trancribed mRNA before translation. In contrast, no bacterial gene is known which contains an intron, and so it is likely that bacteria do not contain the proper machinery for such splicing. This suggests that eukaryotic genomic DNA may not be used as a source of the gene for expression. This problem is overcome by using cDNA. Thus far, there are several cases where eukaryotic cDNA has been used to express genes in *E. coli* (Baty *et al.*, 1981; Guarente *et al.*, 1980).

Transcriptional signals in eukaryotic genes are different from those of prokaryotes (Corden *et al.*, 1980). Moreover, the eukaryotic mRNA structure is also different tothe bacterial counterpart. For instance, eukaryotic mRNA is usually polyadenylated at the 3′ end and capped at 5′ end and does not have the equivalent sequence of the bacterial SD region (Kozak, 1981).

Thus transcription of a eukaryotic gene is usually controlled by inserting the DNA adjacent to a strong prokaryotic promoter in an expression vector. Several prokaryotic promoters are used for this purpose; the *lac* promoter, the trp promoter, bacteriophage λ early promoters, P_L and P_R (Caulcott and Rhodes, 1986) and β-lactamase promoter. The expression is increased by using a high copy number plasmid, which increases the gene dosage in the cell (Gelfand *et al.*, 1978). Efficient termination of transcription may also increase the expression of the gene. This is ensured by placing a termination site after the cloned gene (Nakamura and Inouye, 1982). Such transcriptional stops are rrnB terminator of *E. coli* and phage fd terminator.

To achieve the translation of the cloned eukaryotic gene, it is usually

fused to a prokaryotic gene in the correct translational reading frame. This uses the rbs and initiation codon of the prokaryotic gene. For this purpose *E. coli* β-galactosidase is widely used. Translation also starts from the initiation codon of the foreign gene if it is placed in front of prokaryotic rbs (Backman *et al.*, 1976)

A second potential problem in heterologous expression is that of codon usage. Analysis of coding sequences of highly expressed *E. coli* mRNA showed that there is marked preference for particular codons for some amino acids (Grosjean and Fiers, 1982). Ikemura (1981) showed such bias correlates with the abundance of particular tRNA species. In accordance with this, A/T rich *Plasmodium* genes show a preference for A/T containing codons whereever possible. Scaife, J (1988) showed by comparing the codon frequency of sequenced Plasmodium genes with that of the *E. coli* genes, that several codons preferred by *P. falciparum*, like TTA, ATA and AGA, are rarely used in *E. coli* (see also Hyde *et al.*, 1989). Table 1.3 shows comparison of codon usage for some amino acids, which highlights the difference between the two organisms. It was suggested that such differential codon bias may reduce the efficiency with which a *Plasmodium* protein is made in *E. coli*, due to the reduced availability of the certain tRNAs.

Moreover, several eukaryotic proteins undergo different types of post-translational modifications, which may be important for the activity of the protein. Many of these modifications do not occur in *E. coli*. Finally, in *E. coli*, protein synthesis is in a state of rapid turnover. To keep the metabolic system going, new proteins are synthesised and old proteins degrade . Such a system is highly selective, It has been shown that abnormal proteins are preferentially recognised and degrade more efficiently (Goldberg and S. John, 1976). The machinary for protein degradation in *E. coli* is not clearly understood. Bukhari

Codons	Amino acid	P.f.	E.c.
TTA	Leu	67	3
TCA	Ser	33	2
ATA	Ile	48	1
ACA	Thr	56	5
AAT	Asn	78	8
AGT	Ser	34	4
AGA	Arg	58	1
GGA	Gly	50	1

Table 1.3 Codon preference of *P. falciparum*. The table shows codons for some amino acids which are rarely used in *E. coli* (Grosjean and Fiers, 1982). The numbers are percentage of the codon usage for the particular amino acid (From Scaife, 1988).

and Zipser (1973) showed that mutation in the *lon* gene, which codes for an ATP-dependent protease, reduces the rate of degradation of nonsense polypeptides. Itakura *et al.*, (1977) and Goeddel *et al.*, (1979) showed that short foreign peptides are unstable in *E. coli*. A higher expression was achieved by linking the eukaryotic gene with a large prokaryotic gene, i.e. β-galactosidase so that fused protein was synthesised.

Fusion of a eukaryotic gene with a bacterial protein also facilitates the purification of the recombinant protein. β-galactosidase fusion proteins can be purified by affinity column chromatography either by using an antiβ-galactosidase antibody column or a substrate affinity column (Marston, 1987). Efficient purification of the target protein may also be obtained by fusing with maltose binding protein (Maina *et al.*, 1988).

However, in general the eukaryotic polypeptide alone is required for further studies, and therefore a strategy is needed for correct cleavage of the fusion protein. The strategy used in this project to allow this was to construct the fusion protein with a cleavage site placed between the bacterial sequence and the foreign protein. A number of cleavage sequences have been reported thus far, which can be broadly divided into two types, chemical and enzymatic. Although chemical cleavage was successfully used for small foreign proteins (Goeddel *et al.*, 1979; Szoka *et al.*, 1980) it is quite unsuitable for large proteins. In contrast enzymatic cleavage is far more specific and widely used. For instance Endoproteinase lys C (Allen *et al.* 1985) Enterokinase (Belagaje *et al* . 1984) and Blood factor Xa (Nagai *et al.* 1985) have been successfully used.

Another problem with producing eukaryotic proteins in *E. coli* is that the product frequently accumulates as insoluble aggregates (inclusion bodies) within the producing bacterium when overexpressed (Wittrup *et al.*, 1988). These inclusion bodies are usually resistant to mild detergents and the proteins

can only be released by using strong denaturing agents, such as 6-8 M urea or 6 M guanidine hydrochloride. Such harsh treatment often denatures the protein irreversibly and recovery of active protein by refolding is often difficult or impossible for a large protein (Schein, 1989). The mechanism of inclusion body formation is not clearly understood. However, it has been shown that expression at a lower temperature reduces the degree of inclusion body formation. For example, increased solubility was achieved for Diptheria toxin (Bishai *et al.*, 1987) and basic fibroblast growth factor (Squires *et al.*, 1988) at 30°C as compared to 37°C. For details about the production of soluble eukaryotic protein. See the review by Schein (1989).

Direct expression of a eukaryotic gene in *E. coli* has several advantages over expression as a fusion protein. Native protein can be obtained in sufficient amount for biochemical studies and the expressed protein is often functionally active (Persico *et al.*, 1989). Such protein can be used to study structure activity relations of the protein by *in vitro* mutagenesis and X-ray crytallography.

1.17 Working Plan.

To achieve the goal described in the 'project proposal' the following strategy is planned.

Firstly, to avoid degradation and facilitate the subsequent purification of the bacterially synthesised PfHPRT, the protein will be expressed as an *E. coli* β-galactosidase fusion protein. This can be done by fusing the *hprt* gene with the *lacZ* gene in a suitable expression vector.

The fusion protein will then be purified from the induced bacterial lysate. To achieve this, classical protein purification methods, like gel chromatography and affinity chromatography will be employed. To check whether the expressed

protein retains the enzymatic activity, the fusion protein will be assayed for both β-galactosidase and HPRT activity. An attempt will then be made to release the PfHPRT domain of the fusion protein. The purified protein will be used to make antibody against the PfHPRT protein which will be an important tool to probe the protein *in vivo* and *in vitro*.

In the second stage, PfHPRT will be expressed directly in *E. coli*. A non-fusion expression vector will be used for this purpose. The expressed protein will be assayed for activity. This information will be very important since the active protein can then be used to complement a bacterial hpt mutation. Moreover, the protein will be a source for obtaining large amounts of the parasite protein for structural studies by X-ray crystallography etc.

Successful complementation will result in a bacterial strain which can be used to screen potential antimalarial drugs for activity against PfHPRT. This bacterium will also allow us to study the biochemical parameters of the parasite enzyme.

Finally the antibodies will be checked for specificity and then will be used to probe the native protein in western blot analysis and used *in vivo* to study possible-stage specific expression of the enzyme in the parasite and to study the location of the protein *in vivo*. This will be done by indirect immunofluorescent microscopy and immunogold microscopy. Such information will help in designing an effective drug against parasite HPRT.

Chapter 2

Materials and methods.

2.1 Microbiological methods.

2.1.1 Strains and plasmids.

The bacterial strains and plasmid vectors used in this study are listed in Table 2.1. The *S. typhimurium* strain KP1684 was a gift from Kaz Jensen. Vector pMS1S was obtained as a member of the series of 3 expression vectors containing, pMS1S, pMS2S and pMS3S and was a generous gift from Michael Schrieber, Germany. Expression vector pJLA503 was bought from MODEM, U.S.A. Other bacterial strains and plasmid vectors are being used in our lab for several years.

2.1.2 Media.

L-broth: Tryptone (Difco Bacto) 10 gms, Yeast extract (Difco Bacto) 5 gm, NaCl 10 gm dissolved in 800 ml of distilled water and pH adjusted to 7.2 with 1M NaOH and finally the volume was top up to 1 litre. Sterilized by autoclaving at 121°C and 15 psi pressure.

L-agar: Same as L-broth but supplemented with agar (Difco) 15 g/L.

Minimal medium A [1] : This medium contains 1 x M9 salt, 2% glucose, 10 mM $MgSO_4$, 0.1 mM $CaCl_2$, 1 µg/ml vitamin B1. For solid minimal medium, the above is supplemented with 1.5% agar (Bacto).

M9 salt: Na_2HPO_4 60 gm, KH_2PO_4 30 gms, NaCl 5 gm and NH_4Cl 10 gm dissolved in 1 litre of distilled water.

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[1] for *E. coli*

TABLE 2.1

Bacterial strain	Reference
Escherichia coli NM 522	Gough & Murray, 1983
Escherichia coli HB 101	Boyer *et al.*, 1969
Escherichia coli NM 654	Noreen Murray Unpublished
Salmonella typhimurium KP 1684	Houlberg & Jensen, 1983
Salmonella typhimurium LB 5000	Bullas & Ryo, 1983
Parasite strain	
Plasmodium falciparum K1	Thaithong and Beale, 1981
Plasmid vectors	
pMS	Schreiber M,. unpublished
pJLA503	Schauder *et al.*, 1987

Made as 4 x stock solution and sterilized by autoclaving.

Minimal medium B [2] — Tris 12 gm, KCl 2 gm, NH_4Cl 2 gm, $MgCl_2.6H_2O$, 0.5 g, $NaSO_4$ 50 mg, Na_2HPO_4 50 mg dissolved in 800 ml distilled water. The pH is adjusted to 7.6 with dilute HCl and the volume to 1 litre with distilled water. After autoclaving $ZnCl_2$ (0.001 mM), glucose (1 %) and vitamin B1, 1 µg/ml are added (Houlberg et al., 1983).

Antibiotic: — Liquid or molten agar medium was supplemented with 50 µg/ml ampicillin when appropriate.

2.2 DNA methods.

2.2.1 Preparation of plasmid DNA.

Small scale preparation.

This method was routinely used for checking transformant strains and initially adapted from that of Birnboim & Doly (1979). Cells from 1.5 ml overnight culture were spun down for 1 min and resuspended in 80 µl GTE solution (25 mM Tris-HCL pH 8.0, 10 mM EDTA, 50 mM glucose) by vortexing. Lysogen solution (stock 10 mg/ml in GTE), 20 µl, was added to the tube, mixed and left on ice for 30 min. After addition of 200 µl of alkaline SDS solution (0.2 M NaOH, 1% SDS), the solution was kept on ice for further 5 min. This was followed by addition of 150 µl of 3M Na-acetate, pH 5.0 and one more hour on ice. The precipitate thus formed was removed by a 10 min centrifugation in an Eppendorf microfuge. To the clear supernatant, 1 ml cold absolute ethanol was

[2] for *S. typhi*

added, mixed and kept in -20 °C for 30 min. DNA was pelleted by a 10 min centrifugation and resuspended in 100 µl of 0.3 M Na-acetate pH 5.0 and reprecipitated with 200 µl of cold absolute ethanol by incubating at -20°C for 15 min. After pelleting the DNA again, These were dried under vacuum and finally, resuspended in 50 µl of 1 x TE buffer (0.1 M Tris-HCl pH 8.2, 0.01 M EDTA).

Large scale plasmid preparation.

An 500 ml L-broth, containing 50 µg/ml ampicillin, was inoculated with 1 ml of overnight culture and incubated for 12 to 14 hours with shaking. The cells were then separated by spinning at 5000 rpm in Sorval RC5B centrifuge with GSA rotor for 20 min and resuspended in 6 ml SET solution (25% sucrose, 50 mM Tris-HCl pH 8.1, 40 mM EDTA). To this 1 ml lysozyme (stock 10 mg/ml in SET) and 1 ml 0.5 M EDTA were added. The mixture was kept on ice for 5 min with occasional swirling. Thirteen ml of Triton mix (2 ml 10% Triton X-100, 25 ml 0.5 M EDTA, 10 ml 1M Tris-HCl pH 8.1, water to 200 ml) was added. The solution was kept on ice for 10 min and mixed by swirling. The lysate was centrifuged for 30 min at 15000 rpm on Sorval RC5B centrifuge, with SS-34 rotor. Clear amber-coloured supernatant was decanted and CsCl (0.95 g/ml) and ethidium bromide (0.5 ml of 5 mg/ml) were added. The mixture was centrifuged to equilibrium at 38k rpm for 72 hours at 18°C. Plasmid band can be visualized by long wave UV light, and removed by side puncture (Maniatis et al., 1982). The ethidium bromide was removed by extracting with TE saturated butan-2-ol and then dialysed against TE buffer to remove CsCl. the DNA was then precipitated with ethanol (see below).

2.2.2 Ethanol precipitation of DNA.

Ethanol precipitation of DNA was performed routinely. For this 1/10 volume 3 M Na-acetate was added followed by 2.5 volume cold 100% ethanol.

The mixture was kept at -20°C for 30 min and the DNA pelleted by centrifuging for 10 min in an Eppendorf microfuge. The supernatant s were discarded and the pellets washed with 70% ethanol in water and centrifuged again. Finally the pellets were dried under vacuum for 5 min. The DNA was resuspended in 1 x TE and stored in -20°C.

2.2.3 Transformation of plasmid.

A 20 ml L-broth was inoculated with 200 µl of overnight culture of the bacterium to be transformed with plasmid, and grown at 37°C with shaking for 1.5 h to early log phase and chilled on ice for 10 min. The cells were then spun down at 4000xg at 4°C and resuspended in 10 ml ice-cold 50 mM $CaCl_2$ and left on ice for 45 min before spinning down. The pelleted cells were then again resuspended in 2 ml on ice-cold 50 mM $CaCl_2$ and called the competent cell. DNA to be transformed, is mixed with 200 µl portions of the competent cell and left on ice for 45 min. This was then heat shocked in a 42°C water bath for 3 min. To this mixture, 0.5 ml of prewarmed L-broth was added and incubated at 37°C unless otherwise stated. Finally the cells were plated on L-agar plates, supplemented with ampicillin and incubated overnight at 37°C, unless any other temperature stated.

2.2.4 Digestion of DNA with restriction enzymes.

DNA was digested routinely to completion, according to the condition suggested by the suppliers (Boehringer-Mannheim, New England Biolabs. U.S.A.). Double digestions were carried out simultaneously in one of the 5 buffers (A, B, L, M and H) as described by Boehringer-Mannheim. Digestion s were terminated by heating at 65°C for 10 minutes and subsequent precipitation with ethanol where appropriate.

2.2.5 Klenow filling of 5′ protruding ends of DNA.

To fill 5′ protruding ends of restriction enzyme digested DNA, Klenow fragment of *E. coli* DNA polymerase I (Boehringer-Manheim) was used as described by Maniatis et al., (1982). In brief, 2 units of the enzyme was mixed with 1 µl DNA with 5′ protruding ends (1 µg) and 2.5 µl 10x Klenow buffer. To this 1 µl dNTP mix (2mM) and water to final volume 25 µl were added and mixed. The mixtures were incubated for 30 minutes at room temperature. The reaction mixtures were then extracted once with phenol:chloroform and ethanol precipitated. 10x Klenow buffer contained 0.5 M Tris-HCl pH 7.2, 0.1 M $MgSO_4$, 1 mM dithiothreitol (DTT) and 0.5 mg/ml BSA.

2.2.6 Ligation of DNA.

Both steaky and blunt ended ligations were performed using the same buffer condition, only in case of blunt ended ligation 2 to 3 times more ATP were added. In detail, the DNAs to be ligated were mixed and suspended in ligation buffer (66 mM Tris-HCl pH 7.2, 1 mM EDTA, 10 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 0.1 mM ATP). To this mixture 5 units of T4 DNA ligase were added and incubated overnight (16-18 h). Reaction was stopped by freezing the reaction mixture at $-20^{\circ}C$.

2.2.7 Cloning of DNA.

Cloning of DNA fragments in plasmid vectors were performed as described by Maniatis *et al* (1982) . The plasmid vector and the insert DNA were digested with appropriate restriction enzyme and mixed in a ratio of vector:insert = 3:1 and ligated (see section 2.2.6). The ligated DNA were then transformed into the suitable bacterial host. The transformants were initially selected on L-agar plates containing 50 µg/ml ampicillin and confirmed by isolating plasmid by mini plasmid preparation.

2.2.8 Colony hybridisation.

For colony hybridisation ampicillin resistant colonies were transferred onto a nitrocellulose membrane (Hybond N, Amersham) placed on a fresh L-agar ampicillin plate. The plates were incubated overnight either at 37°C or 30°C. The filters were then peeled off and placed colony side up, on whatman 3MM paper presoaked with denaturing solution (0.5 M NaOH, 1.5 M NaCl) and left for 10 min. Filters were then neutralized with 1 M Tris-HCl pH 7.4 for 10 min with 2 changes and finally, washed once with 2 x SSC and put in a sealed bag for hybridisation.

2.2.9 DNA gels:

Large agarose gels.

DNA was fractionated by electrophoresis through 0.7%, 1.0%, or 1.5% agarose gels as described bellow. Horizontal slab gels (28 x 14 x 0.5 cm), connected by wicks at each end of 500 ml buffer tanks were routinely used. The electrophoresis buffer contains 50 mM Tris-HCl, 20 mM trisodium acetate, 1 mM EDTA, pH adjusted to 8.2, with glacial acetic acid and 0.5 µg/ml ethidium bromide (TAE buffer). Agarose type II (Sigma) was melted in the same buffer, used at concentrations appropriate for resolution of particular fragments (Maniatis et al., 1982).

DNA samples for electrophoresis were prepared by addition of 1/10th volume loading buffer (Ficoll 10% and bromophenol blue 0.025%). Electrophoresis was carried out at 6 volts/cm for 30 min. and then overnight at 3 volts/cm. Marker DNA was HindIII digested λcI875 DNA.

Mini agarose gels.

For rapid checking, small amounts of DNA (0.05 to 0.5 µg) were fractionated using gels (10 x 5 x 0.5 cm) . Gels contained 0.8% agarose in 89 mM Tris-HCl pH 8.2, 89 mM Boric acid, 2 mM EDTA (TBE buffer) and 0.5 µg/ml ethidium bromide. Gels were electrophoresed under the same buffer for 60

min at 150 volts and immediately photographed.

2.2.10 Gel photography.

Gels were photographed under short wave UV light using Ilford HP5 professional 5" x 4" sheet film, and a red filter. Exposures were for 30 sec to 1 min. films were develop for 5 min in Ilford Microphen developer and fix for 5 min. All size measurements were performed directly from the negative.

2.2.11 Southern blotting.

The DNA was first fractionated on large agarose gel and transferred on Hybond N nitrocellulose (Hybond N is the trademark of Amersham) sheets as described by Southern (1975) as follows. The gel was immediately photographed and cut into size (if needed), denatured in 0.5 M NaOH, 1.5 M NaCl (2 x 500 ml; 15 min each) then neutralized in 1 M ammonium acetate and 0.02 M NaOH (2 x 500 ml; 30 min each). The gel is then assembled in a 'sandwitch' structure as follows. Nitrocellulose, cut into the size of the gel and soaked in neutralization buffer was placed on the gel with 6 sheets of 3 MM Whatman filter paper and 2 to 3 inches of paper towels with a heavy weight on top. Transfer was completed in 1 to 2 hours as monitored under UV after restaining with .5 µg/ml ethidium bromide. Filters were washed in 2 x SSC (0.3 M NaCl, 30 mM Na-citrate pH 7.0) for 2 min stored wet in a polyethylene bag at 4°C until used.

2.2.12 Extraction of DNA from agarose gels by electroelution.

To isolate a DNA fragment from an agarose gel, the DNA was separated on a large gel. A trough of 3 mm wide was cut immediately in front of the relevant band. The rear of the trough was lined with a strip of dialysis tubing and the trough was filled and replenished as necessary, with TAE buffer. Electrophoresis was continued at 250 V for 30 min until the band had entered

the trough. The current was reversed for 30 seconds and the buffer trough containing the DNA fragment was removed. The trough was washed out once with the buffer to recover as much of the DNA fragment as possible. The preparation was extracted sequentially with an equal volume of butanol, phenol, phenol:chloroform (1:1 v/v), chloroform and ether (2x), preequilibrated with TE. The DNA was then ethanol precipitated.

2.2.13 Labelling of DNA by nick translation.

DNA used for probing a southern blot was labelled with $\alpha[^{32}P]$-CTP (NEN) of specific activity 410 Ci/mM (0.8 mCi/ml). The labelling reaction was performed using the nick translation kit (BRL) according to the protocol supplied with the kit. Usually 500 ng of DNA were labelled and the activity incorporated was 1-10 x 10^{+6} cpm/µg DNA. The incorporated counts were separated from the unincorporated label by passing the reaction mixture over a sephadex G-50 (fine) 'spun column' (Maniatis et al., 1982). Probes were denatured by boiling for 10 min and diluted into 20 ml of hybridisation solution (below).

2.2.14 Hybridisation.

The filters were preincubated for two hours at 37°C in hybridisation solution (HS): 4 x SSC (0.15 M NaCl, 0.015 M Na_2citrate, pH 7.0), 50% formamide, 0.1% SDS, 1 x Denhardt solution (0.02% BSA, 0.02% polyvinyl pyrolidone, 0.02% ficoll), containing 50 µg/ml sonicated salmon sperm DNA (Sigma). Blots were then hybridized overnight in HS containing the freshly boiled labelled probe at 37 °C with constant shaking.

After hybridisation the filters were washed at 37°C with shaking in 0.1% SDS and 0.1X SSC, 4 x 15 min washes. This was followed by 4 x 15 min washes in 0.1X SSC only. The wet filters were then put in a sealed polyethylene bag. The filters were then mounted in X-ray cassette for

autoradiography.

2.2.15 Autoradiography.

Autoradiography was routinely performed using CRONEX 4 X-ray film (Dupont), cassettes and lightning plus intensifying screens. The films were exposed overnight or longer at -70°C and develped with Photosol xray film developer (Photosol, England.).

2.2.16 Polymerase Chain Reaction (PCR).

PCR was performed by adapting the method as described by (Saiki *et al.*, 1988). In the reaction mixture: 2 µl dNTP solution (10 mM dATP, 10 mM dCTP, 10 mM dTTP, 10 mM dGTP), 10 µl Taq pol buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin), 10 µl oligo-primer 1(1µM), 10 µl oligo-primer 2 (1µM), 10 µl target DNA (50 ng and 100ng) and 58 µl sterile distilled water were added sequentially. To these 2 units of Taq DNA polymerase (Cetus corporation, U.S.A.) were added and mixed and pulse spin. The reaction mixtures were then overlay with 100 µl of sterile mineral oil. The reaction was carried out in a Techne Dri Block PHC-1. using the protocol shown in figure 2.1. At the end of the reaction 10 µl of the reaction mixtures were checked for amplified DNA by a mini agarose gel. Reaction mixtures were left overnight at 4°C before ethanol precipitation and resuspended in 1 x TE buffer.

2.3 Protein methods.

2.3.1 Estimation of protein.

Proteins concentration were estimated routinely by the method of Bradford (1976).

Cycles	Time		
	1st Seg. 92°C	2nd Seg. 55°C	3rd Seg. 70°C
1st	5 min	2 min	5 min
2nd	5 min	2 min	5 min
3rd	3 min	2 min	5 min
4th to 25th	1.5 min	1.5 min	5 min
26th	1.5 min	1.5 min	10 min

Figure 2.1 Temperature cycle programme for the PCR reaction.

preparation of reagent

Coomassie Brilliant Blue G-250, 100mg was dissolve in 50 ml 95% ethanol. To this solution 100 ml 85% (w/v) phosphoric acid was added. The solution was diluted to a final volume of 1 liter. The solution was filtered before used.

Protein assay.

Protein solutions in 0.1 ml were mixed with 5 ml of the protein reagent and mixed by inversion. The absorbance of the solution is measured at 595 nm after 2 min against a reagent blank prepared from 0.1 ml of the appropriate buffer and 5 mls of protein reagent. The amount of protein was estimated by comparing the absorbance with an standard curve, which was prepared simultanuously using bovine serum albumin (BSA) as the standard protein.

2.3.2 SDS-PAGE.

Proteins were routinely fractionated according to their size using the Sodium dodesyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970). In this case 4.75% stacking gel pH 6.8 and 7.5%, 8%, 10% or 12.5% separating gel pH 8.8 were used. Samples were prepared by mixing equal amount of Laemmli sample buffer (0.125 M Tris-HCl pH 6.8, 4% SDS, 10% 2-mercaptoethanol, 20% glycerole and 0.04% bromophenol blue) with the protein solution, mixed and left in room temperature for 5 min and heating in boiling bath for 5 min. Samples were centrifuged for 5 min before loading on gel. Ten to 50 µl of sample were used for electrophoresis. Electrophoresis were carried out with 20 mA constant current for 3 to 4 hours.

The molecular marker used was, prestained high molecular weight standard (Sigma) and contained: γ2-Macroglobulin (180 kD), β-galactosidase (116 kD), Fructose-6-phosphate kinase (84 kD), Pyruvate kinase (58 kD), Fumerase (48 kD), Lactic dehydrogenase (36.5 kD) and Triosephosphate isomerase (26.6 kD).

2.3.3 Analysis of SDS-PAGE for protein profile.

After electrophoresis gels were removed from the apparatus and transferred into a plastic tray containing staining solution (0.1% coomassie Blue R-250, 50% methanol, 10% acetic acid) and stained for 1 h. Gels were then destained overnight with several changes of destaining solution (10% methanol, 10% acetic acid). The protein profiles were then analysed by comparing the protein bands for size and intensity. The relative molecular mass were estimated by comparing with that of the marker proteins.

2.3.4 Western blot.

Western blotting were carried out as described by Tawbin et al (1978) using the Bio-Rad Trans-blot apparatus. After SDS-PAGE gels were removed from the electrophoresis apparatus and washed with transfer buffer (20% methanol, 0.25 M Tris,0.192 M glycine, 0.1% SDS). Hybond N nitrocellulose membrane cut into same size of the gel, soaked with blotting buffer and placed on the gel. The gel with the membrane were then arranged in the gel cassette and place in the transfer chamber. Transfers were performed with 50 volts overnight with cooling.

Filters were blocked with 5% skimmed milk in TS buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl) at room temperature for 1 h with shaking and probed with antisera in same buffer at specified dilution overnight. These were washed with TS buffer for 30 min with 4 changes. The blots were then incubated with alkaline phosphatase conjugated antirabbit or antihuman IgG with 5% milk in TS buffer for 4 h. The blots were again washed for 30 minutes with TS buffer with 4 changes developed with protoblot kit (Promega) as described by the manufacturer.

2.3.5 Assay of PRTase activity

Assay mixtures, in a final volume of 50 µl, contained 225 mM Tris-HCl pH 7.5, 5 mM $MgSO_4$, 0.1% BSA, 2 mM PRPP and 100 µM of ^{3}H-hypoxanthine (Amersham) or ^{14}C-guanine or ^{14}C-xanthine (NEN, Dupont). The reactions were started by adding enzyme extract. The control contained no extract. The reactions were allowed to continue for 10 minutes at 37°C and stopped by heating at 100°C for 5 min. Precipitated proteins were pelleted by centrifugation for 10 minutes. Aliquots of 20 µl of reaction mixtures were applied to Whatman No. 3MM filter paper, previously spotted with a solution containing 0.1 µmole of non-radioactive mixture of purine and it's corresponding ribonucleotide. Chromatograms were developed in an ascending direction at room temperature for 2 h in 5% Na_2HPO_4:isoamyl alcohol, 2:1 v/v. The extent of the conversion of the purines to corresponding nucleotides was determined by cutting out the UV light absorbing spots containing the corresponding ribonucleotide. The radioactivity of each spot was counted (Beckmann, LS7000, liquid scintillation system) in 2 ml Scintillation fluid (4 g PPO, 0.01 g POPOP, 50 ml Triton X-100 in a total volume of 150 ml of toluene).

2.3.6 Assay of β-galactosidase.

For β-galactosidase assay o.1 ml of the extract (diluted if required) were added in 0.9 ml enzyme assay buffer (0.1M Na-phosphate buffer pH 7.0, 10 mM KCl, 1 mM MgSO4, 50 mM 2-mercaptoethanol) and incubated at 28°C for 5 min. The reaction was started by adding 0.2 ml of ONPG (o-nitrophenyl β-D-galactopyranoside, 4 mg/ml) and incubated for 2 min. the reactions were stopped by adding 0.5 ml 1 M Na_2CO_3. The extent of reaction were determined by measuring the optical density of the reaction mixture at 420 nm.

2.3.7 Induction of recombinant cells for protein expression.

Induction for β-gal:PfHPRT fusion protein.

For the induction of the fusion gene under the control of the lac promoter, the recombinant bacteria were grown overnight in 5 ml L-broth with ampicillin (50 µg/ml) at 37°C with shaking. The cultures was diluted 1:20 with fresh medium and the growth continued for 3 to 4 hours before adding IPTG (Isopropyl-thiogalactoside, final conc. 2 mM). The growth were continued for further 2 to 3 hours when the cells were collected by spinning at 6000 rpm for 10 min in Sorval RC5B centrifuge and GSA rotor.

Induction nonfusion PfHPRT in *E. coli*.

To study expression of the recombinant PfHPRT under the control of λ P_L/P_R promoters and repressed by cI857 protein, a 5 ml culture of the bacterium was grown overnight (30°C) in L-broth containing 50 µg/ml ampicillin in a shaking water bath. To induce expression the temperature was shifted to 42°C for 1 hour. A 1.5 ml aliquot was centrifuged, for one minute and the pelleted cells were resuspended in 50 µl of distilled water and processed for SDS-PAGE.

2.3.8 Purification of the fusion protein.

Cell lysis.

For lysis induced cells from 500 ml were suspended in 10 ml lysis buffer (50 mM Tris-HCl pH 8.0, 1 mM EDTA, 100 mM NaCl) by vortexing and kept on ice. To this, 50 µl fresh PMSF (phenylmethylsulphonylfluoride, 50 mM in methanol) and 0.5 ml lysozyme (stock 10 mg/ml) was added and mixed well. The suspension was incubated on ice for 20 min with occasional swirling. Thirty mg deoxycholic acid was added to the mixture, mixed with swirling and transferred into a 37°C water bath and stirred with a glass rod. This time the suspensions became thick and viscous. A 200 µl of deoxyribonuclease (DNAse, 1 mg/ml) was added and incubated at 37°C for 30 min until the suspension

was no longer viscous.

Isolation of inclusion bodies.

The dense inclusion bodies were released from the cells by lysis (above) and then isolated from the cell lysate by differential centrifugation method (Glover, 1987). For this, cell lysates were centrifuged sequentially at speeds of 500, 1000, 1500, 3000 and 12000 xg for 15 min. The pellets of each centrifugation step were washed once with lysis buffer. Finally the pellets and the final supernatant were diluted in SDS-sample buffer and analysed by SDS-PAGE.

Washing inclusion bodies.

To remove coprecipitating meterials, the inclusion bodies were washed with Triton X-100 and urea. Cell lysates were centrifuged at 1000 g for 20 min to remove intact cells and debris. The supernatant was then centrifuged at 15000 rpm for 20 minutes to collect the inclusion bodies. The pellets were first washed once and resuspended in 5 ml lysis buffer. To wash with Triton X-100, pellets of the 100 µl inclusion bodies were suspended in 0.5% Triton X-100 and left in room temperature for 5 minutes and centrifuged for 10 min in a Eppendorf microfuge. The supernatant was removed and the pellets were resuspended in fresh 0.5% Triton X-100 solution. A portion of 10 µl of the pellet was dissolved in SDS sample buffer for analysis. The washing step was repeated for 3 times. The pellets from 3 consecutive washings were analysed for protein profile by SDS-PAGE.

For washing with urea, inclusion bodies from 50 µl suspensions were resuspended in 0.5, 1.0, 2.0, 5.0 M urea solutions in 0.1 M Tris-HCl pH 8.5. The suspensions were mixed, left in room temperature for 5 min and finally centrifuged at 13000 g for 20 minutes. The supernatants and pellets were then analysed by SDS-PAGE to check the extent of purity.

Gel filtration.

For gel filtration, appropriate amount Sephacryl-200 (Pharmacia, Sweden) was washed thoroughly with distilled water and equilibrated with urea buffer (8 M urea, 25 mM Tris-acetate pH 5.0, 1 mM EDTA, 1 mM 2-mercaptoethanol). A 70 x 1.6 cm column (Pharmacia column K16) was prepared with a flow rate of 30 ml/h using a peristaltic pump. The column was equilibrated with 200 ml buffer. The void volume was estimated by running 0.5 ml blue dextran (10 mg/ml) through the column. The outlet of the column was connected with an automatic fraction collector (LKB 2070 ULTRORAC II, LKB, Germany) through a UV monitor (with 280 nm filter,). The UV monitor was connected with a recorder (LKB type 6520-4, LKB, Germany). The sample was prepared by dissolving the inclusion bodies in urea buffer, to a protein concentration of about 10 mg/ml. The sample was then centrifuged for 10 min in Eppendorf microfuge. Before loading on the column the sample was passed through a 0.45 µm filter (Whatman). Two ml of the sample was then chromatographed on the column with the urea buffer, at an elution rate of 20 ml/h. Fractions of 5 ml was collected until the major peak was completely eluted out and the reading on the UV monitor was zero. Aliquot of each sample from the peak was analysed with SDS-PAGE for protein profile. The fractions containing the fusion protein was pooled togather and dialysed against TS buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl).

2.3.9 Purification of the fusion protein from soluble fraction.

Preparation of affinity column.

ECH-Sepharose 4B (Pharmacia Fine Chemicals) was thoroughly washed according to the supplier's recommendation. To 100 ml gel 1 mmol of APTG (p-amino- phenyl-β-D-thiogalactoside (Sigma) in 10 ml distilled water was added. The pH was adjusted to 4.7 and 7.5 mmol of N-acetyl-N'(3-dimethylaminopropylcarbidiimide (Sigma) in 8 ml water were added drop wise

to the mixture. The pH was maintained at 4.7 for 1 h and the reaction was allowed to proceed for 24 h at room temperature by gentle stirring. The gel was thoroughly washed with distilled water and stored at 4°C. A column of 4 x 0.5 cm is prepared with the APTG-coupled gel and equilibrated thoroughly with the equilibrium buffer (20 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 1.6 M NaCl, 10 mM 2-mercaptoethanol).

Affinity chromatography

Bacterial lysate prepared in the equilibrium buffer by sonication and centrifuged at 15000 rpm for 20 min. The supernatant diluted to 10 to 15 mg of protein/ml with the same buffer. The solution was applied to the APTG-ECH-Sepharose affinity column. The extract was passed through the column which retained the fusion proteins. The column was washed with 200 ml of the buffer used for equilibration, no more material absorbing at 280 nm appeared in the flow through. The protein was eluted from the column with 100 mM sodium borate, 10 mM 2-mercaptoethanol, pH 10 and fractions of 1 ml was collected. The elution profile was monitored by measuring absorption of each fraction at 280 nm.

2.3.10 Digestion of the fusion protein with factor Xa.

Digestion of the protein with activated factor Xa was performed as described earlier (Nagai et al., 1984). Two mg of the fusion protein in Factor Xa buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM $CaCl_2$) was mixed with 50 µg of activated factor Xa (Sigma) and incubated for 2 h. The extent of digestion was estimated by electrophoresis of the sample on SDS-polyacrylamide gel.

2.3.11 Production of polyclonal antiserum.

To produce polyclonal antiserum rabbits against purified β-gal:PfHPRT fusion protein, 180 µg of the protein in 2 ml PBS was emulsified in 2 ml of

Freund's complete adjuvant (Bacto labs.). For primary injection, 0.5 ml of the emulsion was given intramuscularly into each of the four limbs of 2 female New Zealand white rabbits (12 weeks old). At the 33rd day a further 100 µg protein was injected through the same route after emulsified with equal volume of Freund's incomplete adjuvant. After 15 days 90 µg protein was injected into each rabbit intramuscularly as booster dose. On 8th day after boosting 10 ml blood was collected from the marginal ear vein of both the rabbits and the serum was tested for immune response by Ouchterlony immunodiffusion test using the fusion protein as antigen. The rabbits were bled by cardiac puncture and the blood was kept at 4°C overnight until the clot retracted. The blood was spinned at 5000 rpm on a benchtop chillspin centrifuge for 15 min. The clear serum was stored at -70 °C and designated anti-β-gal:PfHPRT serum.

2.3.12 Ouchterlony double immunodiffusion.

Ouchterlony immunodiffusion test was used to test the presence of specific antibody in immunized rabbits and performed on microscope slides. 2% agarose in PBS was melted in a boiling water bath and poured on precleaned microscope slide placed on a levelled surface. After setting the gel punch were made using cork borer as shown in figure 4.10. The agarose plugs were removed using a needle. The wells were filled with antiserum or antigens until the meniscus just disappears. The slides were then placed in a humid chamber and incubated overnight at room temperature. Precipitin bands were appeared within 12 hours. The slides were then washed thoroughly with PBS over a period of 8 hours and finally with distilled water to remove unreacted proteins. The gels were dried on the slides in room temperature. The slides were then stained with coomassie blue R250 in 10% acetic acid and washed thoroughly with 50% methanol and 10% acetic acid and finally air dried and photographed.

2.3.13 Preabsorption of antiserum with *E. coli* extract.

Antibodies against *E. coli* proteins and β-galactosidase in the rabbit antiserum were removed by incubating 0.5 ml of the serum with 1 ml sonicate of induced *E. coli* strain NM522/pMS1S. Cells from 200 ml overnight culture were collected and resuspended in fresh 500 L-amp medium after one hour incubation at 37°C IPTG was added to a final concentration of 1 mM and the growth was continued for further 6 hours. The cells were collected and resuspended in 5 ml PBS and sonicated with full power for 2 minutes. The lysate was stored in -20°C as aliqouts of 0.5 ml. For preabsorption 0.5 ml serum was mixed with 0.5 ml sonicated lysate and incubated for 15 minutes at 37°C with shaking. The mixture was centrifuged for 10 min in microfuge centrifuge. The preabsorption step was repeated once more with fresh lysate. Finally, the preabsorbed serum can be stored at 4°C with 0.05 % Na-azide for atleast 1 month. The antiserum was diluted with 5% skimmed milk in PBS to desired concentration.

2.3.14 Preabsorption of antiserum with red cell membrane.

For IFA the serum was also preabsorbed with RBC membrane. Packed red blood cells (2 ml) were resuspended in 15 ml distilled water and left on room temperature for 10 minutes. The RBC ghosts were then collected by spinning at 5000 g and resuspended in 2 ml PBS, and stored at -20 °C in aliquots of 200 µl. For absorption, 500 µl of the antiserum preabsorbed with E. coli lysate, mixed with 200 µl of the red cell membrane and left overnight at 4°C. The membranes were spun down and the clear supernatant use for IFA with suitable dilution.

2.3.15 Indirect immunofluorescence assay (IFA).

The experiment was actually an adaptation of that as described by Voller et al (1980). in detail, for blood stage parasite, smear was done with infected

cells from in vitro cultures with parasitemia about 5% (slides were prepared by Anne McGowen and stores at −70°C). This slides were fixed either with chilled methanol or acetone and quickly dried. The acetone fixed slides of sexual stage parasites were obtained from Dr. David read, protozoan genetics and acetone fixed slides of infected mosquito salivary glands were the source of the sporozoites and obtained from Professor David Walliker, protozoan genetics, University of Edinburgh. These slides were stored at −20 until required. The IFA was carried out at room temperature in a moist chamber. Before adding antibodies the slides were washed with PBS (0.15 M NaCl, M sodium phosphate buffer pH 7.4) for 1 min and air dried. Each slide was then separated into small squires using 'nail polish' paint. Each square was then treated with 20 µl of the antiserum of different dilutions for 30 min. The antiserum was diluted with 5% dried skimmed milk in PBS. The slides were washed with PBS for 3 times and incubated with Fluorescein isothiocyanate (FITC) conjugated antirabbit or antihuman IgG antibodies (Sigma) (final dilution 1 in 1000) for further 30 minutes. Slides were counter stained with DAPI, washed air dried and mounted with Citifluor (Citifuor Ltd. UK) and examined at 1000 times magnification by fluorescence microscopy. Photographs were taken on Kodak gold 400 ASA film or Kodak Tri-x pan film.

Chapter 3
Result 1

Expression of *Plasmodium falciparum* HPRT in *Escherichia coli* as a β-galactosidase fusion protein

A potential problem in the expression of a foreign protein in a bacterial system is proteolytic degradation of the expressed recombinant gene product (Itakura *et al.*, 1977). To avoid this at least in part and to facilitate the subsequent purification of the recombinant protein, the immediate objective of this project was to express the *P. falciparum* HPRT enzyme in *E. coli* as a β-galactosidase fusion protein. There is evidence to suggest that although certain foreign proteins are unstable in *E. coli*, fusing them with a larger *E. coli* protein may impart stability (Goeddel *et al.*, 1979). To date numerous eukaryotic gene products have been successfully expressed as β-galactosidase fusion proteins (Harris, 1983). Furthermore, several techniques are available for the purification of β-galactosidase fusion proteins from total cell extract (Marston, 1986).

The *Pfhprt* gene like many other eukaryotic genes, contains a noncoding intervening sequence (intron) in the structural region (Sullivan et al., 1987; Khan and Scaife, personal communication). It was therefore decided to use the PfHPRT cDNA clone previously isolated and characterised (King and Melton, 1987), for expression studies, since intronic sequences in the structural gene would prevent its expression in *E. coli* (see introduction).

A second problem in the expression of eukaryotic genes in *E. coli* is that the transcriptional signals in eukaryotes are different from those of prokaryotes and are not usually recognised by prokaryotic RNA polymerase (Corden *et al.*, 1980). Moreover, the structure of eukaryotic mRNA is different to bacterial mRNA. For example, eukaryotic mRNA is polyadenylated at the 3′ end and normally capped at the 5′ end. Such structures may be important for ribosome binding or mRNA stability in eukaryotes (Breathnach and Chambon, 1981). On the other hand, eukaryotic mRNA does not seem to have an equivalent of the SD sequence present in prokaryotic mRNA (Kozak, 1981). These features in

eukaryotic mRNA mean that cDNA of eukaryotic origin may need to be manipulated to allow its expression in bacteria.

3.1 Structure of the cDNA of *Pfhprt*.

Figure 3.1 shows the nucleotide sequence of the cDNA clone isolated by King and Melton (1987). The sequence can be broadly divided into three distinct regions; a 5' noncoding sequence, an open reading frame (encoding a protein of predicted molecular mass of 26.392 kD) and a 3' noncoding sequence. The 5' region is 125 bp long and contains 4 translational stop codons in the same phase as the translational reading frame, two of which immediately precede the initiation codon. The expression of this cDNA clone, containing the 5' noncoding sequence, was therefore considered unlikely and may explain the failure of previous attempts to express the gene (King and Melton, 1987). Furthermore, this region is extremely A/T rich (88%), typical of the *P. falciparum* genome. The 3' flanking region of the cDNA clone which is 239 bp long, is also over 87% A/T rich, containing 2 long stretches of A's (27 and 28 bases in length) and a long AT ladder (36 bases long). Such AT rich segments may form stable secondary structure which could interfere with the translation of the recombinant mRNA. Secondary structure may also be responsible for the random deletions and rearrangements of *P. falciparum* insert DNA commonly seen in bacteria. Thus, for expression studies, it was considered necessary to remove from the cDNA clone, both the 5' sequence up to the initiation codon, and the 3' sequence, following the translational stop codon.

As mentioned in the general introduction, the A/T codon bias, observed in all *P. falciparum* genes thus far characterised, is also present in the *PfHPRT* gene. More specifically, a hindrance to the expression of the *P. falciparum*

```
          .         .         .         .         .         .
TAAAAATTTTAATAATATACTTTCACCACACCAAAAACCAAAAATATATATTTAATTCAT
          .         .         .         .         .         .
AATATTAAAGAAAATATATGTTTCTTTGTATATATTATTACTATATATTTATATAATATT
          . ***     .***    ***.         .         .         .
AGAAAATGCCAATACCAAATAATCCAGGAGCTGGTGAAAATGCCTTTGATCCCGTTTTCG
     MetProIleProAsnAsnProGlyAlaGlyGluAsnAlaPheAspProValPheV
          .         .         .         .         .         .
TAAAGGATGACGATGGTTATGACCTTGATTCTTTTATGATCCCTGCACATTATAAAAAAT
alLysAspAspAspGlyTyrAspLeuAspSerPheMetIleProAlaHisTyrLysLysT
          .    ***  .         .  ***    .         .         .
ATCTTACCAAGGTCTTAGTTCCAAATGGTGTCATAAAAAACCGTATTGAGAAATTGGCTT
yrLeuThrLysValLeuValProAsnGlyValIleLysAsnArgIleGluLysLeuAlaT
          .         .***      .         .         .         .
ATGATATTAAAAAGGTGTACAACAATGAAGAGTTTCATATTCTTTGTTTGTTGAAAGGTT
yrAspIleLysLysValTyrAsnAsnGluGluPheHisIleLeuCysLeuLeuLysGlyS
          .         .         .        ******   ***        .
CTCGTGGTTTTTTCACTGCTCTCTTAAAGCATTTAAGTAGAATACATAATTATAGTGCCG
erArgGlyPhePheThrAlaLeuLeuLysHisLeuSerArgIleHisAsnTyrSerAlaV
          .        ***   ***  .         .         .         .
TTGAGATGTCCAAACCATTATTTGGAGAACACTACGTACGTGTGAAATCCTATTGTAATG
alGluMetSerLysProLeuPheGlyGluHisTyrValArgValLysSerTyrCysAsnA
     ******   ******         .         .        ***   ***   .
ACCAATCAACAGGTACATTAGAAATTGTAAGTGAAGATTTATCTTGTTTAAAAGGAAAAC
spGlnSerThrGlyThrLeuGluIleValSerGluAspLeuSerCysLeuLysGlyLysH
     *** .         .         .         . ***      .         .
ATGTATTAATTGTTGAAGATATTATTGATACTGGTAAAACATTAGTAAAGTTTTGTGAAT
isValLeuIleValGluAspIleIleAspThrGlyLysThrLeuValLysPheCysGluT
  ***    .         .         .         .         .        ***.
ACTTAAAGAAATTTGAAATAAAAACCGTTGCCATCGCTTGTCTTTTTATTAAAAGAACAC
yrLeuLysLysPheGluIleLysThrValAlaIleAlaCysLeuPheIleLysArgThrP
        ***       .         .   ***    ***       .         .
CTTTGTGGAATGGTTTTAAAGCTGATTTCGTTGGATTCTCAATTCCTGATCACTTTGTTG
roLeuTrpAsnGlyPheLysAlaAspPheValGlyPheSerIleProAspHisPheValV
          . ***     .***      .         .         .         *
TTGGTTATAGTTTAGACTATAATGAAATTTTCAGAGATCTTGACCATTGTTGTTTGGTTA
alGlyTyrSerLeuAspTyrAsnGluIlePheArgAspLeuAspHisCysCysLeuValA
**       ***       .         .  ******  .         .         .
ATGATGAGGGAAAAAAGAAATATAAAGCAACTTCATTATAAATACATTTATTGAAGTGAT
snAspGluGlyLysLysLysTyrLysAlaThrSerLeuEnd
          .         .         .         .         .         .
CAAAAATGTCACAACCTTTCTATTTATATCAATTTAAAAAAAAAAAAAAAAAAAAAAAA
          .         .         .         .         .         .
AATAGAGAAGAAAATATCTGTACCAAAAATATATATATATATATATATATATATATATAT
          .         .         .         .         .         .
ATATGTATATATATATTTTATGTACATGTTATATATATTTATATATAACTTATAATGAAC
          .         .         .
GTGACTATATAAAAAAAAAAAAAAAAAAAAAAAAAAA
```

Figure 3.1. *Plasmodium falciparum* cDNA (King and Melton, 1987) The *E. coli* rare codons are shown in esteriscs at above and the stop codons in 5' end are underlined.

HPRT protein in bacteria, the cDNA clone contains certain A/T biased codons, e.g.. ATA (Isoleucine), GGA (Glycine) and TTA (Leucine) codons, are rarely, if ever, used in *E. coli* (Scaife, 1988).

The absence of any restriction enzyme sites in suitable positions,(Figure 3.2) in the noncoding regions of the cDNA prevented the removal of the flanking regions, without sacrificing portions coding sequence. To overcome similar problems other workers have digested with Bal31 (Silhavy et al., 1984). However, this technique has drawback. The digestion of DNA can not be properly controlled and therefore generates products of different sizes. Selection of an optimal fragment from this mixture is time consuming. Moreover, the DNA products of such treatment are blunt ended and therefore

difficult to clone in the correct frame and orientation. To avoid these problems, it was decided to use the Polymerase Chain Reaction (PCR) to remove the flanking sequences of the open reading frame of the PfHPRT cDNA and in addition, to help position this in the desired place and frame in expression vector. PCR is used to amplify a particular region from a large piece of DNA (Saiki *et al.*, 1988). In the following sections the strategy, used for the expression of PfHPRT as a β-galactosidase fusion protein will be explained in detail.

3.2 The expression vector and selection of host.

The vector used for the expression of PfHPRT as a β-galactosidase fusion protein in *E. coli* was pMS1S. The vector is one of a series of three, pMS1S, pMS2S and pMS3S, which differ only at the unique EcoRI site, positioned at the 3' end of the β-galactosidase gene, at which the reading frame is shifted.

The main features of the vector are shown in Figure 3.3. The vector contains the complete β-galactosidase gene up to amino acid 1023 which is

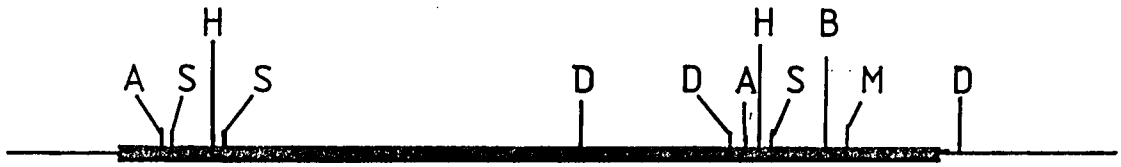
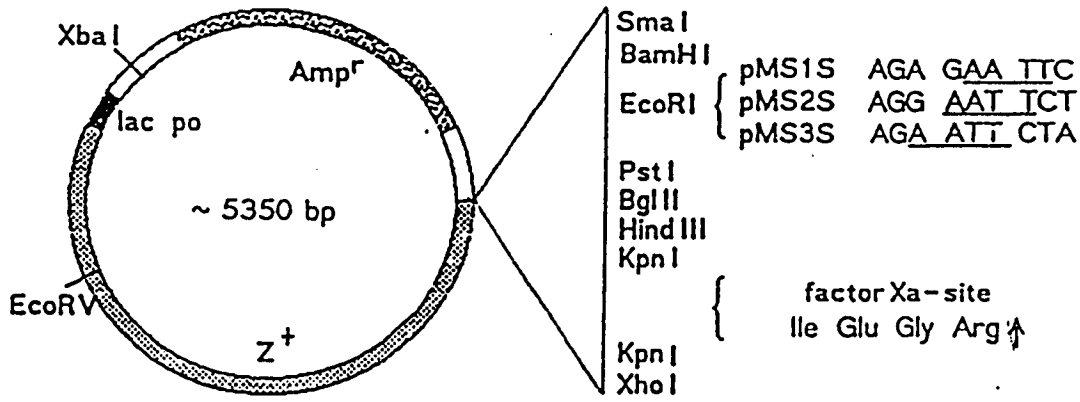


Figure 3.2 Restriction map of *Pfhprt* cDNA. A = AluI, B = BglII, D = DraI, H = HinfI, M = MnlI, S = Sau3AI. Bold line shows Pfhprt open reading frame.



→ lacZ 1023 Xho I Kpn I factor Xa-site Kpn I

5'- CAA AAA CTC GAG GTA CCG ATC GAA GGA CGT ACG GTA CCA

Ile Glu Gly Arg↑

EcoR I

Hind III Bgl II Pst I

AGC TTA CAG ATC TGC AG

pMS 1 S: A GAA TTC BamH I

pMS 2 S: G AAT TCT GAT CCT

pMS 3 S: A ATT CTA

stop - region Sma I

AGT AAT TAA TAG ATA ATA GCC CGG GCT TCC AAA TCG AGT -3'

Polylinker sequence of pMS1S, pMS2S, pMS3S.

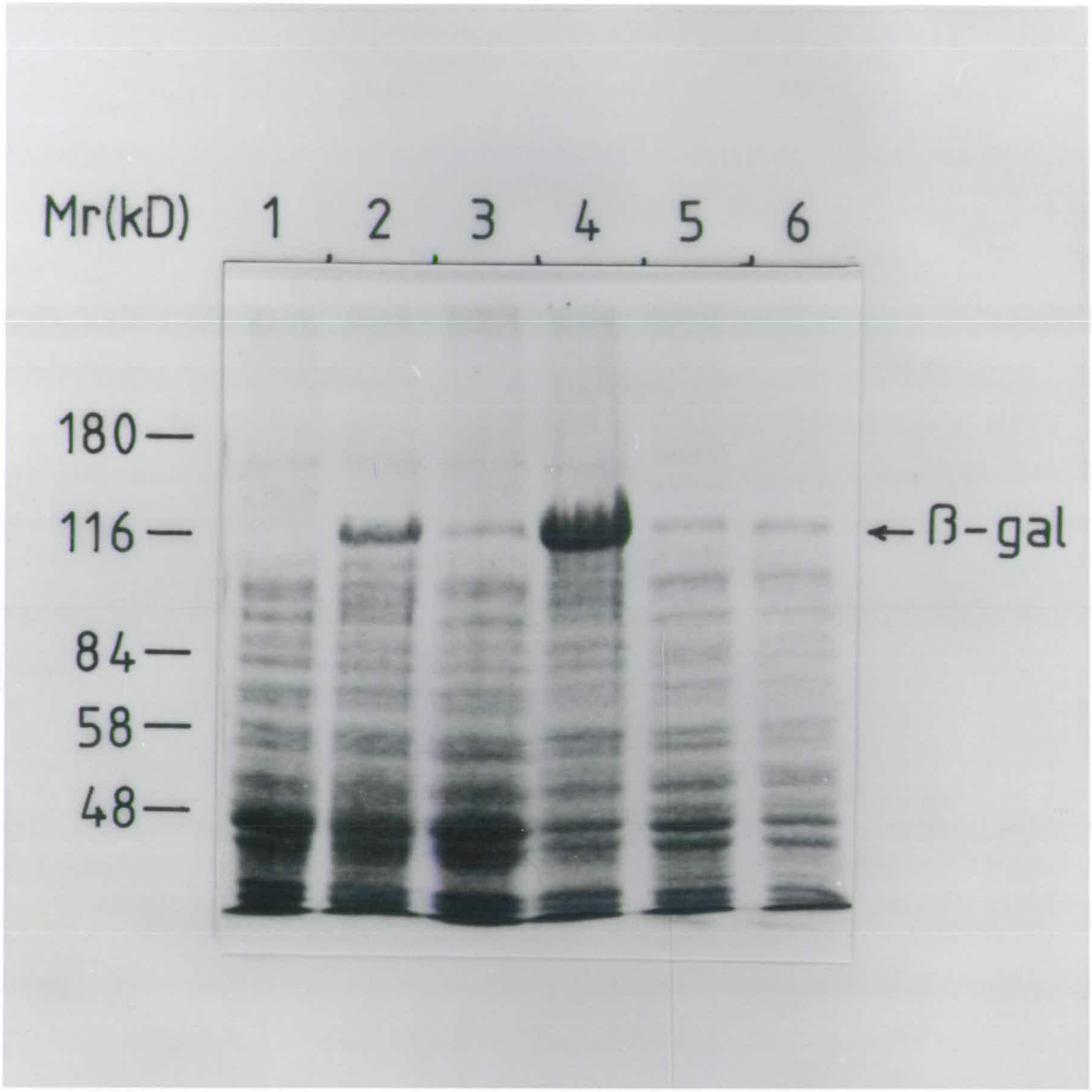
Figure 3.3 Structure of the pMS vector.

regulated under the control of the lac promoter. Immediately downstream of the 1023rd codon of the β-galactosidase, is an XhoI restriction site, followed by 2 KpnI sites which located a small polynucleotide sequence. On translation this sequence produces a tetrapeptide, Ile-Glu-Gly-Arg which is the recognition site for activated blood clotting factor X (factor Xa, Fuzikawa *et al.*, 1972). Following the second KpnI site is a short polylinker region, containing single HindIII, BglII, PstI and EcoRI sites. Translational stops are present in each of the three reading frames downstream of the polylinker region. A fragment of DNA can therefore, inserted into one of the three vectors as same frame with the β-galactosidase gene. When induced the foreign DNA will be translated as a fusion protein linked to the β-gal domain by the tetrapeptide. If this hybrid protein is treated with activated blood factor X, the foreign protein domain will be released (Nagai and Thogersen, 1984). The pMS vectors also contain the β-lactamase gene which confers ampicillin resistance.

The first step of using this vector would be to select a suitable host for it. Expression of the *lacZ* gene is repressed by maintaining the pMS1S plasmid in a host that overproduces lac repressor, i.e. due to the *lacI*^{q} allele. Two strains of *E. coli* carrying the I^{q} mutation, NM522, and TG1 were tested for suitability and efficiency. A strain without *lacI*^{q} allele, *E. coli* HB101 was included as a control. The three strains were transformed with the pMS1S plasmid and transformants were selected by ampicillin resistant phenotype and confirmed by isolating the vector from the selected transformants. The transformed strains were grown, induced and subsequently processed. Treated cell lysates, from a 300 µl culture were electrophoresed on a 7.5% SDS-polyacrylamide gel for protein profiles (method).

Figure 3.4 shows a typical coomassie stained gel. The result demonstrated that the β-galactosidase is expressed in induced TG1/pMS1S

Figure 3.4 Expression of β-galactosidase in different pMS1S transformed *E. coli* strain. (1) uninduced TG1/pMS1S, (2) induced TG1/pMS1S, (3) uninduced NM522/pMS1S, (4) induced NM522/pMS1S, (5) uninduced HB101/pMS1S and (6) induced HB101/pMS1S. The arrow shows *b*-galactosidase protein.

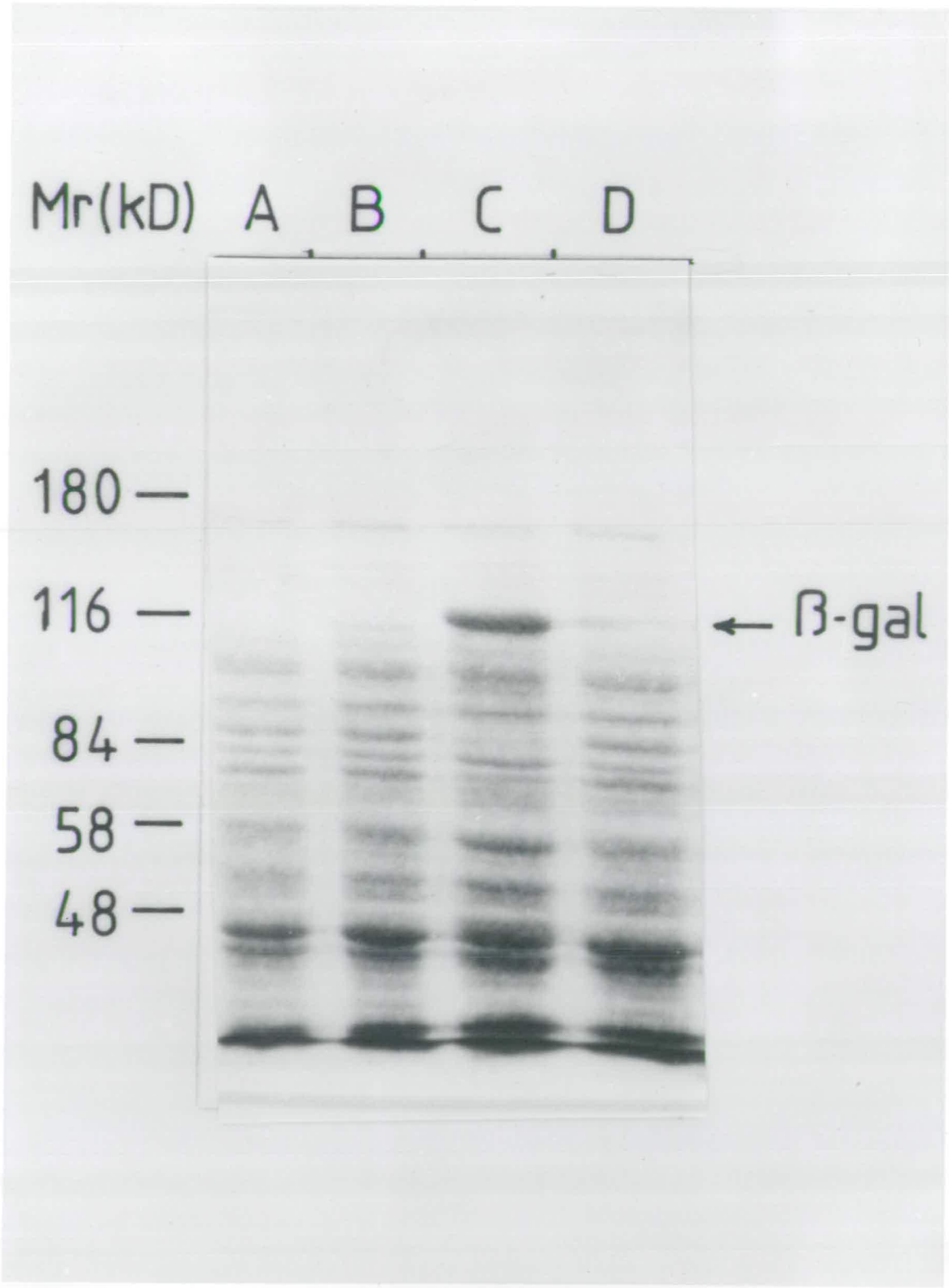

Mr(kD)
1
2
3
4
5
6
180
116
84
58
48
ß-gal

(lane 2) and NM522/pMS1S (lane 4). The protein is not present in the uninduced transformants (lanes 1 and 3), suggesting that the gene is well regulated in these strains. Visual comparison of the protein levels show that the induction of β-galactosidase is at least 4 to 5 times higher in NM522 (lane 4) than that in TG1 (lane 2). Strain NM522 was therefore selected as the host for pMS1S in expression studies. The strain did show some leakage in the regulation of β-gal when uninduced (lane 3), but this was ignored because the high level of expression of the protein, which was considered important for subsequent purification of the fusion protein. The protein is not induced in strain HB101/pMS1S (lane 6) to a high level, rather a low level expression was observed in both induced and uninduced transformants, suggesting the vector gene is not well regulated in this strain as expected from a non *lacI*^{q} mutant strain.

3.3 The origin and efficient induction, of the β-galactosidase was confirmed.

To confirm the origin of the β-galactosidase in induced NM522/pMS1S and to demonstrate the regulation of the vector gene more clearly, the profiles of uninduced and induced NM522 cells only and uninduced and induced NM522/pMS1S were compared in a 7.5% SDS-polyacrylamide gel. The coomassie stained gel shown in Figure 3.5, indicates that neither uninduced nor induced NM522 cells (lane A and B respectively) contain any visible level of β-galactosidase. Similarly no protein is observed in uninduced NM522/pMS1S (lane D). β-galactosidase protein is seen in the induced transformant NM522/pMS1S confirming that it is of vector origin. Comparison of the protein level in induced and uninduced (lanes C and D) NM522/pMS1S also confirms the efficient induction of the vector *lacZ* gene in this strain.

Figure 3.5 Origin of Induced β-galactosidase in *E. coli* NM522/pMS1S. (A) uninduced *E. coli* NM522, (B) induced NM522, (C) induced NM522/pMS1S and (D) uninduced NM522/pMS1S.


Mr(kD)
A
B
C
D
180
116
84
58
48
β-gal

3.4 Removal of 5' and 3' flanking regions of Pfhprt cDNA.

As mentioned before, it was considered important to remove the flanking sequences of the open reading frame (ORF) from the PfHPRT cDNA before inserting the coding region into the expression vector. The Polymerase Chain Reaction (PCR) was used in this study to achieve this. It was decided to insert the ORF of the PfHPRT amplified by PCR into the HindIII - EcoRI site of the vector polylinker to force the orientation. The sequences of the 2 oligonucleotide primers synthesized for the amplification of the *Pfhprt* ORF and their relative positions with respect to the cDNA sequence is shown in the Figure 3.6. Primer 1 (26 bases) was designed to incorporate the first 18 bases of the ORF starting from the ATG. Eight nucleotides, containing a sequence AAAGCTTA (HindIII linker) was included at the 5' end of the primer to facilitate the subsequent cloning of the PCR product. The 'A' introduced between the ATG and the HindIII linker placed the ORF of the PfHPRT in frame with the vector *lacZ* gene.

Primer 2 (22 bases) was designed from the antisense strand, spanned from position 818 to 839 in the cDNA (see Fig 3.1). This included the stop codon TAA. Two bases at position 836 and 838 was changed to create an EcoRI site at the 3' end of the amplified product. The DNA used as the template in the PCR reaction was the recombinant plasmid containing PfHPRT cDNA inserted into the EcoRI site of pUC8 plasmid (King and Melton, 1987). The plasmid was first linearised with HindIII and the PCR reaction was carried out according to the protocol described section.

After 20 cycles, 10 µl of the reaction mixtures were analysed by 0.8% agarose gel. The gel shown in Figure 3.7 indicates that a DNA fragment of about 700 bp (lanes B and C) was amplified from the 3.8 kb plasmid (lane A), which is of the expected size for the amplified ORF (720 bp). The

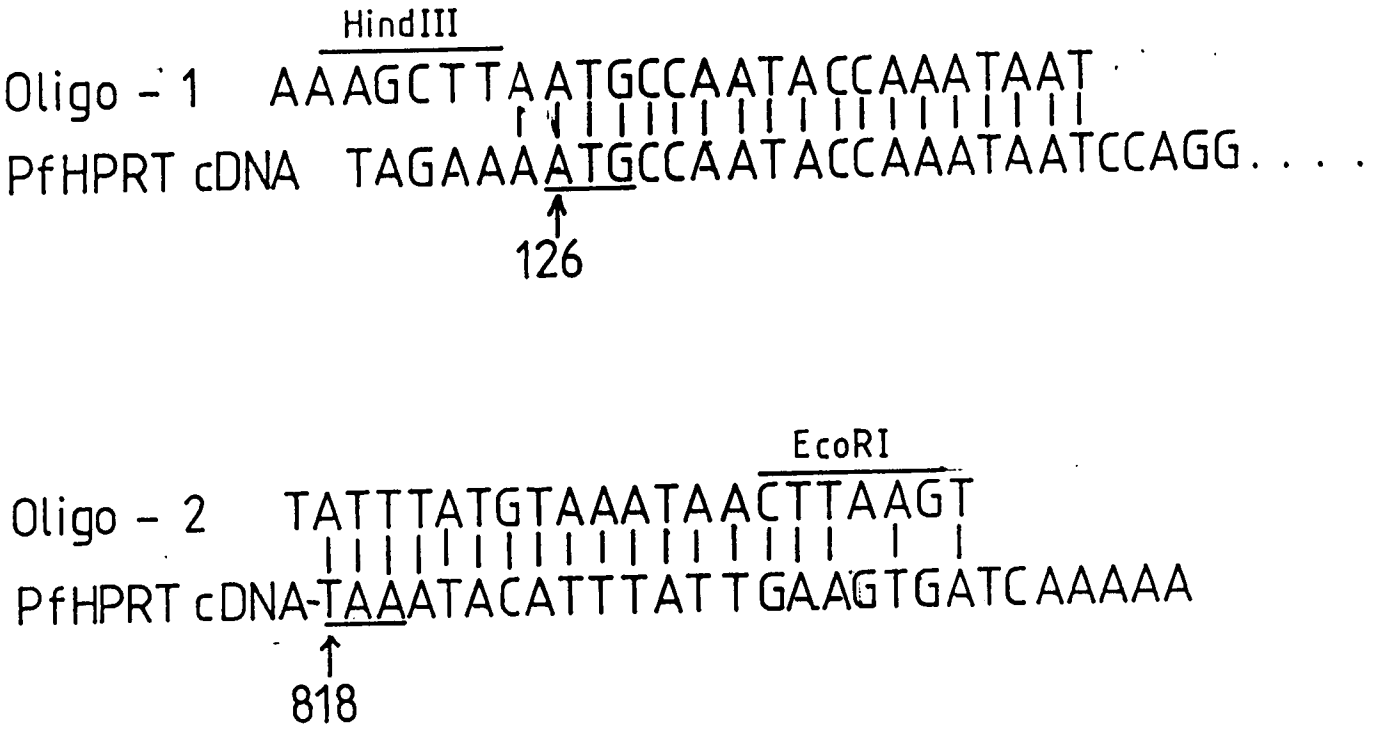
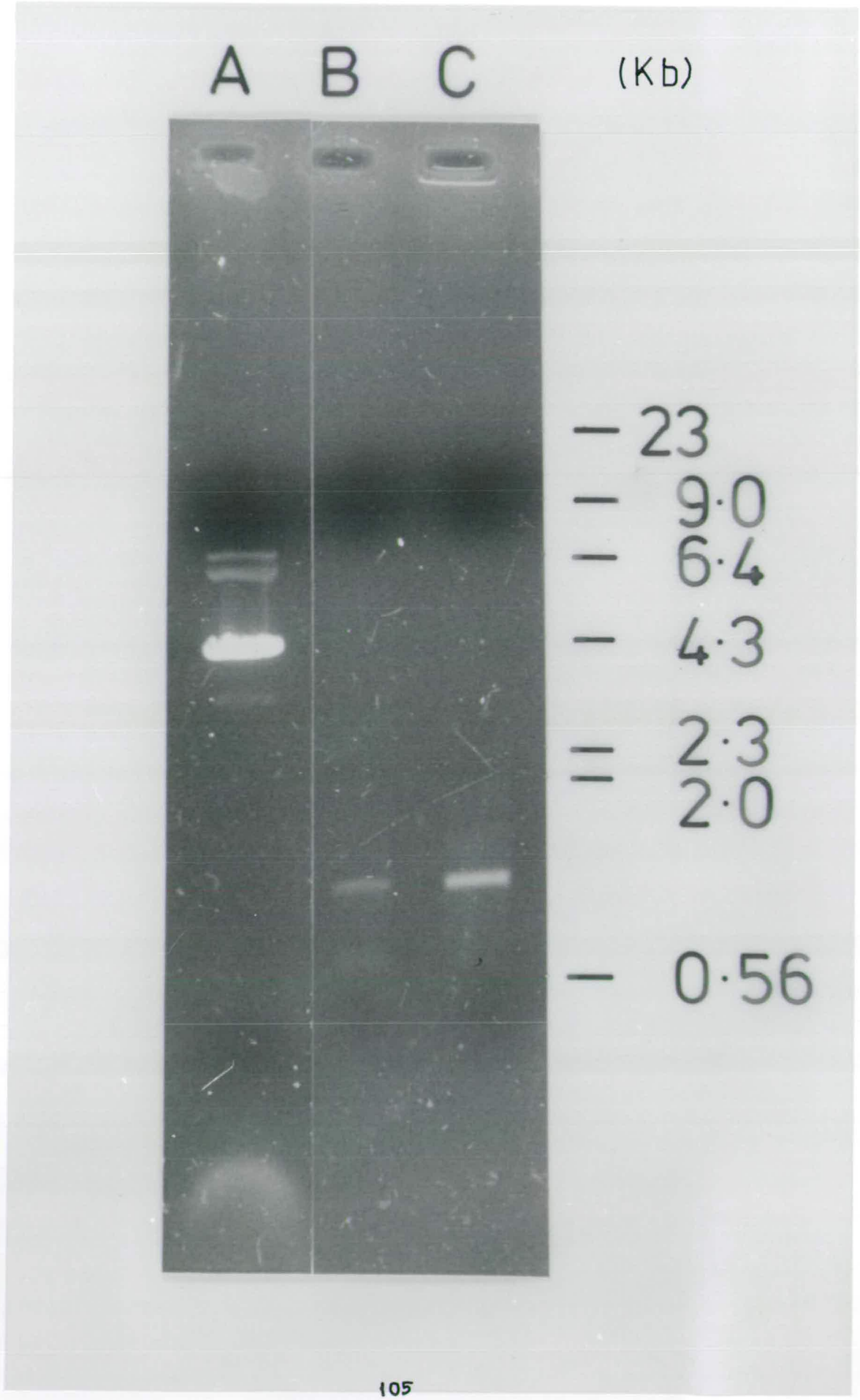


Figure 3.6 Oligonucleotide primers for PCR reaction and its relative position in *Pfhprt* cDNA (fig. 3.1).

Figure 3.7 PCR amplification of *Pfhprt* open reading frame from pUC8-*Pfhprt* cDNA clone. (A) HindIII cut plasmid, (B) 10 µl PCR reaction mix with 50 ng target DNA and (C) same as (B) except with 100 ng of target DNA.


A
B
C
(Kb)
23
9·0
6·4
4·3
2·3
2·0
0·56

concentration of the product was higher where twice the amount of target plasmid was used, indicating that the amplification was proceeded at an proportionate rate. To confirm that the amplified product of the PCR reaction contained the predicted fragment, the gel was southern blotted and probed with labelled *PfHPRT* cDNA insert of the pUC8 clone (above). The result in Figure 3.8, shows that the amplified fragment was recognised by the cDNA probe. The PCR reaction mix was subsequently extracted with phenol:chloroform and ethanol precipitated.

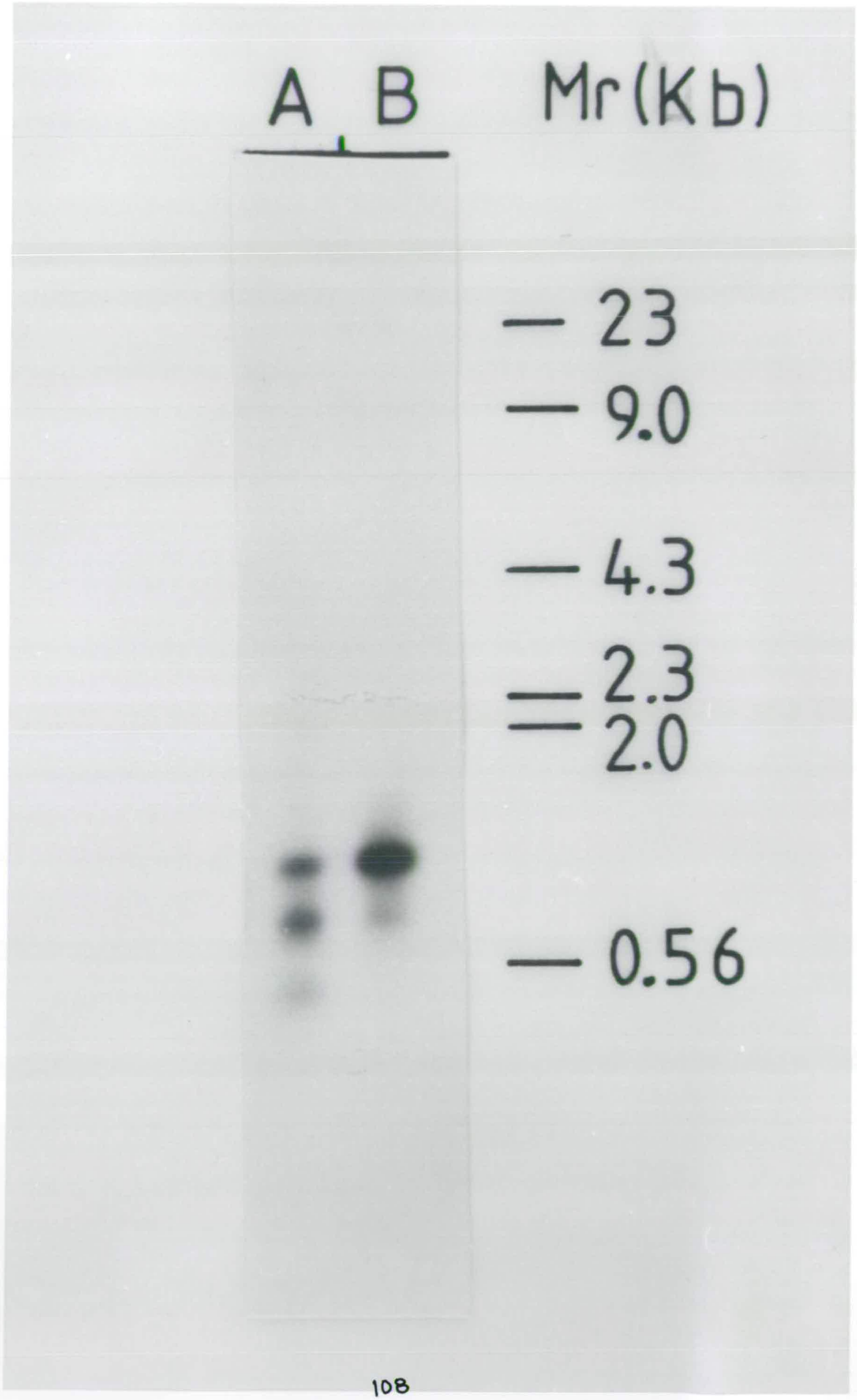
3.5 Construction of the lacZ:Pfhprt fusion plasmid.

Detail of the construction strategy of the recombinant plasmid are shown in Figure 3.9. The amplified DNA product of the PCR reaction is predicted to be blunt ended and contain a complete ORF from ATG to TAA with a HindIII site preceding the ATG and an EcoRI site following the TAA. To insert into the HindIII-EcoRI site of the expression vector pMS1S, the PCR product was digested with both HindIII and EcoRI and ethanol precipitated. The vector DNA was also digested with HindIII and EcoRI and subsequently purified from 0.8% agarose gel. Gel purification of the linear vector removed the small polynucleotide linker (19 bases), thus preventing selfligation of the vector in the subsequent reaction. The vector was ligated with the digested PCR product in a vector:insert ratio of 1:5 .

3.6 Transformation of the recombinant plasmid into E. coli NM522.

The ligated mixture was transformed into the selected host E. coli NM522 and the transformation mixture were spread on minimal-agar plates with ampicillin and incubated overnight at 37 C. Ampicillin resistant bacteria were

Figure 3.8 Hybridization of the PCR product with labelled Pfhprt cDNA. (A) and (B) PCR products from two different reactions.


A B
Mr(Kb)
23
9.0
4.3
2.3
2.0
0.56

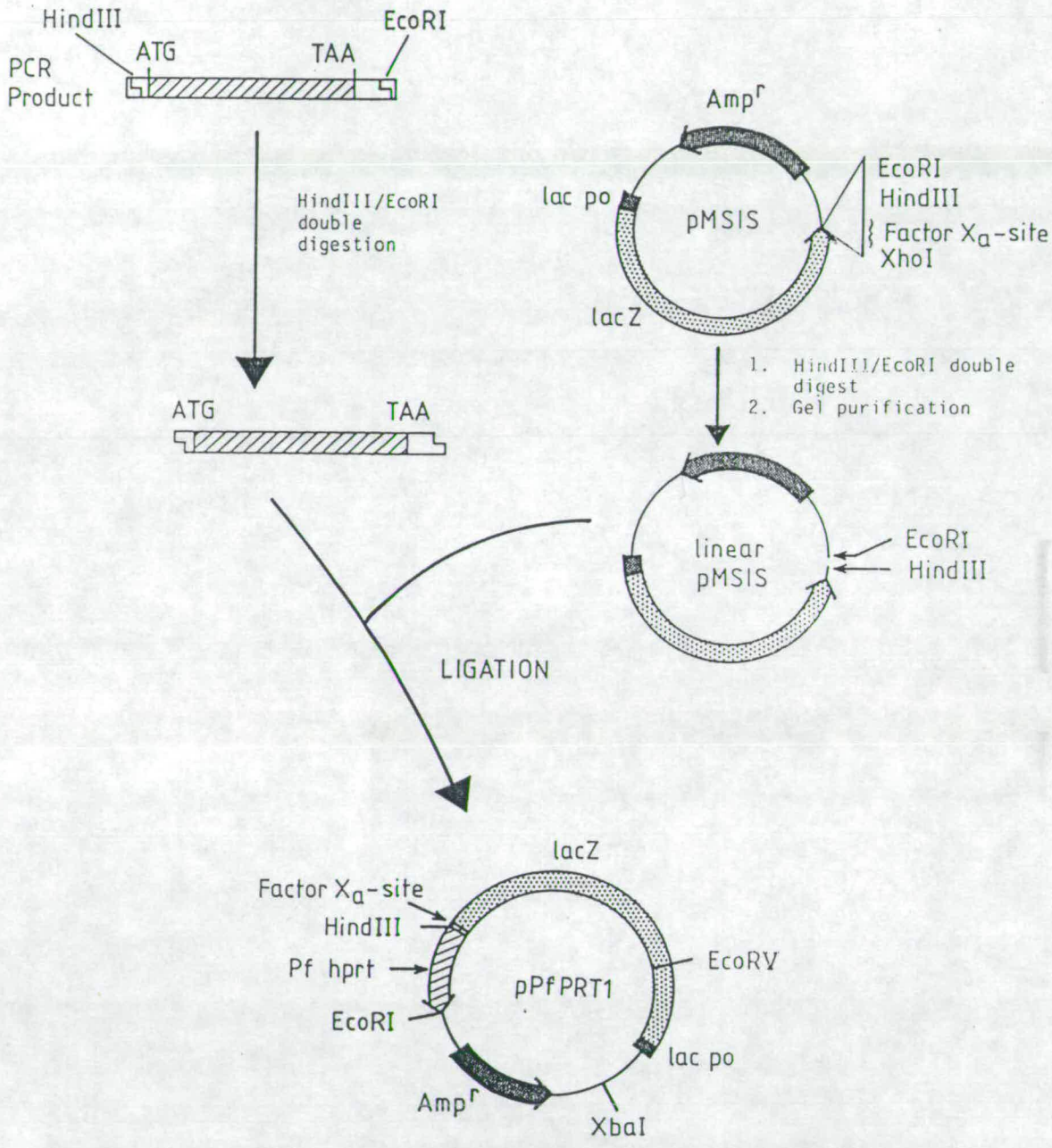


Figure 3.9 Construction of the recombinant plasmid, pPfPRT1.(see Text)

screened for the presence of *Pfhprt* gene by colony hybridisation using labelled purified PfHPRT cDNA. The blot is shown in Figure 3.10. The frequency of positive clones identified was approximately one in 15 ampicillin resistant colonies. One of the positive clones were selected for further study, and called strain **SH1**.

3.7 Confirmation of the structure of the recombinant plasmid.

To confirm the structure, the recombinant plasmid was isolated from strain SH1 by mini plasmid preparation and digested with HindIII and EcoRI or EcoRI only and analysed on a 0.8% agarose gel. Figure 3.11 shows the results. Upon digestion with HindIII and EcoRI a DNA fragment is released of the predicted size, about 700 bp (Lane A). Lane B shows the recombinant plasmid digested with EcoRI only. The recombinant plasmid was named **pPfPRT1**.

3.8 Expression of the fusion protein in SH1.

The strain SH1 was then studied for the expression of the recombinant protein. The bacterium was grown, induced and cells from 1.5 ml culture were processed for SDS-PAGE [method].

Protein profiles of the induced SH1, uninduced SH1 and pMS1S transformed NM522 were analysed on 7.5% SDS-polyacrylamide gel. Figure 3.12 shows a typical coomassie stained gel. The result shows that as expected β- galactosidase was induced from the pMS1S transformed NM522 cells (lane C). In contrast, a protein of approximately 140 kD was expressed in induced SH1 cells (lane B). This is a predicted size of the β-galactosidase:PfHPRT fusion protein (26.4 kD plus 116 kD). This protein was neither present in uninduced SH1 (lane A) nor in the induced pMS1S transformed NM522 (lane C).

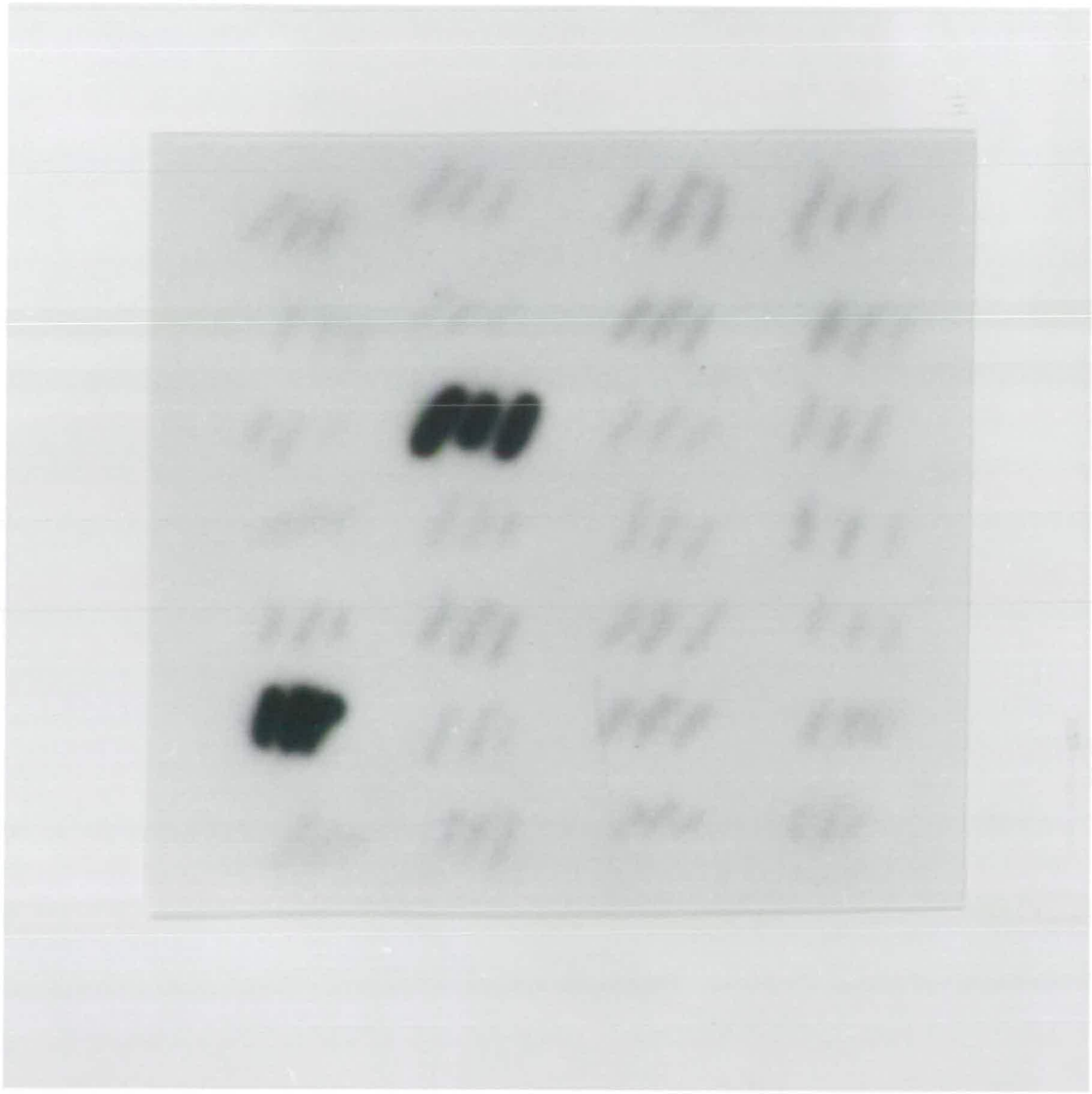


Figure 3.11 Release of insert from pPfPRT1. (A) EcoRI and HindIII digested and (B) only EcoRI digested pPfPRT1.

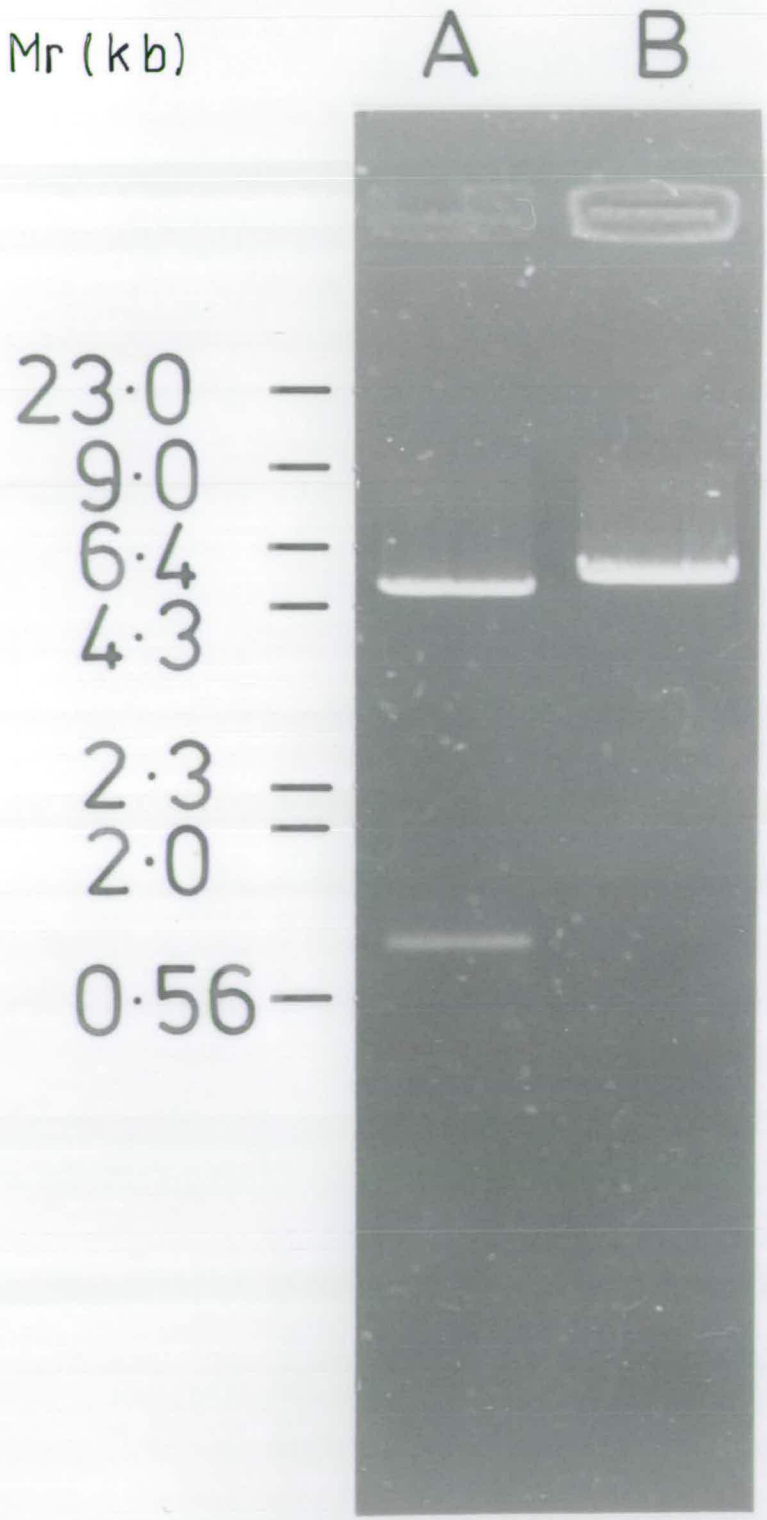
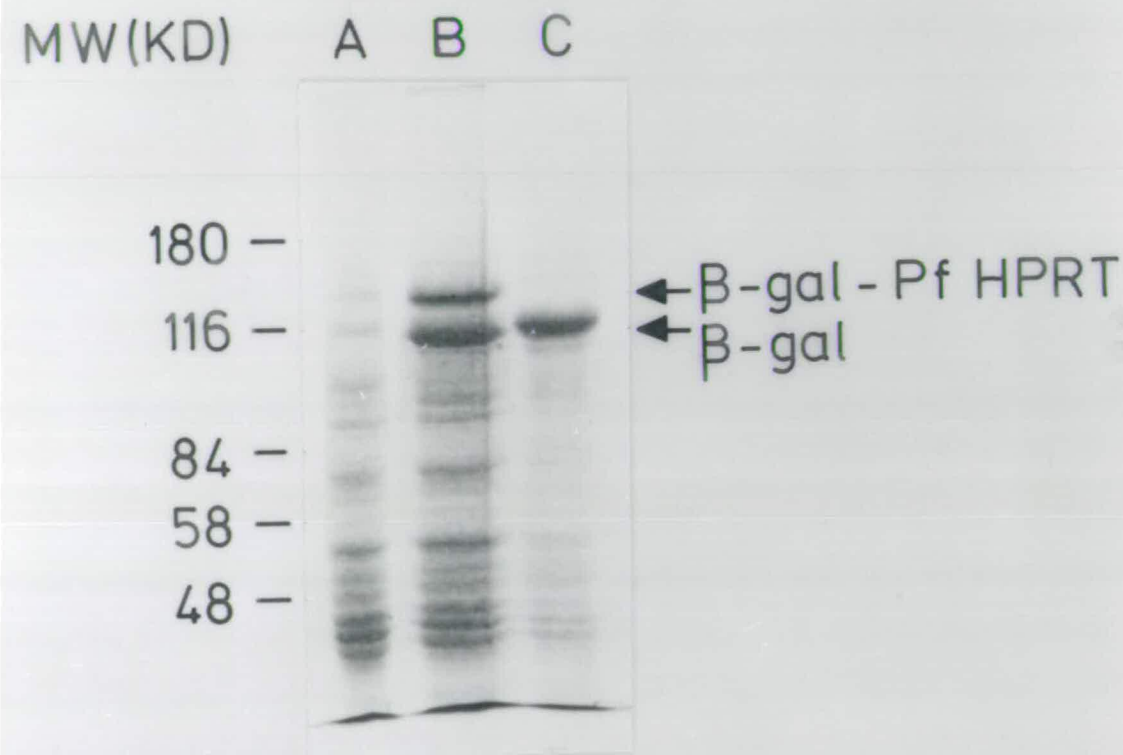

Mr (kb)
A
B
23·0
9·0
6·4
4·3
2·3
2·0
0·56

Figure 3.12 Expression of β-galactosidase:PfHPRT fusion protein. (A) Uninduced SH1 (*E. coli* NM522/pPfPRT1), (B) induced SH1 and (C) induced NM522/pMS1S.


MW(KD)
A
B
C
180
116
84
58
48
β-gal - Pf HPRT
β-gal

3.9 Enzymatic activity of the fusion protein.

One of the ways of proving the authenticity of the expressed fusion protein, is to show that it possesses both β-galactosidase and HPRT activity. This depends on the correct folding of the protein in E. coli and the effect of the β-galactosidase domain on the folding process of the HPRT domain and vice versa. There is evidence that β-galactosidase fusion proteins retain the activity of the foreign protein. To study this possibility cell free extracts of uninduced SH1, induced SH1 and induced NM522 were assayed for the HPRT activity. Activity of induced SH1 extracts was found to be more than 15 times greater than that present in extracts of uninduced SH1 (Figure 3.13 bar 3/4). Induced SH1 extract showed 20 times more HPRT activity than the extracts of induced NM522 (bar 2/3).

Similar result was obtained when the extracts were assayed for β-galactosidase activity. Results shows that the induced NM522 did not have any activity for this enzyme (bar 1) whereas, induced SH1 showed at least 50 times more activity than the uninduced SH1 (data not shown). This results indicate that the fusion protein is active for both β-galactosidase and HPRT activity.

3.10 Discussion and conclusion.

This chapter has described successful expression of *P. falciparum* HPRT in *E. coli* as β-galactosidase fusion protein.

The selection of an *E. coli* host for the expression vector pMS1S, led us to choose Strain NM522. This strain expressed the vector encoded β-galactosidase more efficiently than strain TG1, which also contains $lacI^{q}$ allele. The reason for such high expression level may be, the maintenance of the

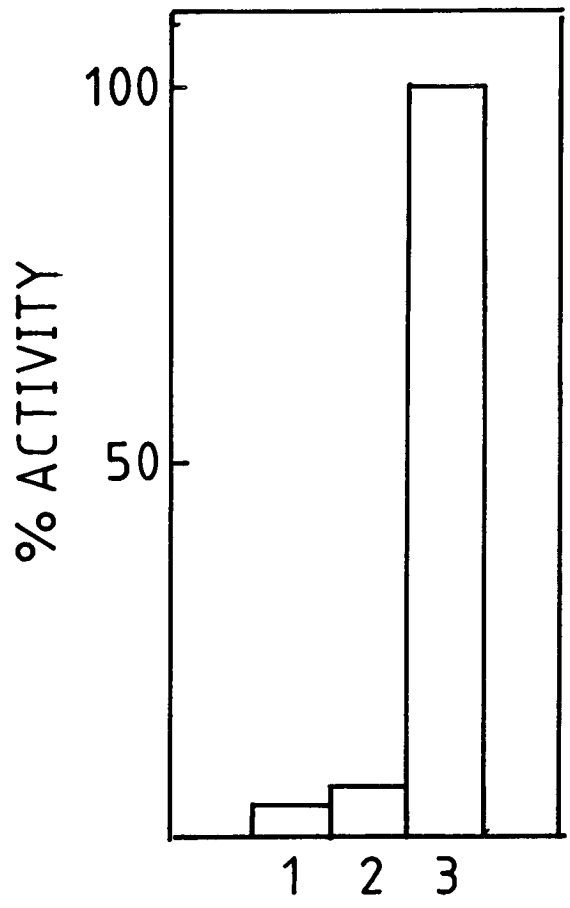


Figure 3.13 HPRT activities in recombinant and parent *E. coli*. Activities in bacterial extracts were measured by following incorporation of ^{3}H-hypoxanthine into IMP (methods). Data are expressed as percentages of the induced recombinant, SH1, (bar 3). (bar 1) induced NM522 and (bar 2) uninduced SH1.

higher copy number of the plasmid in NM522 than in TG1. Effect of high copy number the accumulation of a recombinant protein is described by O'Farrel *et al* (1978). These authors demonstrated increased expression of the plasmid encoded β-galactosidase by increasing the copy number of the plasmid in the cell.

A second possible reason of the high protein level in NM522 is less degradation of the expressed protein compared to that in TG1. Proteolytic degradation of expressed protein in *E. coli* is a normal metabolic process. However, the rate of degradation is different for different proteins. Such degradation is also depends on the genotype of the strain. For example, mutation in the *lon* gene (codes for a protease) reduce the degradation of nonsense polypeptides (Bukhari and Zipser, 1975).

The expression of the plasmid gene in NM522 was found to be well regulated as shown in figure 3.5, lane D. The result showed that no visible level of β-galactosidase was present in the uninduced cell. This property is essential for a good expression vector, because, overexpression of a recombinant protein is a metabolic burden to the cells and results in slow growth rate (Carrier *et al.*, 1983). In such cases, cells which lose their plasmid soon outgrow plasmid-containing cells during cultivation. Tight control of expression allows the cells to be grown first to high biomass before induction of recombinant gene expression.

The analysis of the PfHPRT cDNA revealed that the structure is typical of a eukaryotic cDNA and more precisely that of the plasmodium DNA. For example, the stop codons preceding the translation initiation codon, the poly A stretches and extremely A/T biased codons in the coding region. These led me to modify the cDNA for cloning in the expression vector pMS1S; in particular I wished to remove the flanking sequences of the open reading frame. . The

polymerase chain reaction (PCR) was considered to be the best for this purpose. PCR is a powerful tool for amplifying a specific region, from a large fragment of DNA. By using thermostable *Thermophilus aquaticus* DNA polymerase (Taq DNA polymerase) and two oligonucleotide primers which can bind to the DNA in two positions of the opposite strands separated by the desired sequence, it is possible to amplify the intervening sequences. This strategy was exploited in this study to remove the flanking sequences from the PfHPRT cDNA.

To force the orientation of the insertion into the vector two different enzyme sites (HindIII and EcoRI) were created at the ends of the *PfHPRT* cDNA ORF. The HindIII site in the vector was chosen for two reasons. Firstly, to force the orientation of the insertion and secondly, to minimize the presence of extra amino acids in N-terminal of the released PfHPRT domain from the fusion protein, after factor Xa digestion.

In the PCR reactions a high concentration of the target DNA were used, compared to the suggested low amount. The rationale was, although the technique is highly sensitive and can use picogram level of target DNA, but this needs large number of cycle to amplify the desired sequence in a usable amount and because it is known that Taq polymerase can incorporate wrong bases during synthesis and these mistakes could amplify during the subsequent amplification steps. It is therefore suggested that use of higher amount DNA may reduce the chance of amplification of such mistakes, because usable amount of target sequence can be produced using minimum number of cycles (3.23).

The desired sequence was successfully amplified, indicated by the size of the fragment and confirmed by the fact that the PCR product was hybridized with the purified and labelled PfHPRT cDNA (Figure 3.8). In the blot there are

two extra DNA bands recognised by the probe. These may be the denatured PCR product as this was not seen when fresh PCR product was separated and probed. This is a phenomenon previously noted with an other small piece of *P. falciparum* DNA (Md. Shahabuddin, unpublished), and probably due to the A/T rich sequences which confer weak hybridization of two strands of DNA compared to G/C rich sequences.

The identification of the recombinant plasmid as selected by the labelled PfHPRT cDNA and the subsequent release of the insert from the plasmid with HindIII and EcoRI double digestion, suggested that the construction procedure had gone according to plan. For instance, the PCR reaction produced DNA fragments containing *PfHPRT* ORF with HindIII and EcoRI site at the ends, the PCR products were then successfully digested and produced sticky ends which were ligated with the double digested vector and produced the recombinant *lacZ:Pfhprt* fusion plasmid, pPfPRT1. the *E. coli* cells harbouring this plasmid is called strain SH1.

Induction of the SH1, with IPTG showed that a protein of 140 kD, similar to the size predicted for the fusion protein (142.3 kD) was expressed only in the induced bacterium. The absence of this protein in induced pMS1S transformed NM522 suggested that the protein is of the recombinant plasmid origin. So, it was concluded that *P. falciparum* HPRT is expressed in The *E. coli* strain SH1 as a β-galactosidase fusion protein.

The expression study also showed an extra protein of about 115 kD is expressed in the induced protein. Although it is difficult to suggest any explanation for this protein at this instance, but later studies using western blot with antiβ-galactosidase and and antiPfHPRT antibodies showed that the protein is a degradation product of the fusion protein. This point will be discussed in detail in the next chapter.

To confirm the authenticity of the expressed fusion protein, the cell free extract of the induced SH1 was assayed for both HPRT and β-galactosidase activity. The result conclusively suggested that the strain SH1 upon induction expressed the fusion protein which is active for both the enzymatic activity. One may argue that the hpt^+ host, NM522 contributed in the higher HPRT activity. However, this possibility was excluded since induced SH1 extract showed 20 times more activity than the extracts of induced pMS1S transformed NM522. The induced recombinant protein also showed high activity of the β-galactosidase whereas the induced host did not show any activity for this enzyme. It may be worth mentioning that the lac operon in NM522 is deleted and a ΔM15 mutant of the *lacZ* gene is present in the F′ episome present in the cell. The protein from this episome encoded enzyme has no enzymatic activity.

The expression of the *P. falciparum* HPRT in *E. coli* suggested that the A/T biased codons, which are comparatively rare in *E. coli* did not stop the bacterium to express the gene as was suggested earlier (king and Melton, 1987). However, It is possible that such codons reduced the rate of expression of the gene in the bacterium. Changing the so called rare codons to abundant codons and comparing the extent of expression, the effect of these codons in the expression of the gene could be revealed.

E. coli is the most studied organism, widely used in the field of biotechnology. Several important proteins of medical importance have been overexpressed in this organism (Harris, 1983). These also include portions of *Plasmodium* antigens (Gentz *et al.*, 1988) and enzymes (Simmons *et al.*, 1985). However, thus far no *Plasmodium* protein has been successfully expressed in full length in *E. coli*. In most of the cases, expression of only part of the protein is reported. This chapter thus demonstrating for the first time the expression of a full length plasmodial protein as a fusion protein. furthermore,

the activity of the recombinant PfHPRT suggest that the expressed protein also fold into the native form in and retain its biological activity. Such successful expression thus suggest that *E. coli* could be a successful system for the expression of plasmodial genes. For example, the production of vaccine antigens.

Chapter 4
Result 2

Characterisation and purification of the β-galactosidase:PfHPRT fusion protein

Once the recombinant PfHPRT is made, the next step is to study the property of the protein in detail and to purify this for making antibodies against PfHPRT. Purification of a recombinant protein may depend on its location in the host bacterium. An increasing number of reports of eukaryotic protein synthesis in *E. coli* suggest that, in general, recombinant proteins accumulate to higher levels than total cell protein in the cytoplasm, when expressed intracellularly. However, many of these proteins aggregate and form insoluble inclusion bodies in the cytoplasm (Wittrup *et al.*, 1988). Purification of the recombinant protein from them does not just involve the application of chromatographic techniques; the primary objective is to recover active and soluble protein. This sometime may need strong denaturing agents, like urea or guanidine hydrochloride.

Such harsh treatments often unfold the polypeptide to inactive form. So, the next step is the adjustment of conditions to refold the protein back to active form. Finally chemical or enzymatic cleavage of the fusion protein is performed to recover the recombinant foreign protein domain. Once the polypeptides are solubilised, conventional chromatographic techniques can be applied to purify the protein.

Some recombinant proteins however, remain predominantly in soluble form, examples include β-galactosidase fusions with HBV preS2 region (Offensperger, *et al.*, 1985) and Cholera toxin CTP3 (Jacobs, *et al.*, 1985). Occasionally, this depends on the host and the temperature at which the recombinant bacterium is grown (Schein, 1989). In some cases only a fraction of the expressed protein is soluble, the remainder being located in inclusion bodies. Usually, the purification of such soluble fusion proteins is quite straightforward employing an affinity chromatography step; either immunopurification or substrate affinity chromatography (Ullmann, 1984).

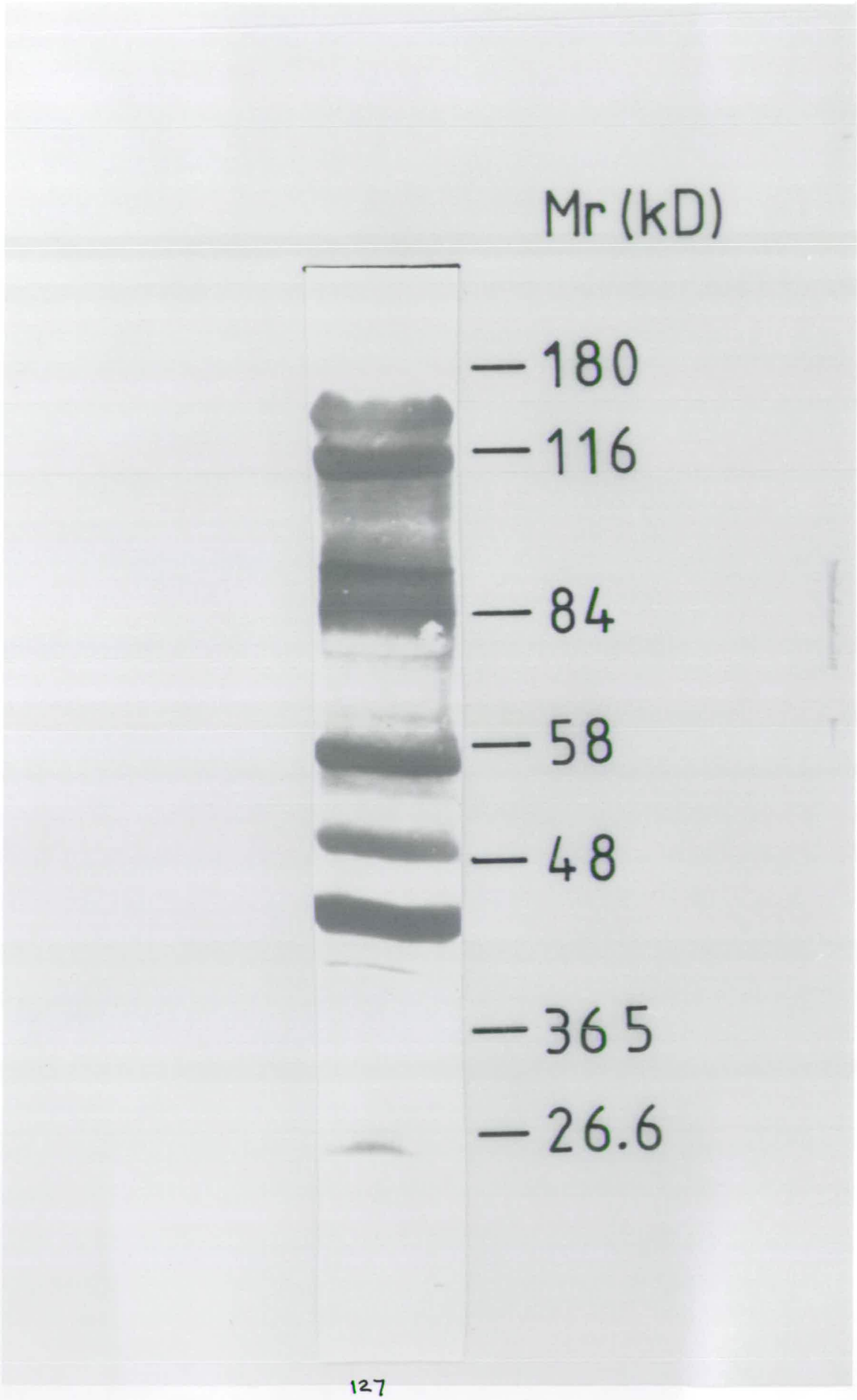
Moreover, degradation of the a recombinant protein can occur in vivo, leading to the major product that is not full length. Similar result can also be observed if the translation of the recombinant mRNA starts at an internal initiation codon (Hope I , 1984). To reduce the chances of misleading results, western blot analysis of the β-gal fusion protein lysate is employed with anti-β-galactosidase antibody to detect any minor products present. In this chapter, the fusion protein will be studied for its degradation pattern and location in the host bacterium, then varius methods will be employed to purify this from the cell extract. After that, attempt will be taken to release the PfHPRT domain from the fusion protein. Finally, purified protein will be used to make antibodies against PfHPRT.

4.1 Anti-β-gal antibody recognised the fusion protein.

To study the degradation pattern of the fusion protein in the cell, anti β-galactosidase antibody was used to probe the separated protein profile in an western blot analyse.

Lysates of whole induced cells from 300 µl culture were separated on a 10% SDS-polyacrylamide gel [method]. The separated proteins were then western blotted and probed with a polyclonal antiβ-galactosidase antibody. The result (Figure 4.1) suggests that the fusion protein is recognised by the fusion protein as expected. In addition to this, the result also shows several other proteins of size ranging from 20 - 115 kD are recognised by the antibody, suggesting that the recombinant protein is severely degraded in the bacterium.

Figure 4.1 Recognition of β-gal:PfHPRT fusion protein by antiβ-galactosidase antibody. Proteins from induced SH1 were separated on a 10% SDS-polyacrylamide gel, western blotted (method) and probed with polyclonal antiβ-gal antibodies.


Mr (kD)
180
116
84
58
48
36.5
26.6

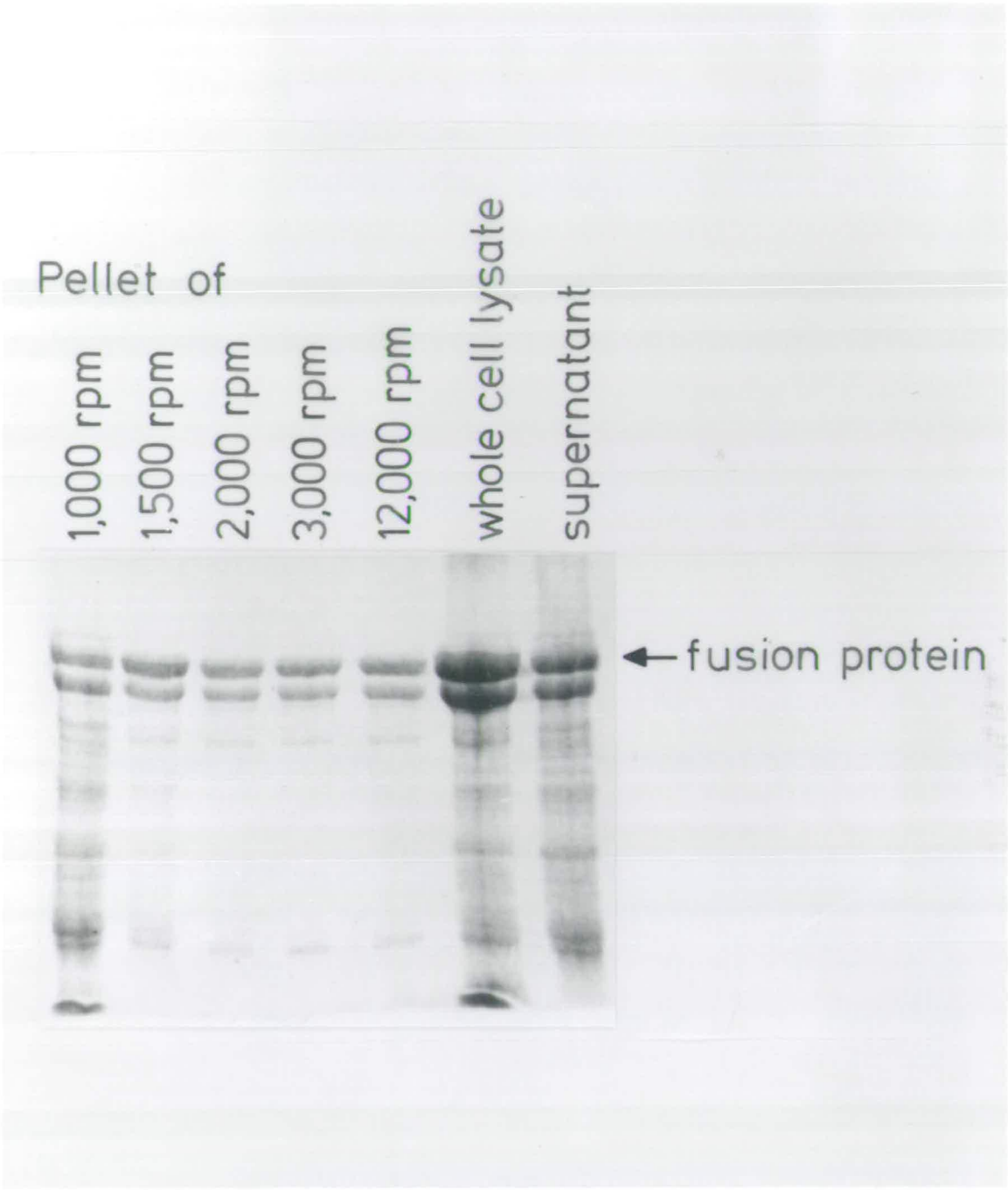
4.2 The fusion protein forms insoluble inclusion bodies in the cell.

As mentioned at the begining of this chapter that β-gal fusion proteins often aggregate and form insoluble inclusion bodies inside cells. Such aggregated forms are sometime useful for the purpose of purification of the fusion protein. The inclusion bodies are dense and sediment readily with low speed centrifugation (Schoner *et al.*, 1985). Speeds as low as 500xg can pellet such aggregates but for some up to 12000xg is used (Marston 1987). Fusion proteins may be purified from isolated inclusion bodies and used without any further treatment (Cabradilla *et al.*, 1986). Kleid et al (1981) used intact fusion proteins in the development of vaccines for FMDV(VP1).

To check whether the β-gal:PfHPRT fusion protein forms such inclusion bodies, following experiment was designed. Lysates of induced SH1 cells from a 500 ml culture were prepared and subjected to differential centrifugation [method]. Briefly, the lysate was centrifuged at 1000 rpm for 20 minutes in a bench top Chillspin centrifuge, to remove the unlysed cells and the cell wall debris. The supernatant was then centrifuged sequentially at 1500, 2000, 3000 and finally 12000 rpm for 15 minutes each. Pellets from each centrifugation steps were washed with the lysis buffer once and stored at -20°C. Aliquots from the pellet samples and final supernatant were analysed on a 8% SDS-PAGE for their protein profile and compared with that of the whole cell lysate. The result, shown in Figure 4.2, demonstrated that at each centrifugation speed the fusion protein is pelleted and the amount of the pelleted fusion protein was found to be similar in each pellet.

The major outcome of this experiment is that the pellets of 2000 rpm, 3000 rpm and 12000 rpm seems to contain more than 80 to 90% pure fusion protein. This suggests that the inclusion bodies can be isolated with a

Figure 4.2 differential centrifugation of sonicated induced SH1 lysate. Proteins from the pellets of different centrifugal forces, whole cell lysate and the final supernatant were separated with by SDS-PAGE (method) and stained with coomassie blue.


Pellet of
1,000 rpm
1,500 rpm
2,000 rpm
3,000 rpm
12,000 rpm
whole cell lysate
supernatant
fusion protein

considerable extent of purity simply by centrifugation. Nevertheless, much of the fusion protein was present in the soluble portion of the cells and could be recovered in the supernatant fraction of the lysate.

4.3 Purification of the recombinant fusion protein.

Several methods has been described in the literature to purify fusion proteins (Marston, 1986). The isolation of the inclusion bodies is quite simple. However, these are often resistant to mild detergents like, Triton X-100, and recovery of native fusion protein from them need stringent chemical treatment. On the other hand, purification from the soluble fraction of the cell lysate is lengthy and time consuming. The overall strategy used to purify β-gal:PfHPRT fusion proteins is described in the following sections and shown in Figure 4.3.

4.3.1 Purification of the fusion protein from the inclusion bodies.

To purify the protein from the inclusion bodies cell lysate from the cells from 500 ml culture of induced SH1 were prepared as described in method section. The lysate was then centrifuges at 1500 rpm for 15 minutes in an SS34 rotor at 4°C. The pellets were discarded and the cloudy supernatant were subjected to high speed centrifugation at 10000 rpm for 15 minutes to collect the inclusion bodies. The pellet thus obtained was washed twice with and resuspended in 1 ml lysis buffer. Such inclusion bodies are presumably free from soluble protein and looked like a creamy-white amorphous powder. On SDS-PAGE the preparation appeared to contain mainly the fusion protein (data not shown).

Contaminating materials do co-precipitate with the inclusion bodies. They may be other proteins of cell or other cellular materials, which could affect the efficiency of the solubilisation, cleavage and refolding. Washing of inclusion bodies with dilute nonionic detergents often removes such contaminants

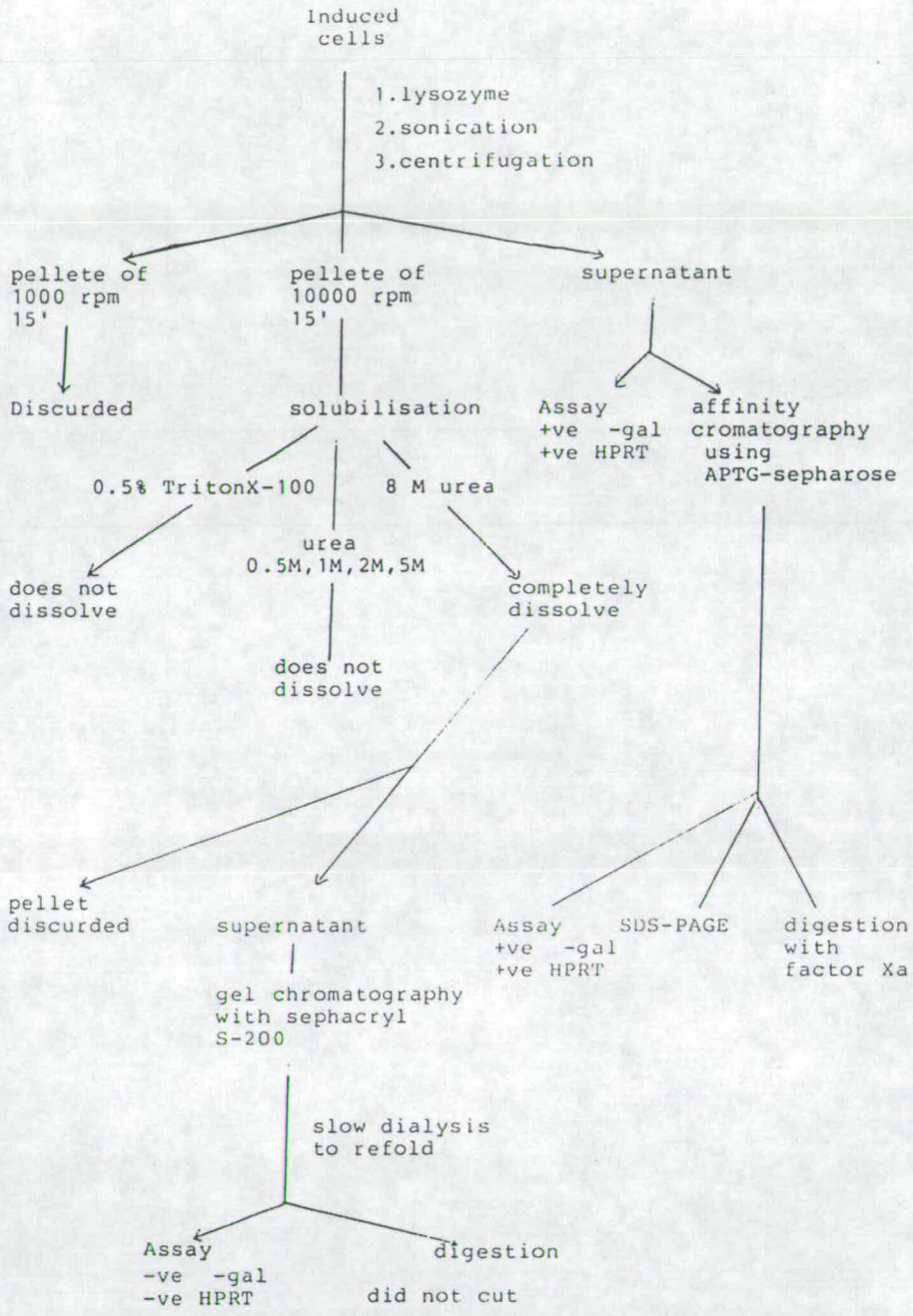


Figure 4.3 Overall scheme used for the purification of β-gal:PfHPRT fusion protein from induced SH1 cell.

(Marston, 1984).

The inclusion bodies thus obtained were washed three times with 0.5% Triton X-100 [Methods]. Fractions of the pellets were then analysed on 8% SDS-polyacrylamide gel. Figure 4.4 shows the coomassie stained gel. The result showed that washing with Triton X-100 did not improve the purity of the inclusion bodies significantly as there was no change in the protein profile. The major degradation product (115 kD protein) of the fusion protein was associated with the fusion protein with the same proportion.

In the second step of the purification, attempt was taken to solubilise the inclusion body. The idea was to separate the fusion protein by gel chromatography. Solubilisation of the insoluble inclusion bodies was necessary to release the protein from the aggregates, because treatment with mild detergent like, Triton X-100 did not release the protein. As mentioned before, inclusion bodies often need high concentration of detergents to be solubilised. The most widely used detergents are urea and guanidine hydrochloride in a wide range of concentrations.

Accordingly, inclusion bodies were spundown and resuspended in 3 ml of 0.5M, 1M, 2M or 5M urea solutions in lysis buffer sequentially by vortexing. In each step, resuspended inclusion bodies were stand on room temperature for 5 minutes before centrifugation for 10 minutes [method]. A portion of the pellets obtained in each step of the urea treatment were analysed by 8% SDS-polyacrylamide gel. The result, shown in Figure 4.5, indicated that the inclusion bodies are quite insoluble in 0.5M and 1M urea and very sparingly soluble in 2M and 5M urea concentrations. So, this concentrations were thought to be unsuitable for obtaining soluble protein. A higher concentration of urea was used to solubilise the inclusion bodies.

This time the pelleted inclusion bodies were resuspended in 5 mls of 8M

Figure 4.4 Protein profile of the Triton X-100 washed inclusion bodies from induced SH1 cells. Lanes (A), (B) and (C) are the inclusion bodies after first, second and third wash respectively. The arrow shows the fusion protein.

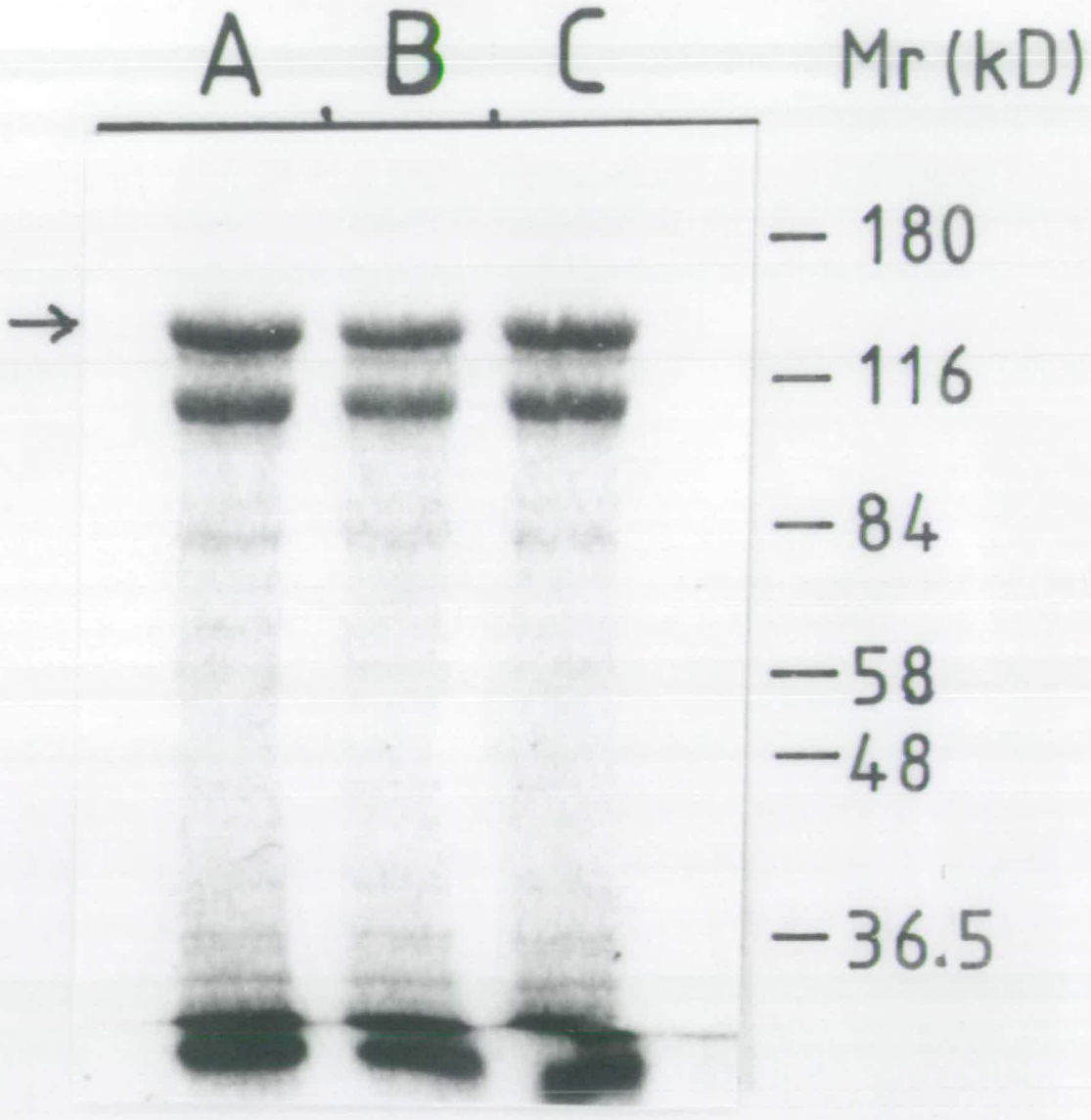
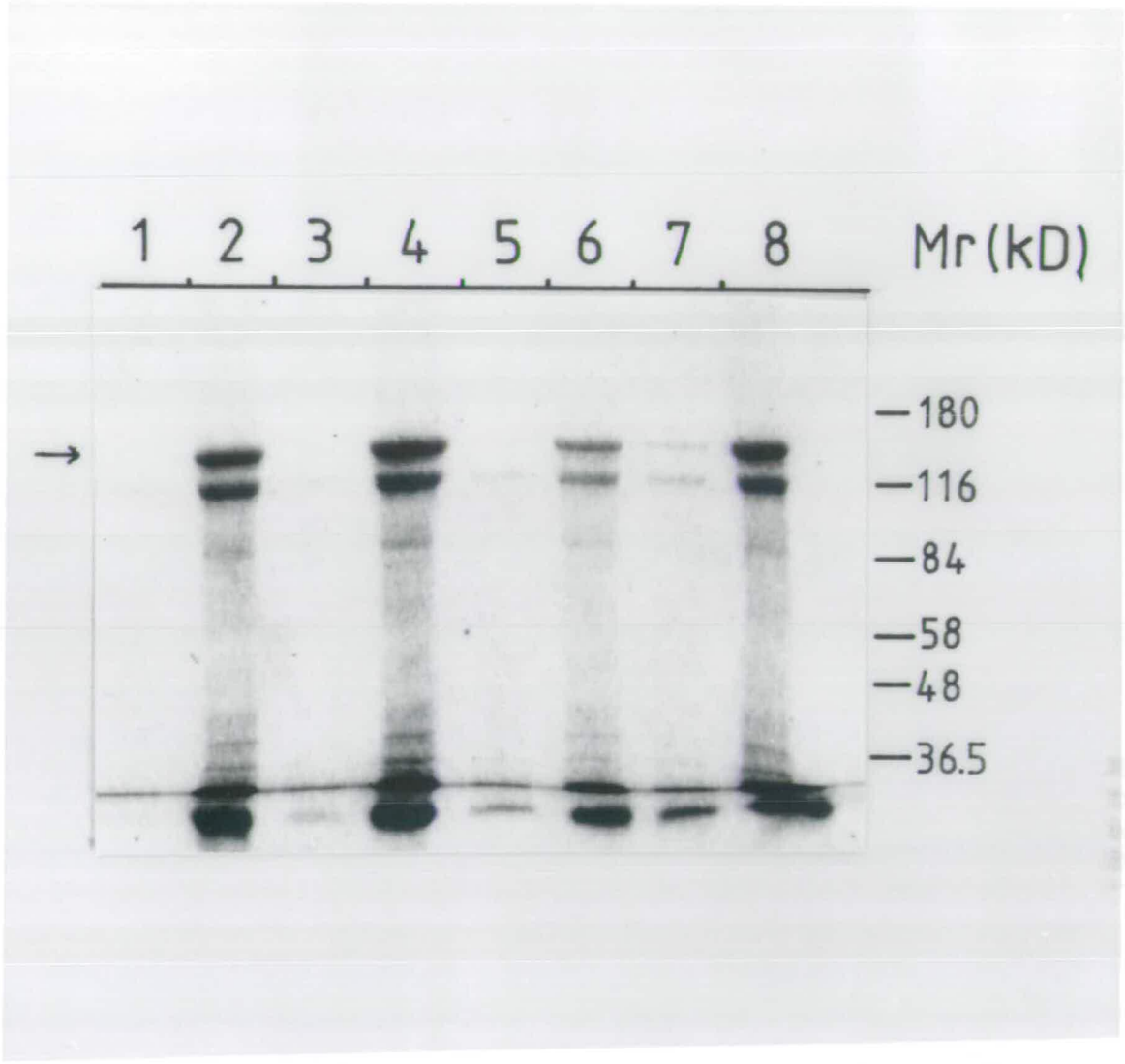

A
B
C
Mr(kD)
180
116
84
58
48
36.5

Figure 4.5 Urea solubilisation of the inclusion bodies. Figure shows the protein profiles of supernatant and pellets after treatment of the inclusion bodies with different concentrations of urea. (1) and (2) Supernatant and pellets of 0.5 M, (3) and (4) those of 1 M, (5) and (6) those of 2 M and (7) and (8) those of 5 M urea respectively. The arrow shows the fusion protein.


1 2 3 4 5 6 7 8
Mr(kD)
→
—180
—116
—84
—58
—48
—36.5

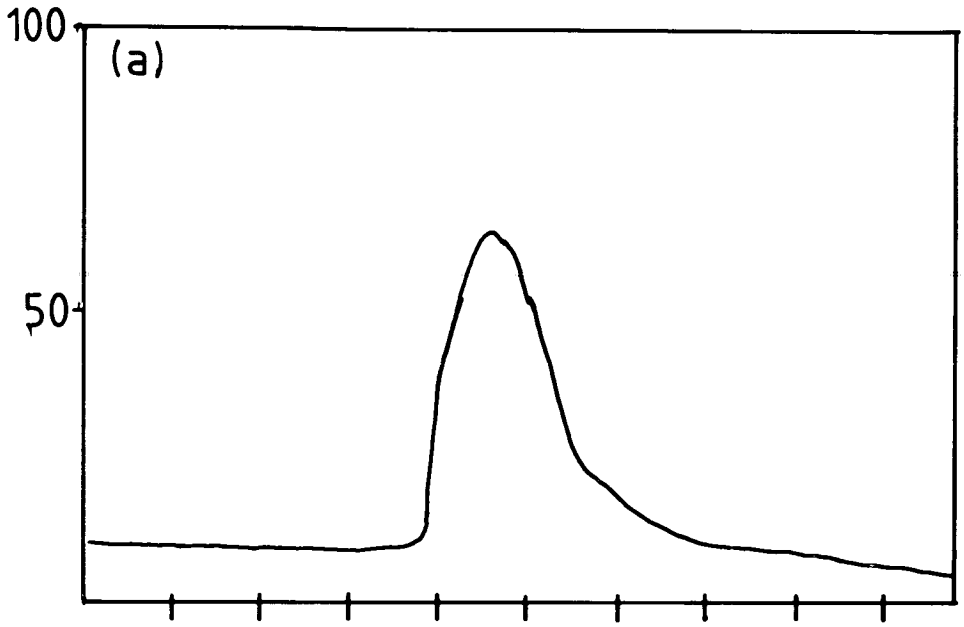
urea, mixed and stand on room temperature. Within 2 to 3 minutes the inclusion bodies were dissolved and produced an amber coloured solution. This was then centrifuges at 19000 rpm for 60 minutes at 4°C to remove the insoluble meterials and the supernatant was then immediately subjected to the gel filtration as described in the method section.

For gel filtration, a sephacryl S-200 (Pharmacia) column of dimension 1.6 cm X 70 cm was prepared. The bed volume and the void volume of the column were 140 ml and 56 ml respectively. Before loading the column was equilibrated with 10 bed volumes of 8M urea in the equilibrium buffer. Seventy mg protein in 5 ml solution of 8M urea was loaded on the gel and eluted with a flow rate of 30 ml/hr. Fractions of 5 ml volume were collected from immediately after loading the column. The elution profile of the protein is shown in the Figure 4.6a. A major peak of the protein was eluted at the volume of 72 ml. Aliquots of 50 µl from each fractions of the major peak were then analysed by SDS-PAGE. Figure 4.6b shows the protein profile of the eluted major peak. The result suggested that the fusion protein was not separated from the major degradation product of the protein by this chromatographic condition. However, other contaminating proteins were removed by this method.

4.3.2 Refolding and factor Xa digestion of the eluted protein.

Two different methods are commonly used to refold a denatured protein. In one method the denatured protein solution is diluted with buffer of high pH and incubated. This allows the partial folding of the protein and then the pH is adjusted to near neutrality to complete the folding (Marston *et al.*, 1984)). In the other method the protein solution is slowly dialysed against the buffer with decreasing concentration of the urea. This allows slow refolding of the protein. Thus from the pooled fractions of the major peak (20 ml) of the gel

Figure 4.6 Sephacryl S-200 gel filtration of the solubilized inclusion bodies (method). (a) shows the major peak of the elution profile and (b) shows the protein profiles of the fractions of the major peak. The lane numbers correspond with the eluted fraction number of the gel chromatography.



(b)

Mr(kD)

—180
—116
—84
—58
—48
—36.5

chromatography, urea was removed slowly by dialysis and concentrated to 5 ml [method]. The extent of refolding was then measured by assaying the protein for β-gal and HPRT activity. The protein did not show any activity for either enzyme (data not shown). This may indicate that the urea denaturation was irreversible or the refolding to the native state was not possible in the condition used.

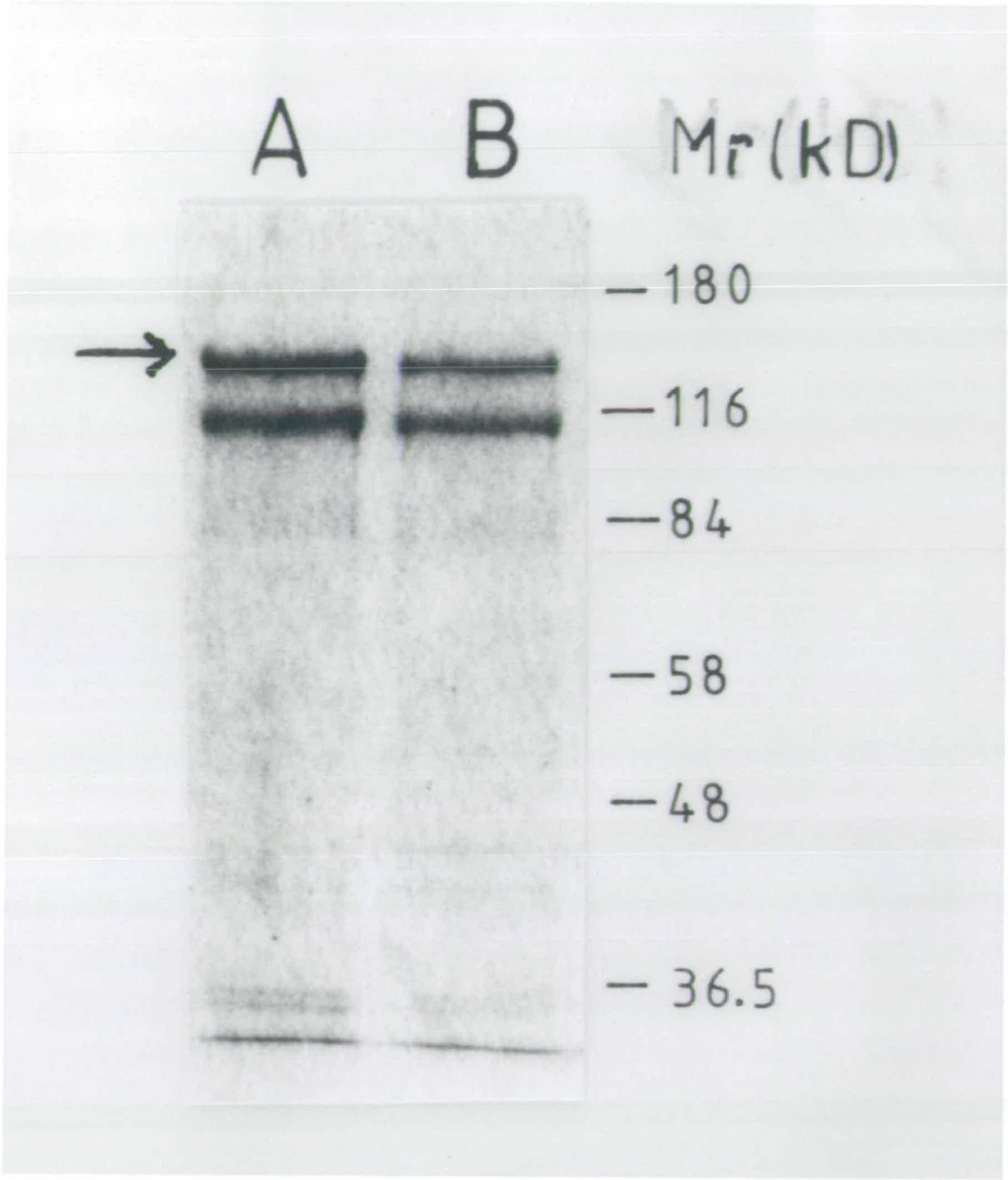
Although the refolding was not complete, the junction of the fusion protein may still be digestible with factor Xa. A 200 µl aliquot of the concentrated protein was digested [method] and the digested protein was then analysed by 8% SDS-PAGE. The result is shown in Figure 4.7. No cleavage of the fusion protein was observed.

4.3.3 Purification of the fusion protein from soluble fraction.

As it has been shown that the purified fusion protein is still contaminated with the major degradation product, the denaturation by urea were irreversible and the PfHPRT domain of the fusion could not be released by digestion with factor Xa, it was decided to purify the protein from the soluble fraction of the cell extract. It has been shown in section 4.2 that, a large amount of fusion protein were still present in the soluble form (Figure 4.2), and that the soluble fusion protein possesses some degree of activity for both HPRT and β-galactosidase activity (section 3.9). The main reasoning behind the purification of the fusion protein from soluble fraction was that, may be the two domains of the fusion protein fold separately and the junction point (target peptide for factor Xa) is accessible to the enzyme, in the native fusion protein.

There are several methods for the purification of β-galactosidase fusion proteins. In this study because the fusion protein was active for β-galactosidase activity, the substrate analogue affinity chromatography was chosen to purify it. An p-aminophenyl thiogalatopyranoside-sepharose (APTG-

Figure 4.7 Factor Xa digestion of the refolded fusion protein eluted from the gel chromatography column. (A) Protein profile of the digestion mix without activated factor X and (B) that with activated factor X.


A
B
Mr(kD)
180
116
84
58
48
36.5

ECH-sepharose) column of bed volume 4 ml were prepared [method] and equilibrated by the method described in the method section. The column was equilibrated with 200 ml equilibrium buffer before loading with protein. Protein sample was prepared sonication and centrifugation of cell lysate from 500 ml induced culture [method]. The clear supernatant was then subjected to affinity chromatography [method]. The elution profile of the protein is shown in Figure 4.8.

Each fraction in the protein peak were then assayed for β-gal activity and alternate fractions for HPRT activity. The activity profile of both the enzymes are also shown in the Figure 4.8. The profiles show that the fusion protein was efficiently bound and eluted from the column. Some activity of both the enzymes were eluted during laoding the sample on the column.

Comparison of the elution and enzyme activity profiles confirms that the fusion protein is active for HPRT activity. This is because the HPRT of the bacterium can not bound to the column proved that the activity must has come from the fusion protein.

4.3.4 Purity of the fusion protein.

To check the purity of the fusion protein, eluted from the APTG column, 20 µl from each fractions (nos. 3 to 8) of the eluted peak were checked by SDS-PAGE on a 8% gel (Figure 4.9). The eluted fractions (lane 1-6) contain the fusion protein free from most of the soluble proteins of the cells (see lane 7). However, two other proteins are also co-purified. These peptides may be integral part of the enzymatically active fusion protein.

4.3.5 Cleavage of the native fusion protein with factor Xa.

To check whether the cleavage site in the fusion protein is accessible to the activated blood factor X, in other words, to release the HPRT domain of the fusion protein, the purified protein, without any further purification, were

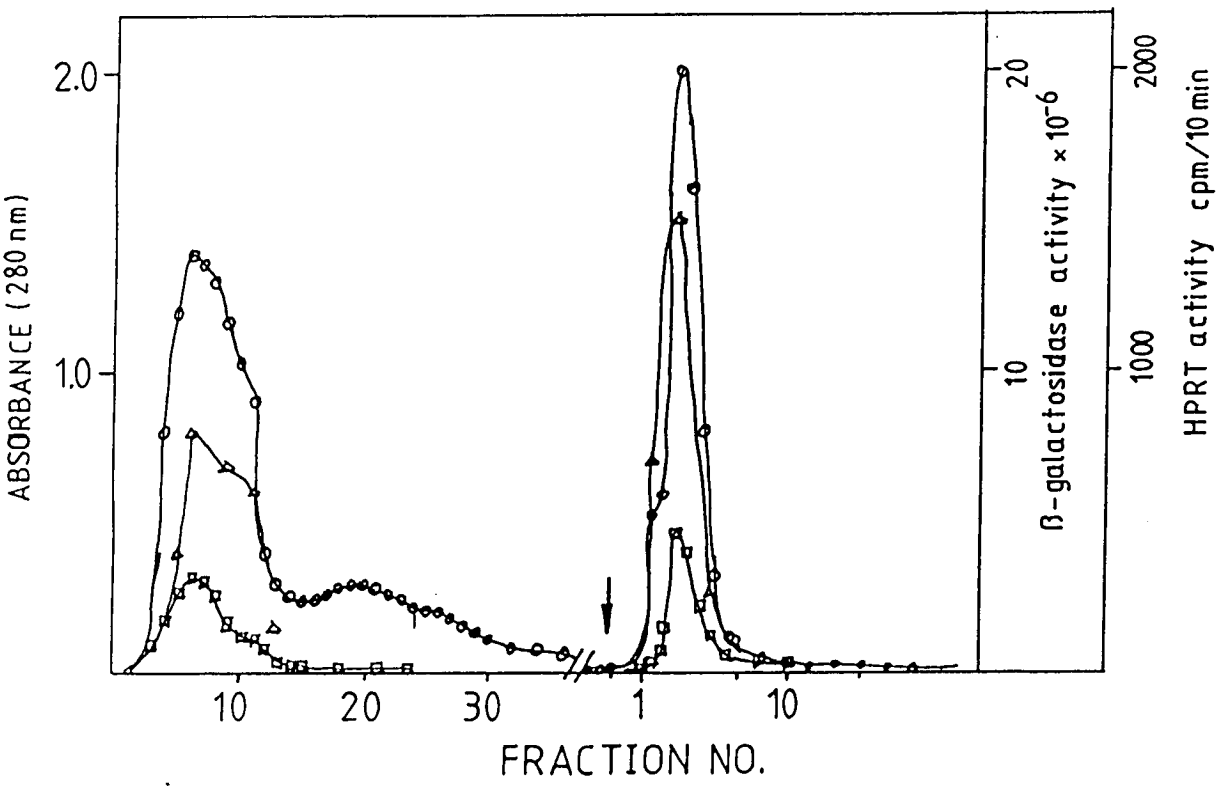
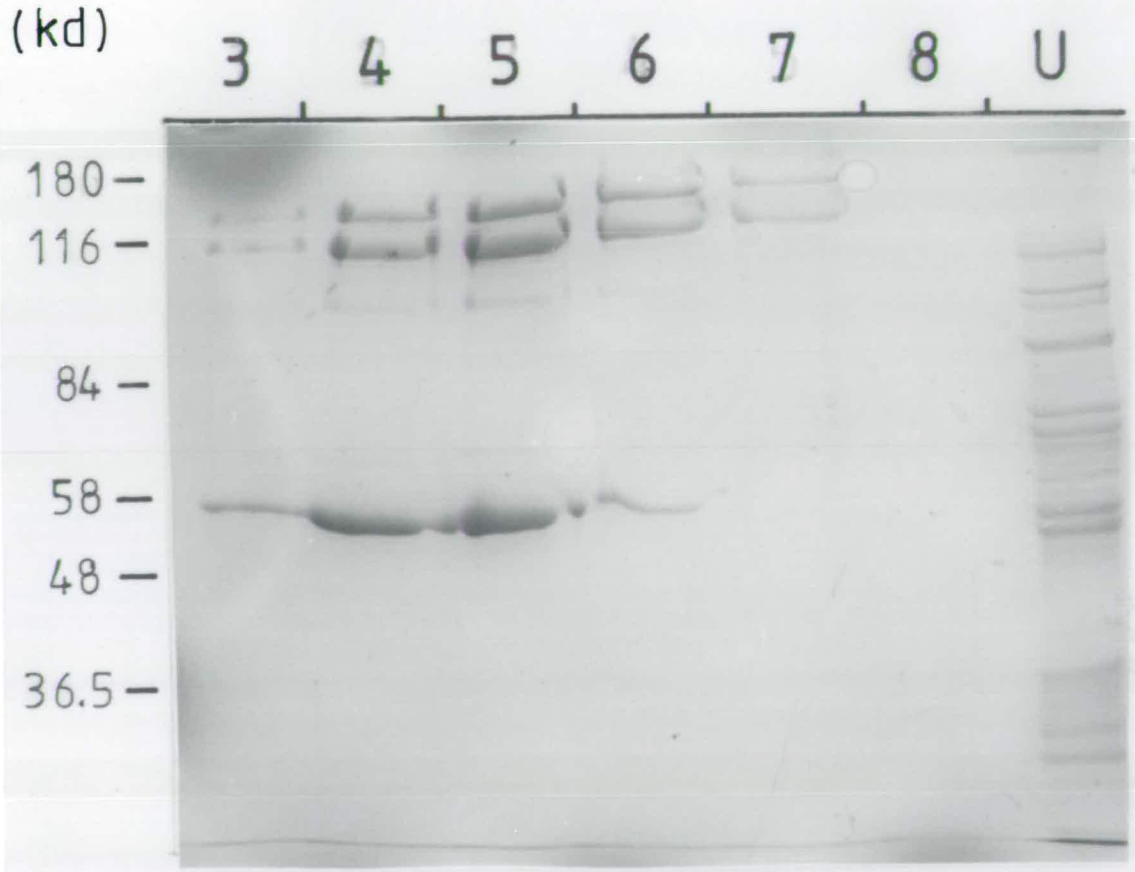


figure 4.8 APTG-affinity chromatography for the purification of the fusion protein from the soluble fraction of the induced SH1 sonicate. Figure shows the elution profile of protein (o—o), HPRT activity (△—△) and β-galactosidase activity (□—□). Arrow shows the point, at which the elution of the column bound protein start.

Figure 4.9 protein profiles of the eluted fractions from the APTG column. The numbers represents the numbers of the eluted fractions (fig. 4.8) and (U) shows the profile of the unbound proteins to the APTG column.


(kd)
3
4
5
6
7
8
U
180
116
84
58
48
36.5

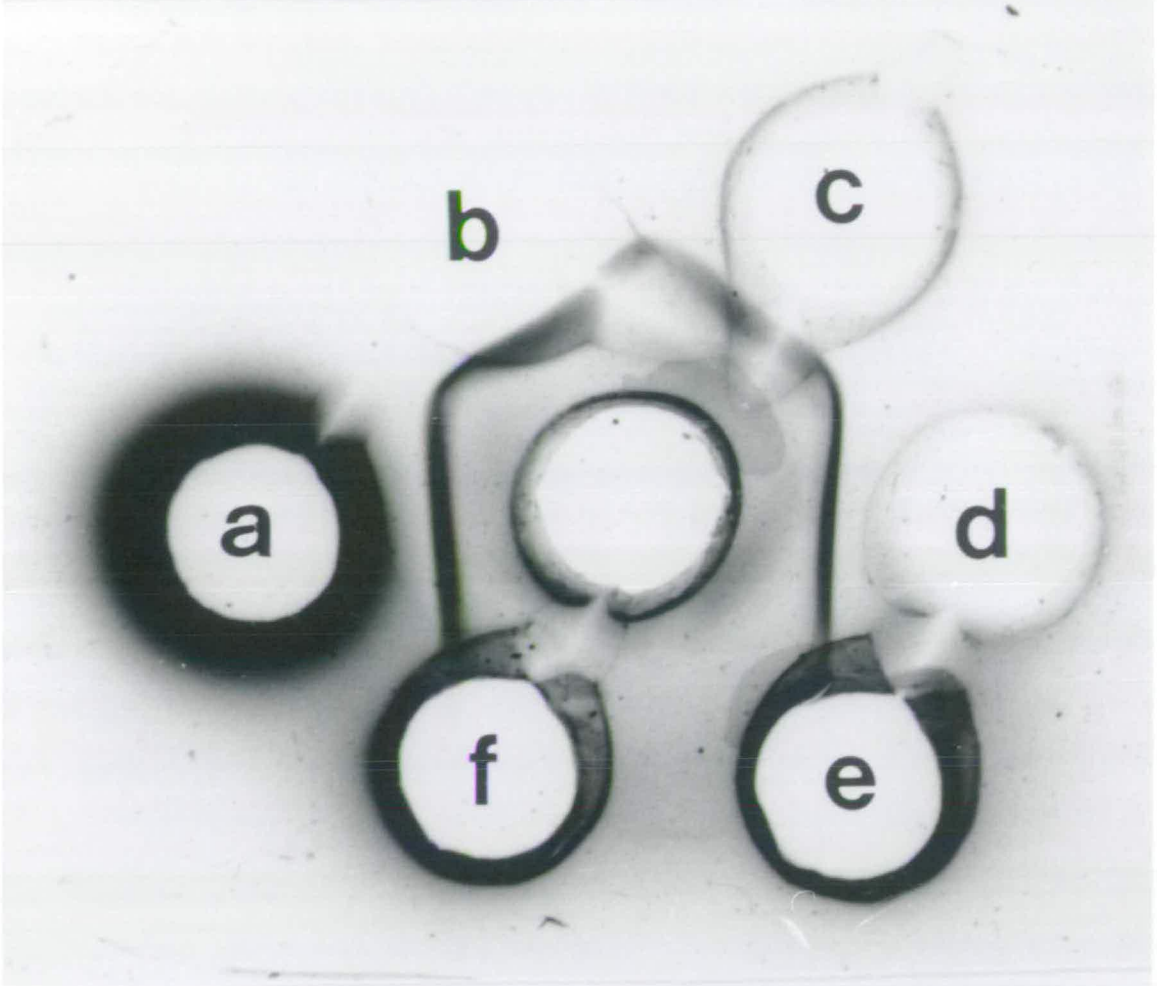
digested with factor Xa and the digested mixture was analysed with SDS-PAGE (data not shown). The result demonstrated that even the native protein is not digestible by the enzyme, suggesting that may be the two domains of the fusion protein are very close to each other and the junction tetrapeptide is hidden inside the fusion protein and not accessible by the cleaving enzyme.

4.4 The antibody against the fusion protein has been made.

Because the HPRT domain was found to be impossible to cleave from the fusion protein, I decided to use the whole affinity column purified native fusion protein for making antibody. Two rabbits of weight 1.5 kg were immunized each with 90 µg of the fusion protein [method]. At the eighth day of boosting the sera of both the rabbits were tested for the presence of anti-fusion protein antibodies by Ouchterlony immunodiffusion technique [method]. The result is shown in Figure 4.10.

The centre of the rosette contained 20 µl antiserum. It is seen from the figure that the Whole cell extract of induced SH1(a)and the soluble protein fraction of the cell (d) gave a strong precipitin band with the immune serum. Similar band also seen with the pure fusion protein (b) and with whole cell extract of induced pMS1S containing host NM522 (c) , which contained β-galactosidase instead of the fusion protein. No such precipitin band was seen between preimmune sera (f) and the whole cell extract (a) of the induced SH1 or between the immune sera (centre) and the whole cell extract of the uninduced host NM522 (e). The result suggests that the immune serum indeed contained antibody against the fusion protein which also contained anti β-gal antibody. this also suggest that such antibodies were not present in the preimmune rabbits. And also that the immune sera may contain antibody against other *E. coli* proteins as indicated by the faint bands with a,c,d and e.

Figure 4.10 Ouchterlony test for the presence of the antibody against the fusion protein. Centre well contained the rabbit serum to be tested. (a) whole cell lysate of induced SH1, (b) purified fusion protein from APTG column, (c) lysate of induced NM522/pMS1S, (d) supernatant of the induced SH1 lysate, (e) lysate of NM522 and (f) preimmune rabbit serum.


b
c
a
d
f
e

The titer of such antibodies may be very low.

4.5 The antiserum recognised the fusion protein.

To check the quality and specificity the antiserum was used in western blot analysis. Proteins from induced NM522, induced pMS1S transformed NM522 and both uninduced and induced SH1 were separated on a 8% SDS-polyacrylamide gel and western blotted and probed with the serum (method). The result is shown in Figure 4.11, suggested that the antiserum does recognize the β- gal in a control extract of a pMS1S transformed strain (NM522) (Lane B) and the fusion protein (lane D) in the induced SH1. The antisera also contained antibodies against other *E. coli* proteins as seen in the induced NM522 (lane A). It is also noticeable that the uninduced SH1 also contained small amount of the fusion protein. This may be due to leaky expression of the high copy number of the plasmid.

4.6 E. coli specific antibodies were removed by preabsorption.

Western blot with whole antiserum showed that, it contains antibodies against *E. coli* proteins other than the β-galactosidase or the fusion protein. Thus it was necessary to remove the antibodies against both β-galactosidase and other *E. coli* proteins from the serum. One very common technique used for this purpose is to preabsorb the serum with whole *E. coli* cell extract.

Thus to remove the *E. coli* and β-galactosidase specific antibodies from the serum, it was preincubated with extracts of induced pMS1S transformed NM522 [method]. Western blot was performed with the extracts as described in the previous section and blot was probed with the preabsorbed serum. The protein profile recognised by the serum was compared with that with the unabsorbed serum. The result shown in Figure 4.12 clearly suggested that the

Figure 4.11 Recognition of the fusion protein by immunized rabbit serum in western blot analysis. Figure shows the protein profiles identified by the immune rabbit serum in recombinant and parent *E. coli*. (A) uninduced NM522/pMS1S, (B) induced NM522/pMS1S, (C) uninduced SH1 and (D) induced SH1. The arrow shows the fusion protein.

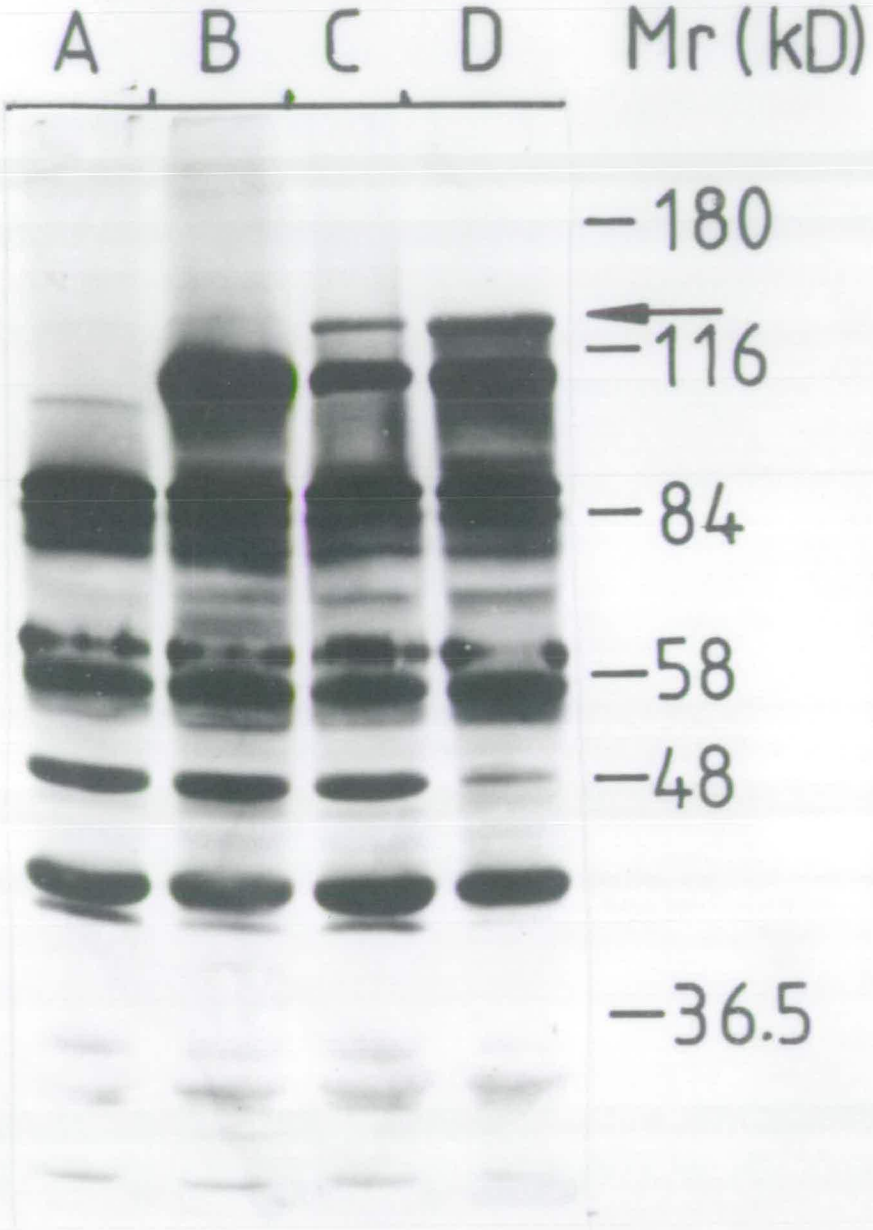
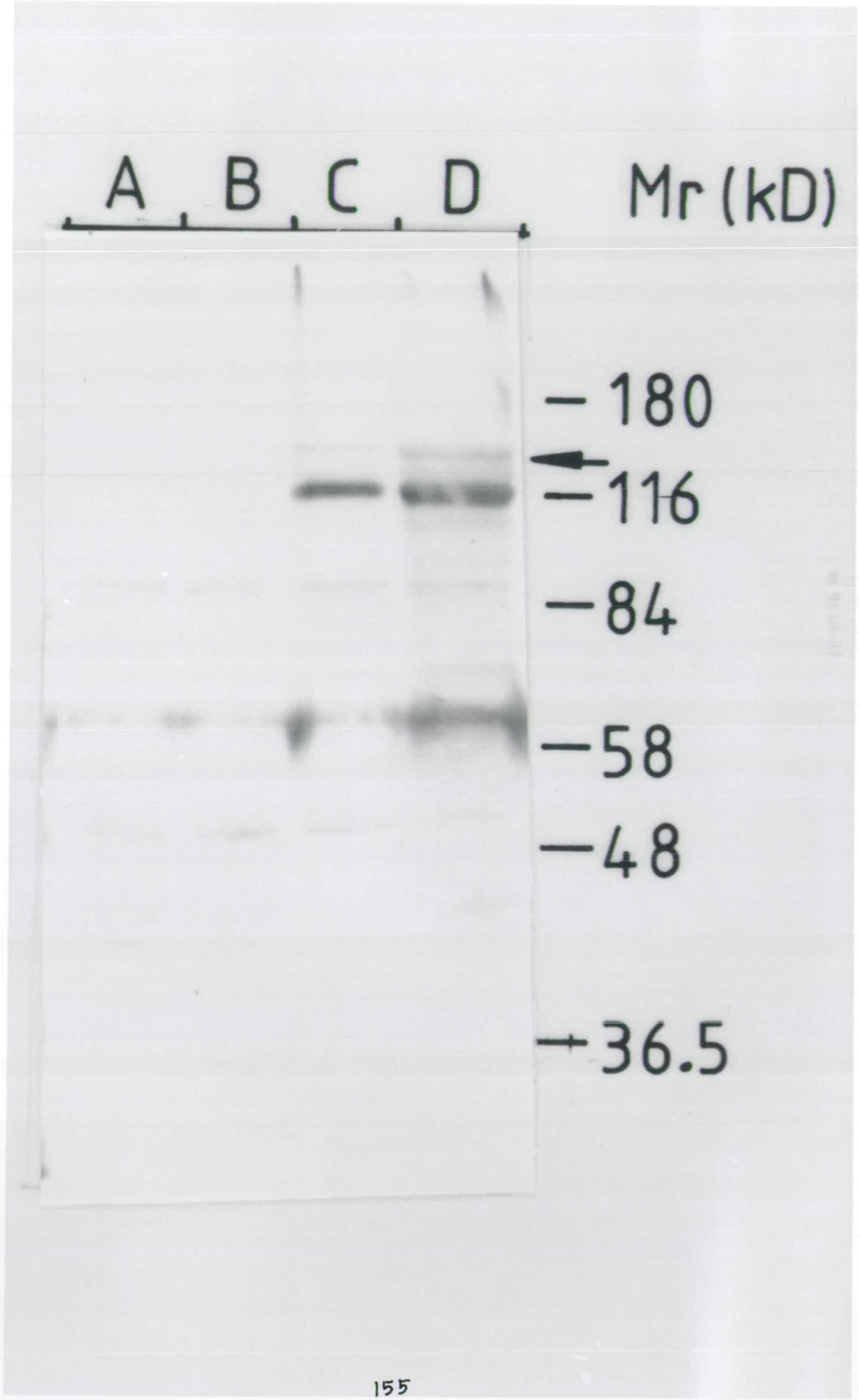

A
B
C
D
Mr(kD)
180
116
84
58
48
36.5

Figure 4.12 Recognition of the fusion protein with the immune rabbit serum preabsorbed with induced NM522/pMS1S cell lysate (method) in induced SH1 (lane D). Lane A, uninduced NM522/pMS1S, lane B, induced NM522/pMS1S and lane C shows the uninduced SH1 cell lysate respectively.


A
B
C
D
Mr (kD)
180
116
84
58
48
36.5

preabsorption step did successfully remove most of the anti*E. coli* and antiβ-gal antibodies. The β-gal in the induced pMS1S transformed NM522 (lane B) and other *E. coli* proteins (lane A and B) were not recognised by the preabsorbed serum. However, the serum still contained antibodies against fusion protein which are presumably against the PfHPRT domain. The major 115 kD protein was also recognised by the serum indicating the presence of the PfHPRT domain in this polypeptide. It was concluded that the preabsorbed serum mostly contains anti-PfHPRT antibodies.

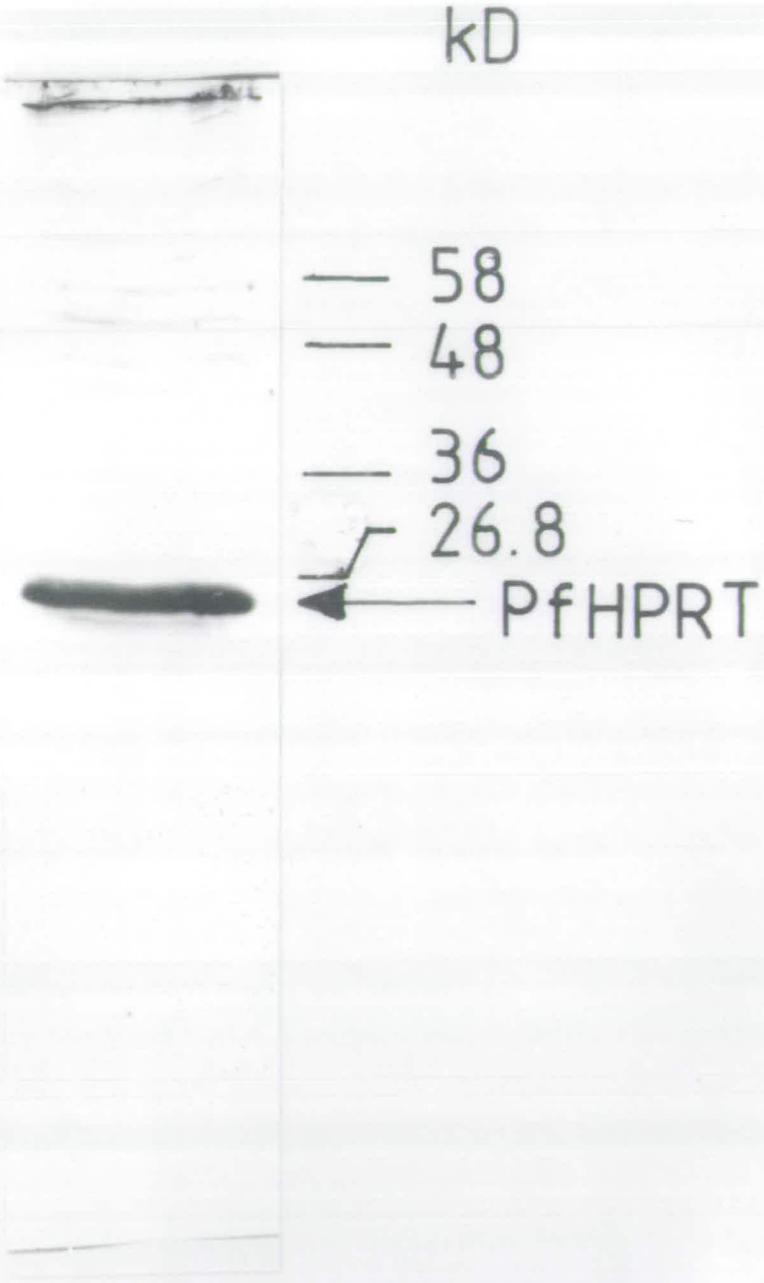
4.7 The preabsorbed serum recognised native PfHPRT.

To confirm that preabsorbed sera indeed contain antiPfHPRT antibody, the whole cell extract of the *P. falciparum* strain K1 were probed with the preabsorbed anti-fusion serum. Sample for SDS-PAGE was prepared with 50 µl of whole parasite and separated on a 12.5% gel. The proteins were first western blotted and then probed with the preabsorbed serum with an overall dilution of the antiserum of 100. The result shown in Figure 4.13, that only one protein of size of about 26.5 Kd is recognised in the whole parasite protein. This is the predicted size of the *P. falciparum* HPRT (King & Melton, 1987). No other protein in the parasite protein profile was found to be recognised by the serum, suggesting the specificity of the serum to the parasite HPRT. This result also indicated that HPRT is an abundant protein in the parasite, at least in merozoite stage.

4.8 Discussion and conclusions.

The major theme of this chapter was to understand the β-gal:PfHPRT fusion protein in molecular level and to purify the protein to make antibody against the *P. falciparum* HPRT.

Figure 4.13 Western blot analysis of the whole *P. falciparum*, strain K1, cell extract with the antiserum against the fusion protein. The serum recognized a protein of size 26.5 kD, similar as the predicted size of the PfHPRT (King and Melton, 1987).


kD
58
48
36
26.8
PfHPRT

The degradation of the product by host bacterium proteases is one of the major problems associated with the production of heterologous proteins in bacteria (Hellebust *et al* 1989). It has been suggested that many eukaryotic foreign peptides are recognised as abnormal proteins and consequently are degraded (Goldschmidt R., 1970). For example, Talmadge and Gilbert (1982) reported that the half life of human proinsulin in E.. coli is only 2 minutes. Such degradation may cause lower yield during the purification steps. Understanding the degradation pattern of a protein may help in designing better purification scheme. For instance, purification of a stable protein may need some special reagent in the buffer or arrangement of particular apparatus.

To understand the β-gal:PfPHRT fusion protein degradation pattern and to show that the authenticity of the fusion protein, the whole cell extract of the induced recombinant SH1 was probed in an western blot with anti β-gal antibodies. The result of this experiment confirmed the authenticity of the fusion protein. Moreover, The antibody recognised many other degradation product of the fusion protein. The size range of these protein suggested that the protein is severely degraded in the cell. In addition, this experiment also showed that the second major protein (about 115 kD) in the induced recombinant was also recognised by the antibody. This may suggest that the protein is a degradation product of the fusion protein as discussed in chapter 3.

The capacity of the proteolytic efficiency is limited for a cell. So, when the rate of expression becomes more than the rate of degradation the newly synthesized proteins aggregate and form inclusion bodies inside the cell. The mechanism of such inclusion body formation is not known.

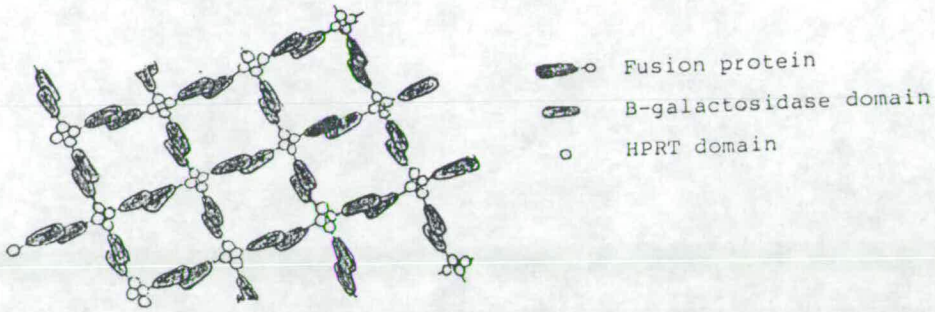
Like many recombinant proteins, β-gal:PfHPRT fusion protein also aggregates and form inclusion bodies in the cell. When a lysozyme and

deoxycholate treated cell lysate was centrifuged with different centrifugal forces the protein pellet of the lowest speed showed an almost identical pattern to the whole cell lysate, indicating the presence of the membrane debris and unlysed cells. In contrast the profile of the pellets of the higher speeds, contains mostly the fusion protein with its major degradation product. The absence of any other major proteins suggested that the protein possibly forms discrete inclusion bodies. By transmission electron micrography Schoner *et al*(1985) also showed that inclusion bodies are not membrane associated or enclosed in a membrane. The presence of the protein in pellets of different centrifugal forces may indicate the size variation of the inclusion bodies. One may argue that this is due to the insufficient centrifugation at any particular speed. However, this possibility can be ruled out by the fact that the amount of protein present in each pellet are almost similar, if the size of the inclusion bodies would have been identical, a large amount of protein would be obtained in a particular speed.

The analysis of the protein profile by SDS-PAGE, revealed that the inclusion bodies are consist of two major proteins, the fusion protein and the major degradation product of it. However, although there is some other proteins were present in small amount, the purification of the fusion protein was greatly achieved by simply isolating the inclusion bodies.

Washing with dilute Triton X-100 and urea solutions did not help significantly to remove minor contaminating proteins or the release of the fusion protein from the aggregates. This may indicate that the intramolecular interactions between the fusion proteins are quite big. One fact may be interesting to remember at this point that both the β- galactosidase and HPRT are multimeric proteins. So interaction of two domain may give rise to a large multimeric network as shown below schematically and it would probably be

very difficult to break such network without denaturing the two active domains.



This may indicate the strong intermolecular binding force in the inclusion bodies. Thus strong denaturing agent, 8M urea was needed to release the fusion proteins from these aggregated bodies. However, this treatment destroyed the special structure of the protein as indicated by the loss of the enzymatic activities for both β-galactosidase and HPRT.

The main idea of the solubilisation was to obtain the protein in pure form, particularly free from the degradation product associated with the fusion protein in the inclusion bodies, by gel filtration. But gel filtration with sephacryl S-200 eluted both the protein in the same peak. Moreover, the eluted protein failed to refold into enzymatically active form. This may be a result of several reasons. Firstly, the denatured fusion protein was not in pure form. The contaminating degradation product may interfere with the refolding mechanism. Secondly, during the denaturing process some important amino acid might have been modified. Occasionally special treatment is needed to protect such modification. For example, sulphonation was used to protect the essential cystein residues of recombinant insulin chains (Goeddel *et al.*, 1979). Finally, refolding is a complex process. different techniques have been employed for different proteins. The step wise dialysis method used in this case may not be ideal for this protein. Detail understanding of the structure of the fusion protein and experimentation with several techniques may result in the correct refolding of the protein.

Active fusion protein was purified from the soluble fraction of the cell lysate. This procedure exploited the β-galactosidase activity of the fusion protein. The protein profile of the purified protein showed that apart from the fusion protein two other proteins also bound to the column and eluted. Since, the column was only able to bind active β- galactosidase proteins, it may suggest that these two proteins are probably integral part of the multimeric enzyme complex. This is possible, because both β-galactosidase and HPRT are oligomeric enzymes and may be true in the case of the 115 kD protein as it also co-aggregates with the fusion protein, in the inclusion bodies. Moreover, we know that β- galactosidase protein with deletion in the N-terminal can make complex with the full length enzyme (α-complementation). The 58 kD protein may not be a part of the enzyme complex, rather a degradation product of the fusion protein occurs when eluted with the high pH buffer. Otherwise, the protein would have been present in the inclusion bodies.

The affinity column purified native fusion protein was used to make antibody. After the first boost the antisera from both the rabbits were found to be high enough to get a precipitin band in immuno-doublediffusion test. In western blot a 1:100 dilution of the pooled serum gave intense bands, indicating the high titre of the antibodies. However, the western blot of induced SH1 profile with this antiserum recognised several bands, in addition to the fusion protein. This may be in part due to the degradation of the fusion protein as we have seen with antiβ- galactosidase antibodies. However, it may be also due to presence of minute amount of the contaminating *E. coli* protein in the affinity purified fusion proteins which was used as the immunogen to make antibodies. These proteins might have raised antibodies against them and recognized the E. coli proteins in the western blot.

Most of these unnecessary antibodies including the antiβ-gal antibodies

were successfully removed by preabsorbing with *E. coli* extracts containing overexpressed β-galactosidase. The preabsorbed antiserum recognised only the fusion protein and also the major degradation product. this suggests that this degradation product also contains HPRT epitope(s).

The preabsorbed serum contained antibodies against PfHPRT is shown by the result that it exclusively recognised a PfHPRT size protein as predicted from the cDNA sequence data (King & Melton, 1987). No other protein in the parasite recognised by the antiserum, indicates that no other protein in parasite cross-react with PfHPRT antibodies. The intensity of the HPRT band in the parasite protein profile may also suggest that the protein is an abundant protein in the parasite. Thus chapter described the successful preparation of the PfHPRT specific antibodies. This antiserum can now be used to probe the protein in vivo or in vitro and be of an important tool to investigate the location of the protein in parasite. And can also be used to purify parasite by affinity chromatography for molecular studies.

Chapter 5
Result 3

Direct expression of the Plasmodium falciparum HPRT in E. coli.

The results in chapter 3 and 4 showed that PfHPRT can be expressed in E. coli, at least as a fusion protein with β-galactosidase, and also that the PfHPRT domain is even able to fold into the active form . The next logical step was therefore, to seek direct expression of the native parasite protein in *E. coli*, because one of the main objectives of this project was to study the biochemistry and molecular biology of the enzyme. A nonfusion full length enzyme is obviously the first choice for such studies.

Production of foreign proteins in bacteria using a direct expression vector has several advantages over production by a fusion vector. In *E. coli* when a protein is expressed directly, it folds into active form and mimics most, if not all of its biochemical properties. Non-abundant proteins of the parasite, can be over-expressed by using direct expression vector and the protein can then be purified and studied by conventional methods.

As discussed in the general introduction, for direct expression, a bacterial promoter and terminators are required for the transcription of the foreign gene. In addition the gene must follow an initiation codon (usually an ATG or sometimes a GTG) for translation.

To facilitate direct expression of PfHPRT, the insert in pPfPRT1 (see chapter 3) was recloned. It contains the complete PfHPRT ORF and can be easily recovered by digestion with HindIII and EcoRI. Moreover the ORF can be manipulated for forced orientation in an expression vector, due to the presence of two different restriction enzyme sites in 5′ and 3′ ends.

5.1 Vector pJLA503 was chosen for the expression.

The vector used for the direct expression of *P. falciparum* hypoxanthine phosphoribosyl transferase was pJLA503 (Schauder *et al.*, 1987). The detail of the vector is shown in Figure 5.1. In brief, the 4.9 kb vector contains

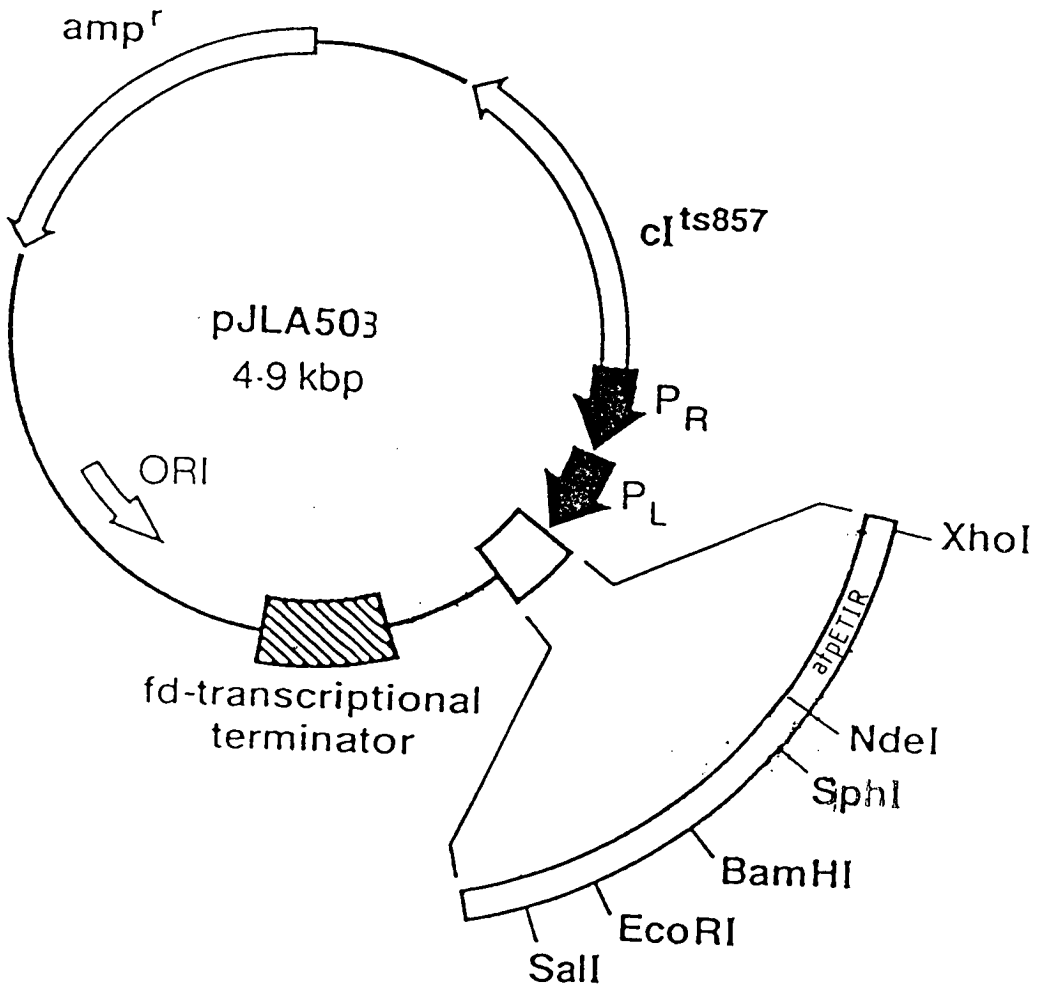


Figure 5.1 Structure of the vector pJLA503 (Schauder et al., 1987).

bacteriophage λ promoters P_R, P_L in tandem. The promoters are regulated by the product of the temperature sensitive mutant of cI repressor, cIts857 present in the vector. The promoters are followed by a polylinker region. A 51 bases long synthetic polynucleotide sequence from -50 to +1 bases of the *atpE* gene, called the *atpE*TIR (*atpE* translation initiation region) is placed in between the promoters and the polylinker region. The *atpE* gene is a highly expressing gene encoding the C subunit of the multisubunit enzyme ATP synthase. The strong transcriptional terminator from phage fd is present in the vector following the polylinker region, to stop the readthrough transcription from the vector, which may interfere with the mRNA stability and also with expression of other genes in the vector (see introduction). The drug resistant marker used in this vector is the ampicillin resistant gene. This vector was used to express several other eukaryotic and prokaryotic genes (Schauder *et al.*, 1987). The *E. coli* strain NM654 was used as a host for the pJLA503 and its recombinant vectors in the expression studies.

5.2 Construction of the nonfusion recombinant vector.

The overall construction strategy of the recombinant vector is shown in Figure 5.2. In brief, the HindIII-EcoRI fragment of the pPfPRT1 containing the ORF, from initiation codon to stop codon was inserted into the NdeI-EcoRI site of the pJLA503. The NdeI site contains the ATG, the initiation codon of the *atpE* gene. Placing the ATG of PfHPRT near or at this position wil put the ORF at the correct context of the bacterial control elements. The EcoRI site was chosen for forced orientation of the gene in the vector.

For subcloning, the fusion expression vector, pPfPRT1 was digested at the unique HindIII site preceding the ATG of the gene, and the ends were made blunt by DNA polymerase I (klenow fragment) and dNTPs. The blunt ended

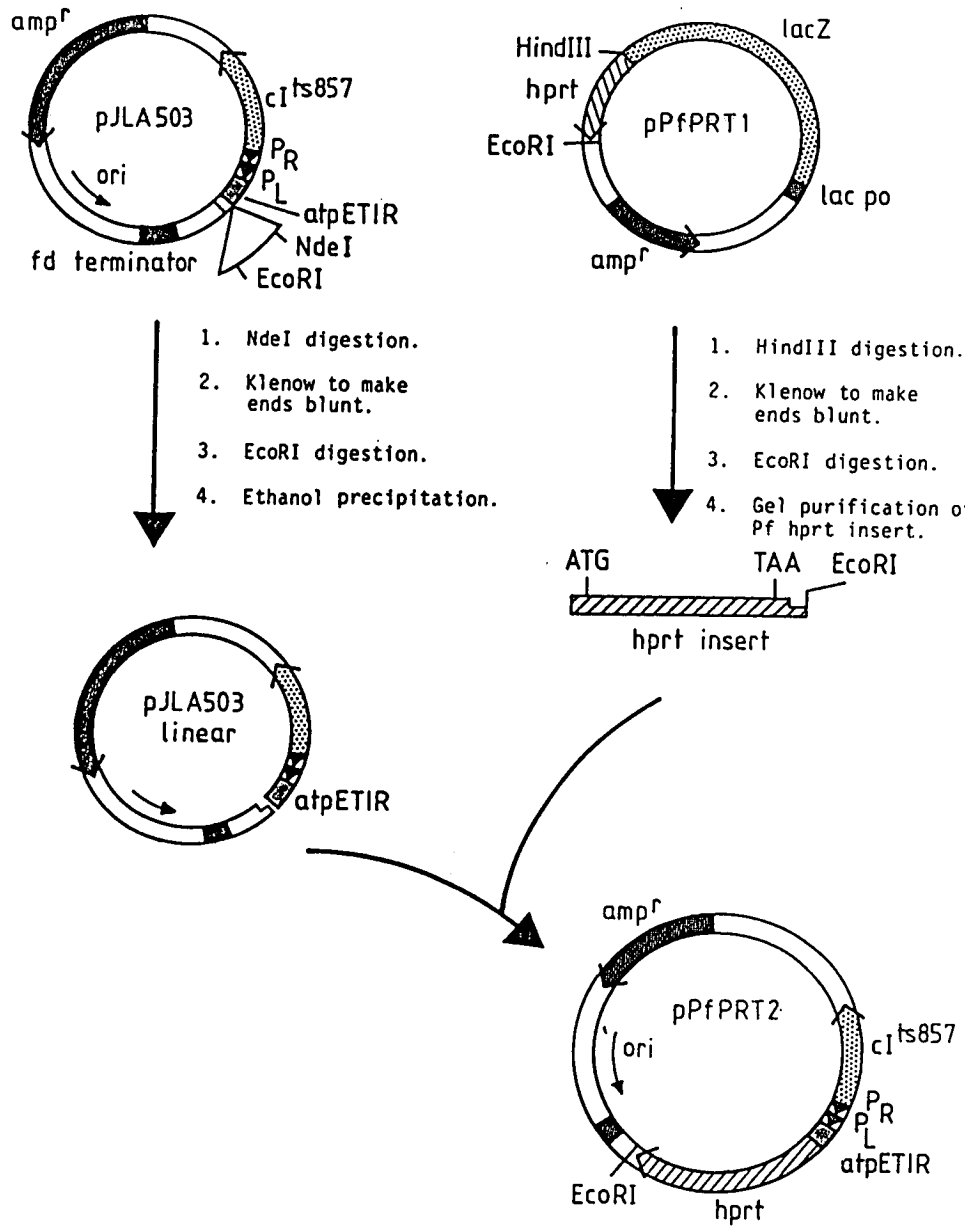


Figure 5.2 Construction of the recombinant plasmid pPfPRT2 (for detail of pPfPRT1 see text of chapter 3).

linear vector were then digested with EcoRI and the released fragment were purified from 8% agarose gel (method). Similarly the vector, pJLA503 The vector was digested at the unique NdeI site and the ends were made blunt. The blunt ended linear vector was then digested with EcoRI and the released polynucleotide, were removed by precipitating the digested vector with ethanol. The precipitated vector was finally resuspended in 1 X TE. For ligation, insert and vector DNA were mixed in 3:1 ratio and ligated using T4 DNA ligase [method].

5.3 The constructed vector was transformed into E. coli.

The ligation mixtures were transformed into *E. coli* NM654 [method]. The transformation mixtures plated on L-amp plates and incubated overnight at 30°C. Primarily the ampicillin resistant colonies were picked and then screened for the PfHPRT recombinants, by colony blot [method], using the purified PfHPRT cDNA as the probe. Figure 5.3 shows the blot. About one positive per 12 ampicillin resistant colonies were obtained.

5.4 The recombinant bacteria were checked for the expression.

The two colonies thus obtained were checked for their ability to express the recombinant protein. The cells were induced for expression [section]and then processed for SDS-PAGE [method]. To check the expression, protein profiles of cells from 300 µl culture of both the induced recombinant colonies were analysed on a 10% SDS-polyacrylamide gel (method) and compared with that of the uninduced recombinants, induced NM654 and pJLA503 transformed NM654. The result of this experiment shown in Figure 5.4, demonstrated that a protein of about 26.5 kD has expressed in both the induced recombinant colonies (Lane 5 and 7). This is about the similar size of the predicted protein

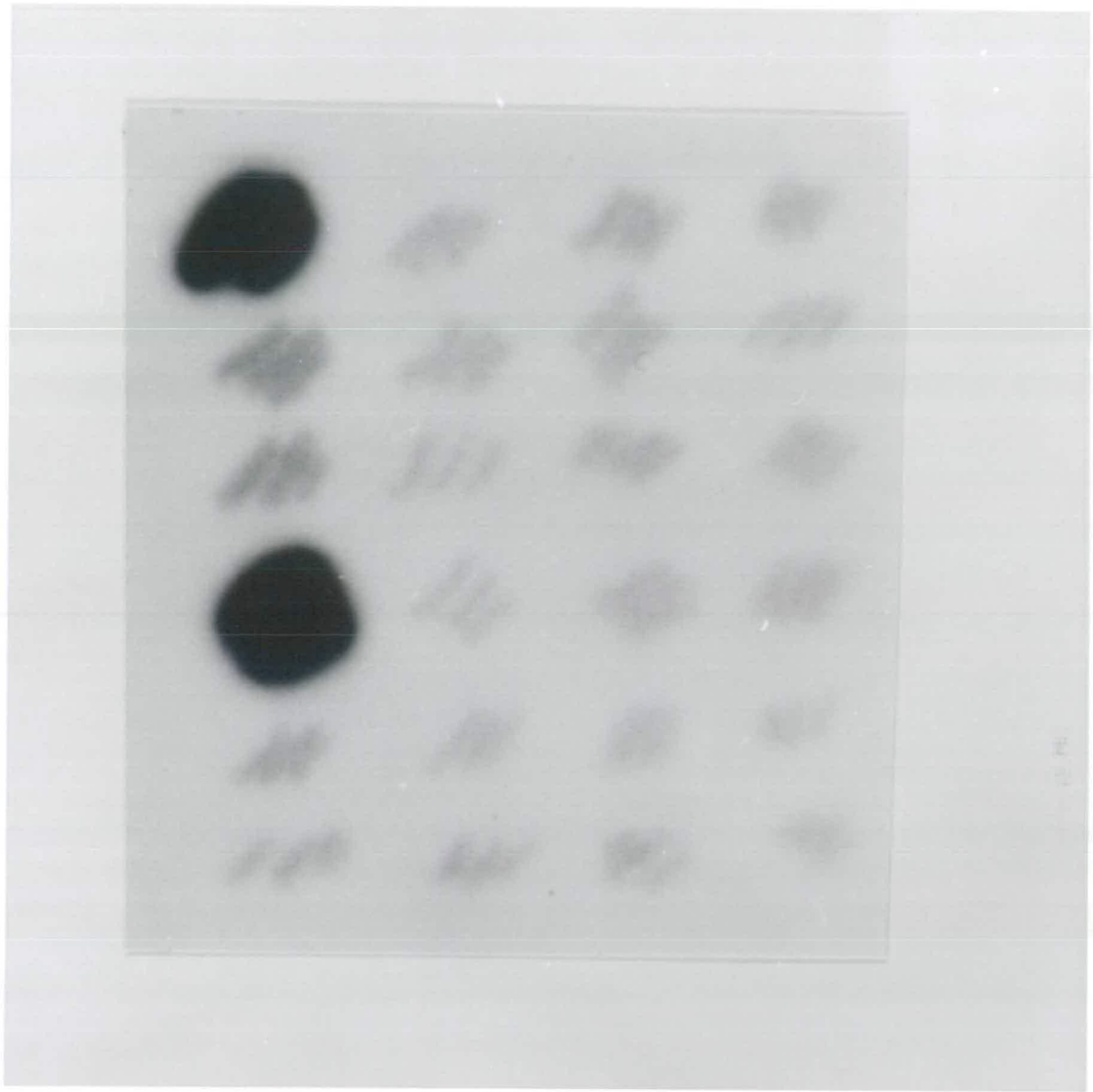
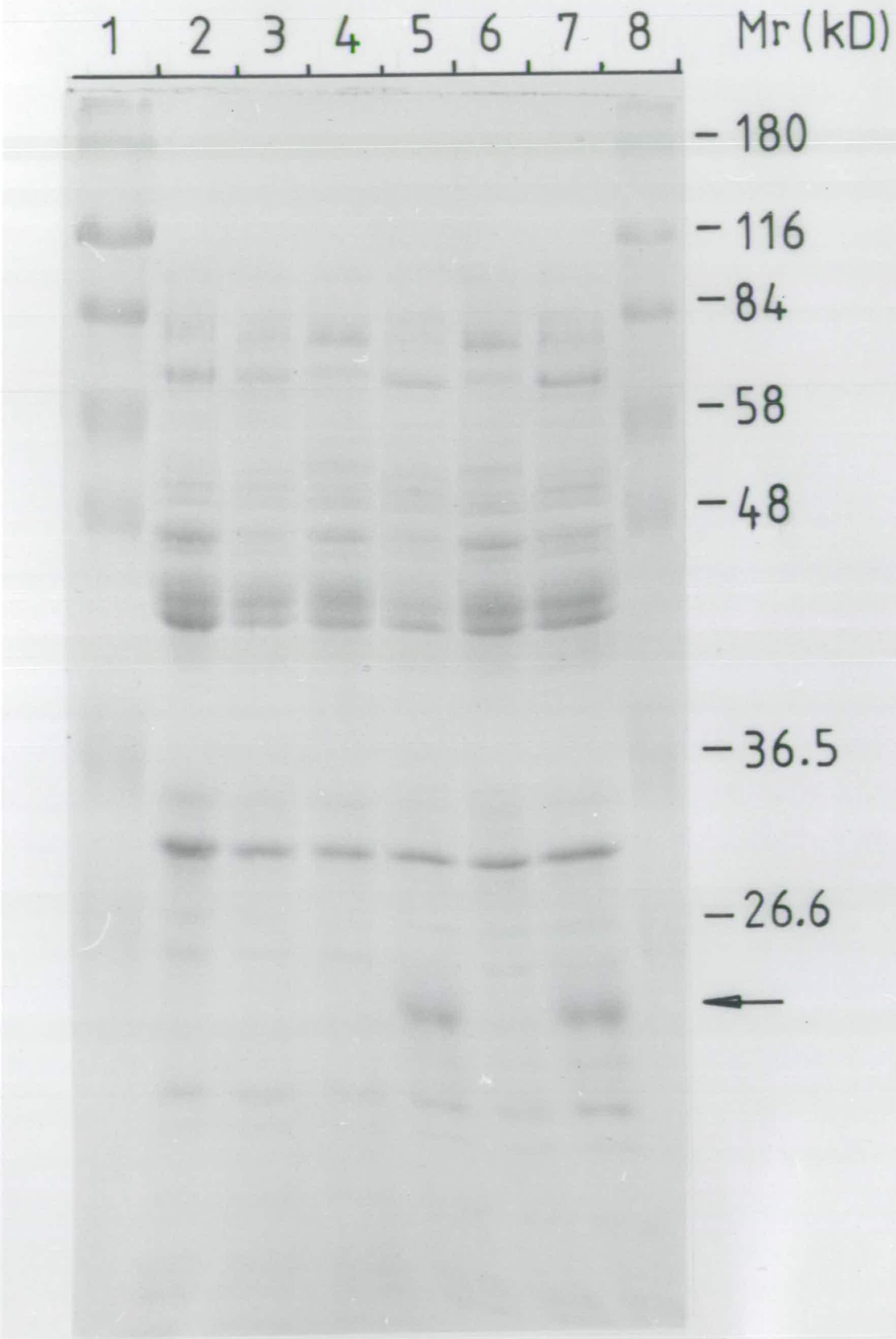


Figure 5.4 Expression study with the pPfPRT2 transformant of NM654. The Figure shows the protein profiles of uninduced (lanes 4 and 6) and induced (lanes 5 and 70 transformants and uninduced (lane 2) and induced (lane 3) NM654/pJLA503. Lanes 1 and 8 show the protein molecular weight markers. The arrow shows the recombinant protein.


1 2 3 4 5 6 7 8
Mr(kD)
180
116
84
58
48
36.5
26.6

from the cDNA of the PfHPRT (26.392 kD). The protein is not present in the uninduced recombinants (Lanes 4 and 6), suggesting the protein is induced by the higher temperature. It is also not present in the induced host NM654 (lane 1) or in the induced pJLA503 transformed NM654 (lane 2). These may suggest that the induced protein was expressed from the recombinant vector. It was concluded that the induced protein is *P. falciparum* HPRT.

One, of the two colonies, was selected for further study and named SH2. To confirm the temperature inducibility of the gene, the selected recombinant SH2, was grown and induced for vector gene expression. The proteins of the induced SH2 were separated on a 12.5% SDS-polyacrylamide gel. The result in the Figure 5.5 shows the comparison of the induced SH2 protein profile (lane 3), with that of the induced host NM654 (lane 1) and uninduced SH2 (lane 2). The result confirmed that the induced protein is of the recombinant vector origin and regulated by the temperature sensitive cI857 repressor.

To check the structure of the recombinant vector, it was purified from the SH2 cells (method), and the position and the orientation of the vector was confirmed by restriction mapping (data not shown). And the vector was named, pPfPRT2.

5.5 The expressed PfHPRT is recognised by the anti-PfHPRT antibody.

The expressed protein was probe in a western blot with the preabsorbed polyclonal antiserum against the β-gal:PfHPRT fusion protein. The proteins from the induced and uninduced SH2 were separated on a 12.5% SDS-polyacrylamide gel and western blotted. The blot was probed with the antiPfHPRT antibody (final dilution 1:100) previously absorbed with *E. coli* extract as described before (method). The result, shown in Figure 5.6, clearly

Figure 5.5 Expression of PfHPRT in *E. coli* SH2 (NM654/pPfPRT2). Figure shows the protein profiles of induced NM654/pJLA503 (lane A), that of the uninduced SH2 (lane B) and induced SH2(lane C). A protein of size 26.5 kD is expressed in the induced SH2 which is similar to the predicted size of the PfHPRT (King and Melton, 1987).

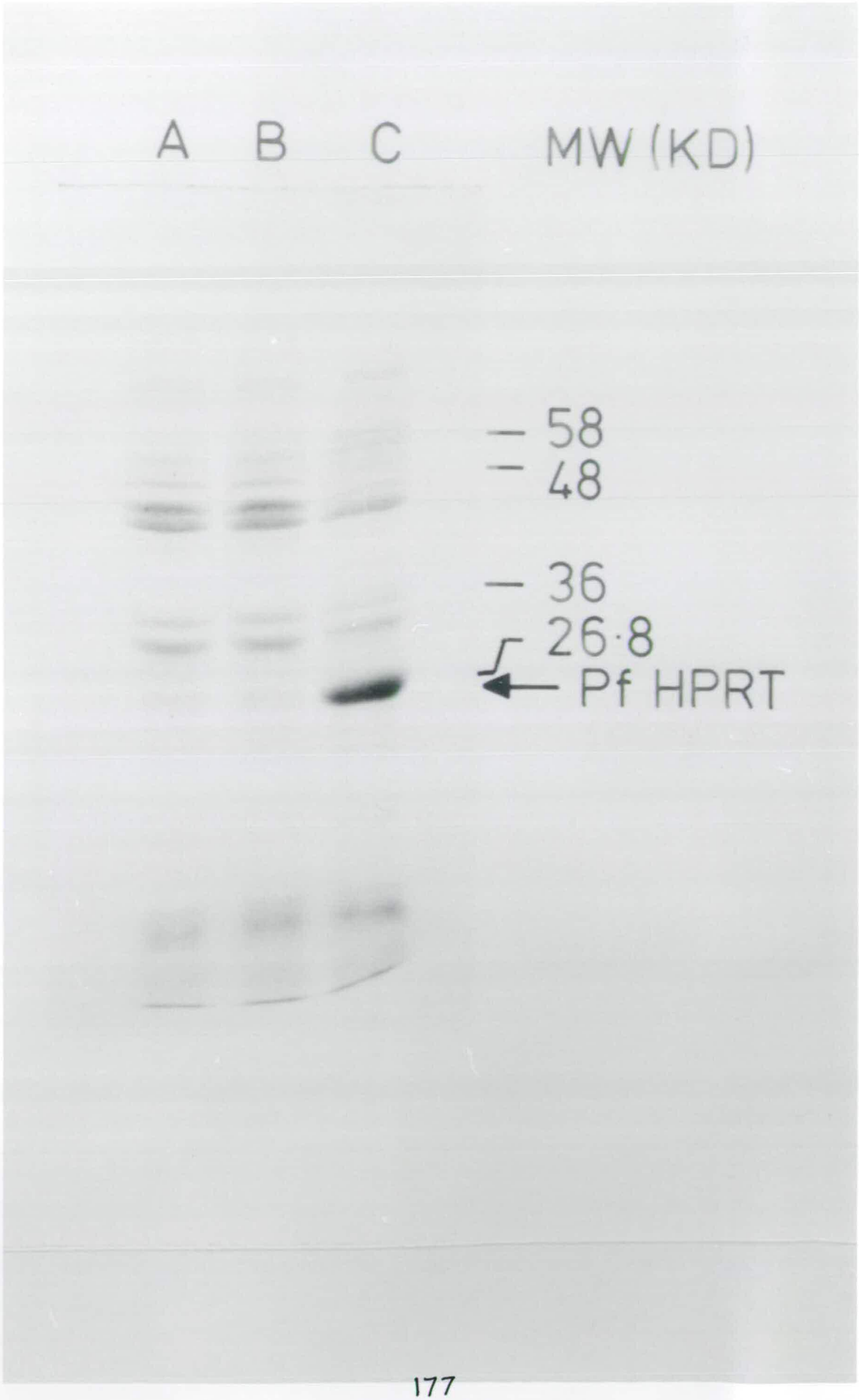
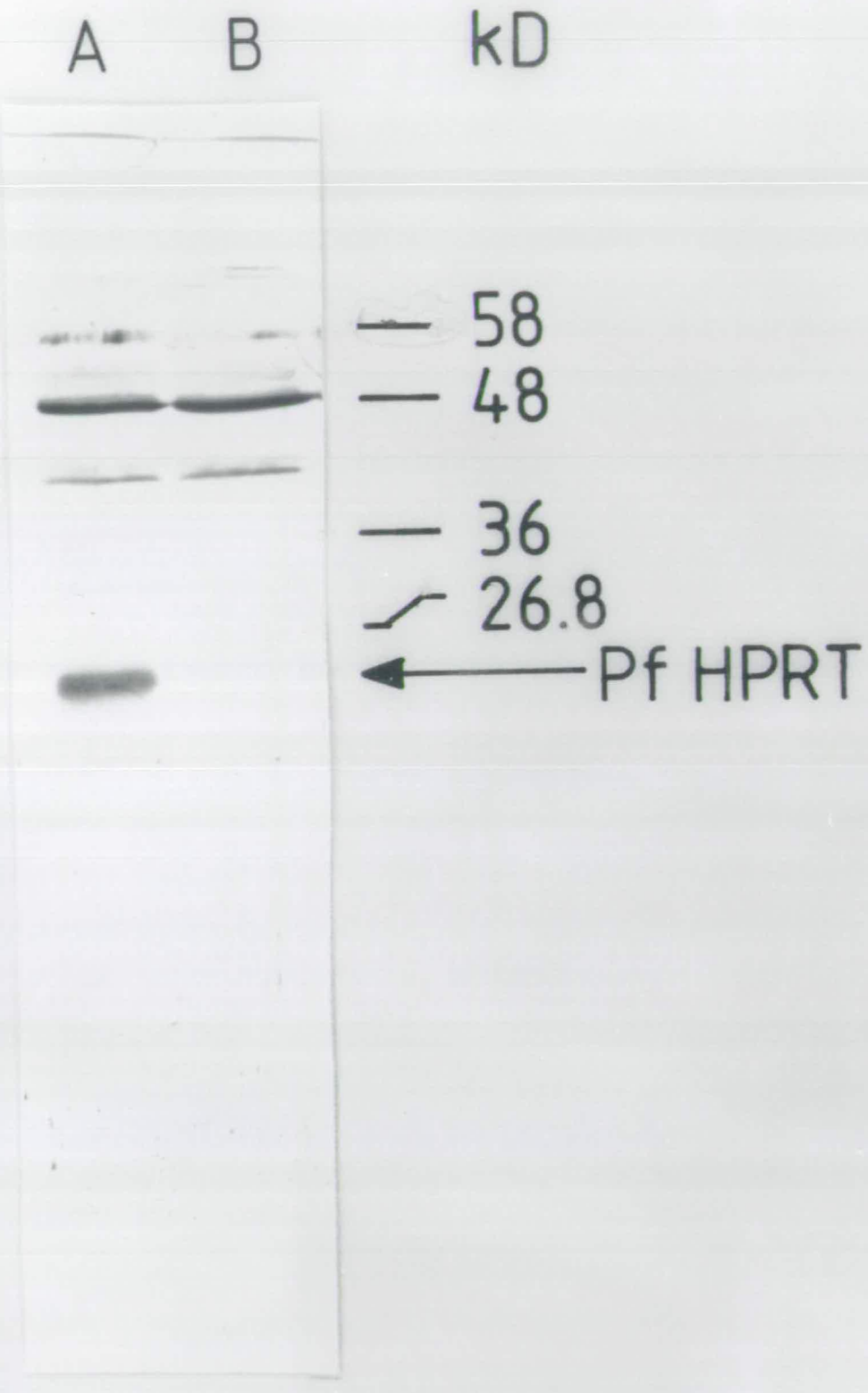

A B C
MW (KD)
58
48
36
26·8
Pf HPRT

Figure 5.6 Recognition of the expressed recombinant protein in SH2 by the immune serum against the β-gal:PfHPRT fusion protein (chapter 3). Lane A shows the recombinant protein in the induced SH2 cells is recognized by the antiserum. Lane B shows no such protein is recognized in uninduced SH2 cells.

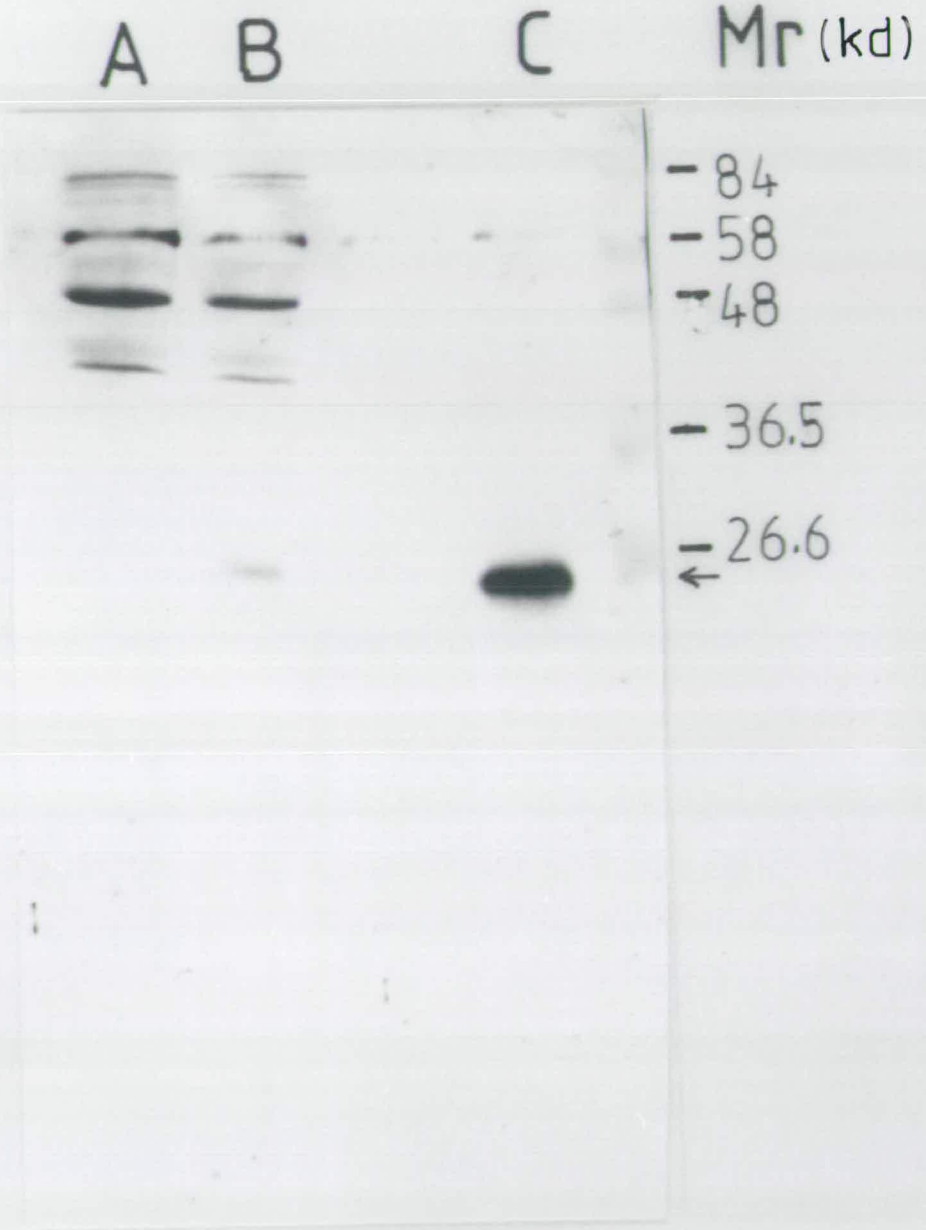

A
B
kD
58
48
36
26.8
Pf HPRT

indicates that the expressed protein in induced SH2 (lane A) is recognised by the antibody, that it has atleast some epitopes of *P. falciparum* HPRT. No such protein is recognised in uninduced SH2 extract (lane B).

5.6 The PfHPRT expressed in E. coli is same size as the native protein.

Following translation many eukaryotic proteins undergo post translational modification, like removal of any Leader(signal) peptides (Blobel & Dobberstein, 1975), glycosylation (Astwell and Morell, 1974), phosphorylation (Rubin and Rosen, 1975) etc. Leader peptides direct the newly synthesizing protein into its target places, for example, to membrane bound vesicles, to plasma membranes for membrane bound proteins. Comparison of the amino acid sequence predicted from the PfHPRT cDNA, with that of the mammalian HPRT, revealed that PfHPRT contains an extra peptide of of 8 amino acids and an extension of 3 amino acids at the C-terminal end. The biological significance of these extra amino acids are not clear at this moment. This raised the question whether this is targeting the protein to any particular part of the cell and removed from the protein afterwards. To answer the second question, the size of the protein synthesized in *E. coli* NM654 and that of the native protein synthesized in vivo were compared in a western blot of the separated protein on a 12.5% SDS-polyacrylamide gel probed with the PfHPRT antiserum. The result of the experiment shown in figure 5.7, indicates that the size of the *E. coli* expressed protein lane 2 is close or identical to that of the native protein (lane 3). Moreover, if the extra peptide is cleaved, a doublet protein band would be expected in the parasite protein profile. These may suggest that no part of the protein is remove after synthesis from spliced mRNA. This is interesting and the question of the significance of the extra amino acids is still remains.

Figure 5.7 Size comparison of the recombinant protein in induced *E. coli* SH2 and native PfHPRT. Lane A shows the protein profile of the uninduced SH1 as recognised by the antiPfHPRT antiserum. Lane B and C shows the same of the induced SH1 and whole *P. falciparum* K1, cell extract respectively. The size of the recombinant protein and the native PfHPRT appears to be the same.


A
B
C
Mr (kd)
84
58
48
36.5
26.6

5.7 The PfHPRT expressed in E. coli is active.

As we have seen in the case of the fusion protein that it may be enzymatically active or correctly folds into active form. If this is true the non fusion protein should fold into the active form even more efficiently. Which can be measured by assaying the activity in the induced SH2 extract. The extracts of the induced cell were prepared and the cell free extract was then assayed for the HPRT activity using hypoxanthine as substrate [method]. Specific activity of HPRT in induced SH2 extract was compared with that of the induced NM654 and uninduced SH2. The result is shown in Figure 5.8, suggests that the specific activity of HPRT is some 50 times more than that of the induced NM654. This result suggests that PfHPRT expressed in *E. coli* is active. However, since the bacterium has its won HPRT, the result is not conclusive. To confirm this conclusion, either the protein need to be purified or the enzyme should be expressed in a HPRT less bacterial strain.

5.8 Discussion and conclusions.

It has been mentioned before that because of unusual instability of the plasmodium enzyme the isolation of the *P. falciparum* HPRT in sufficient quantities for biochemical use has been difficult (Queen *et al.*, 1988) and although important, structural studies were prevented because of the limited supply of the highly purified material. This chapter demonstrated a new approach for recombinant production of this important enzyme. It should now be possible to isolate relatively large amount of pure P. falciparum HPRT, necessary for further biochemical and structural studies, from the SH2 bacterium. This has eliminated the necessity of large scale parasite culture for this purpose.

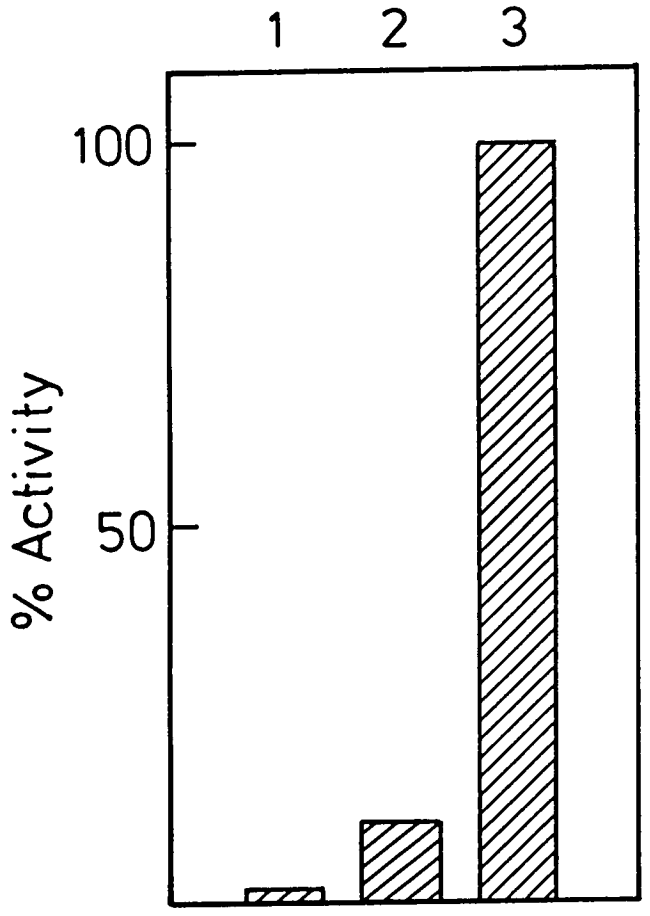


Figure 5.8 HPRT activities in recombinant strain SH2 and parent *E. coli* NM654. Data are expressed as percentages of the induced recombinant, SH2 (bar 3). Bar 1 and bar 2 show the relative activities in induced NM654 and uninduced SH2 respectively.

Plasmid pPfPRT1 was initially constructed as a fusion *E. coli* expression vector (chapter 3). This plasmid contained the complete open reading frame of the PfHPRT which is recoverable by double digestion with HindIII and EcoRI. This ORF is placed in the right context with the Translation initiation region of the *atpE* gene which was present in the vector. This put the gene under control of the λpromoter P_L and P_R and can be regulated by the temperature sensitive cI857 regulatory gene product. This newly constructed plasmid was called the pPfPRT2 and the transformed *E. coli* NM654 is called the SH2.

Upon induction at 42°C, this bacterium produce high level of the PfHPRT protein which is of the similar size (26.4 kD) as predicted from the cDNA. Further confirmation about the authenticity was came from the western blot with the antiPfHPRT antibody prepared with the β-gal:PfHPRT fusion protein and preabsorbed with *E. coli* protein. This antibody was previously shown to recognize PfHPRT in western blot experiments (chapter 4). Visual analysis of coomassie stained gel clearly suggested that PfHPRT is the major protein in the induced SH2 and the expression level is quite high.

When the cell free extract of induced SH2 was extract was assayed for HPRT activity the bacteria showed significantly high activity compared to the uninduced SH2 and induced host NM654 cell extract. The activity in the induced SH2 extract is about 10 times higher than that of the uninduced SH2 whereas the activity is some 50 times higher than the induced host NM654 extract. It is concluded that the *E. coli* SH2 produced PfHPRT is enzymatically active.

Comparison of the size of an in vitro translated protein from purified mRNA with that of the native is commonly used to study posttranslational cleavage of any signal peptide. Hart et al (1987) showed that *invitro* translation product of recognised by the monoclonal antibody against Trypanosomal

aldolase had the same size as the native protein. They concluded that the protein does not undergo any post translational cleavage. Because the PfHPRT had an extra 8 amino acid in the N-terminal which is hypothesized to have some possible role as signal peptide, I have compared the size of the *E. coli* produced PfHPRT (assuming *E. coli* does not have the specific signal peptidase to remove any Plasmodium signal peptide) and *P. falciparum* produced native HPRT in an western blot analysis using the PfHPRT antibody as the probe. The identical size of both the proteins may suggest that The *P. falciparum* HPRT does not undergo any detectable cleavage modification after synthesis. Moreover, If the protein undergoes such cleavage we would have expected a doublet in the western blot analysis. This result suggest that the extra peptide may not be a typical signal peptide.

Now because the the *E. coli* expressed PfHPRT is enzymatically active it can be expected that the enzyme can complement a homologous gene in *E. coli* . If this is possible the complemented strain could be an excellent source for the PfHPRT enzyme for further study. Furthermore, the strain will be a easy tool to screen putative antimalarial drugs directed against *P. falciparum* HPRT.

Chapter 6
Result 4

Biochemistry of the recombinant PfHPRT and Complementation of a bacterial mutation

The significantly high specific activity in induced SH2, in chapter 5 suggested that the PfHPRT expressed in *E. coli* is enzymatically active. However, the possibility that the *E. coli* gene contributes to this activity can not be ruled out. An in vitro complementation of bacterial hpt mutation would enable us to study the enzymatic activity in a HPRT-less environment. Moreover, the determination of the biochemical properties of the expressed enzyme in such a system could give authentic information regarding the biochemical properties of the *P. falciparum* HPRT.

As mentioned in the introduction section that *E. coli* has as same basic de novo pathway for purine synthesis as other organisms and also similar salvage pathway to use preformed purines. *E. coli* has two 6-oxopurine phosphoribosyl transferases, HPRT and XGPRT, both use hypoxanthine and guanine as substrate although at different efficiencies (Hochstadt and Cashel, 1972). Furthermore, adenine and guanine nucleotide may interconvert through the common precursor, IMP. These features suggest that for complementation study with the recombinant PfHPRT, the suitable mutant must have blockage in the de novo biosynthesis of purine, in purine interconversion and also in HPRT and GPRT steps.

The results described in the following sections are an attempt to complement a bacterial hpt mutation with the active recombinant enzyme. This will also study some biochemistry of the recombinant enzyme expressed in the bacterium.

6.1 The S. typhimurium KP1684 was used for complimentation study.

At the begining, *E. coli* was considered for the complementation study. But the hpt mutants available, were not suitable for this purpose. A mutant of

S. typhimurium, called KP1684 was described by Houlberg and Jensen (1983). This bacterium possesses all the required mutations for a suitable strain for the complementation study, and needs adenine and guanosine for its survival. Therefore, it was decided to use this strain for the complementation study.

The genotype of the *S. typhimurium* strain KP1684 is purE *deoD* hpt gpt. Figure 6.1 shows the purine salvage pathway in *S. typhimurium* with the steps blocked in the strain KP1684. The gene *purE*, codes for the enzyme, phosphoribosylaminoimidazole carboxylase. Mutation in this gene, blocks the IMP biosynthesis, so de novo biosynthesis of purine can not occur in this bacterium. The mutation in the *deoD*, the purine nucleoside phosphorylase gene, blocks the interconversion of purines. So, the strain needs preformed both adenine and guanine nucleus. But mutation in both hpt and gpt do not let the strain to use guanine base rather it uses guanosine as the guanine source, which converts into GMP by guanosine kinase. Furthermore, the strain can not use hypoxanthine, due to the hpt mutation, as its sole source of purine, made the strain suitable for the complementation of this mutation with an exogenous HPRT.

If the recombinant PfHPRT is enzymatically active and expressed in the *Salmonella* strain, It would be possible to compensate for the hpt mutation and the strain will then be able to use hypoxanthine as its sole source of purine.

Before going to the complementation study, the growth phenotype as expected from the genotype, of the strain KP1684 was checked. The bacterium was grown in minimal plates with adenine, hypoxanthine, guanine and guanosine in different combinations. The result in Table 6.1 shows that the strain can not grow on minimal plates and hypoxanthine or guanine can not serve as the purine source, neither adenine by itself. The strain only grows when both adenine and guanosine are present in the medium.

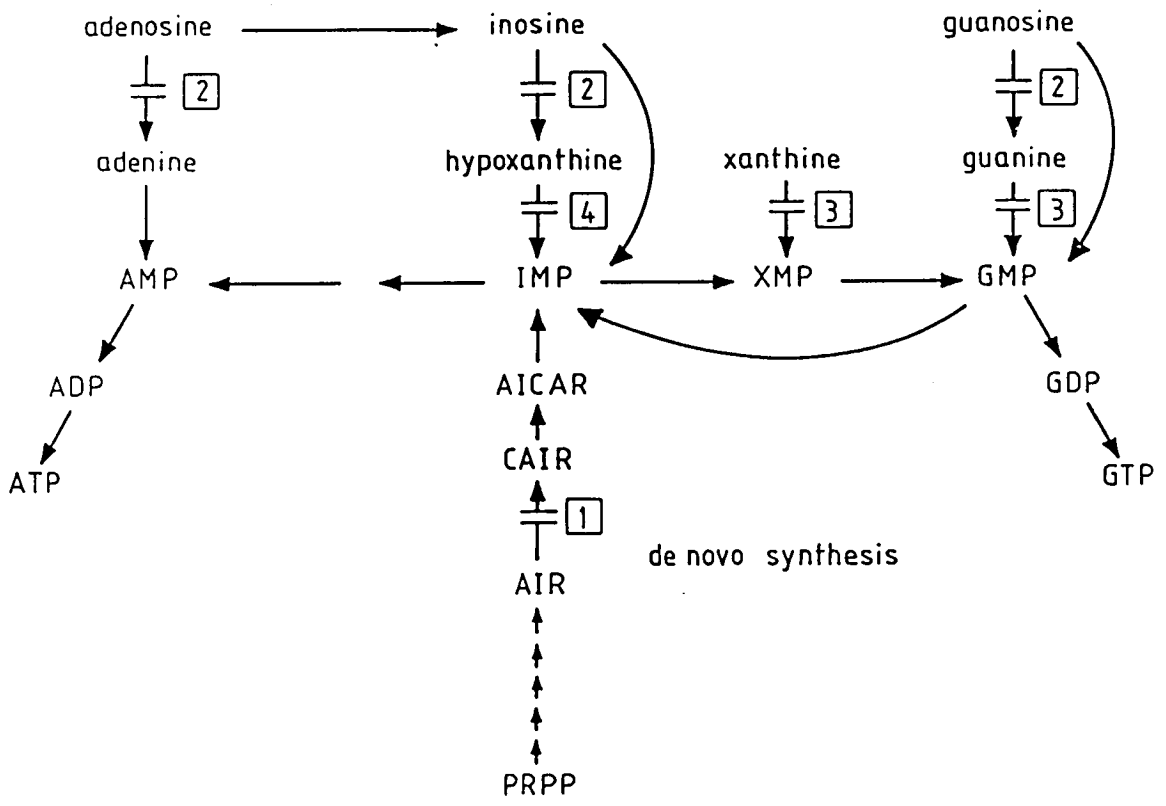


Figure 6.1 The blockages in the biosynthesis, salvage and interconversion pathways of purine in *S. typhimurium* strain KP1684 (*purE deoD hpt gpt*). For detail see text.

strain / medium	LB5000	KP1684
A	++	-
B	++	-
C	++	-
D	++	-
E	+++	+++

Table 6.1 Growth phenotype of *S. typhimurium* KP1684. A = minimal medium, B = A + 30 µg/ml hypoxanthine, C = A + 30 µg/ml adenine, D = A + 60 µg/ml guanosine and E = A + 30 µg/ml adenine + 60 µg/ml guanosine. - = no growth, ++ = small colony and +++ = big colony after overnight incubation at 37°C.

This data fits with the prediction from the genotype of the strain. And seemed suitable for our purpose.

6.2 pPfPRT2 was methylated for Salmonella modification pattern.

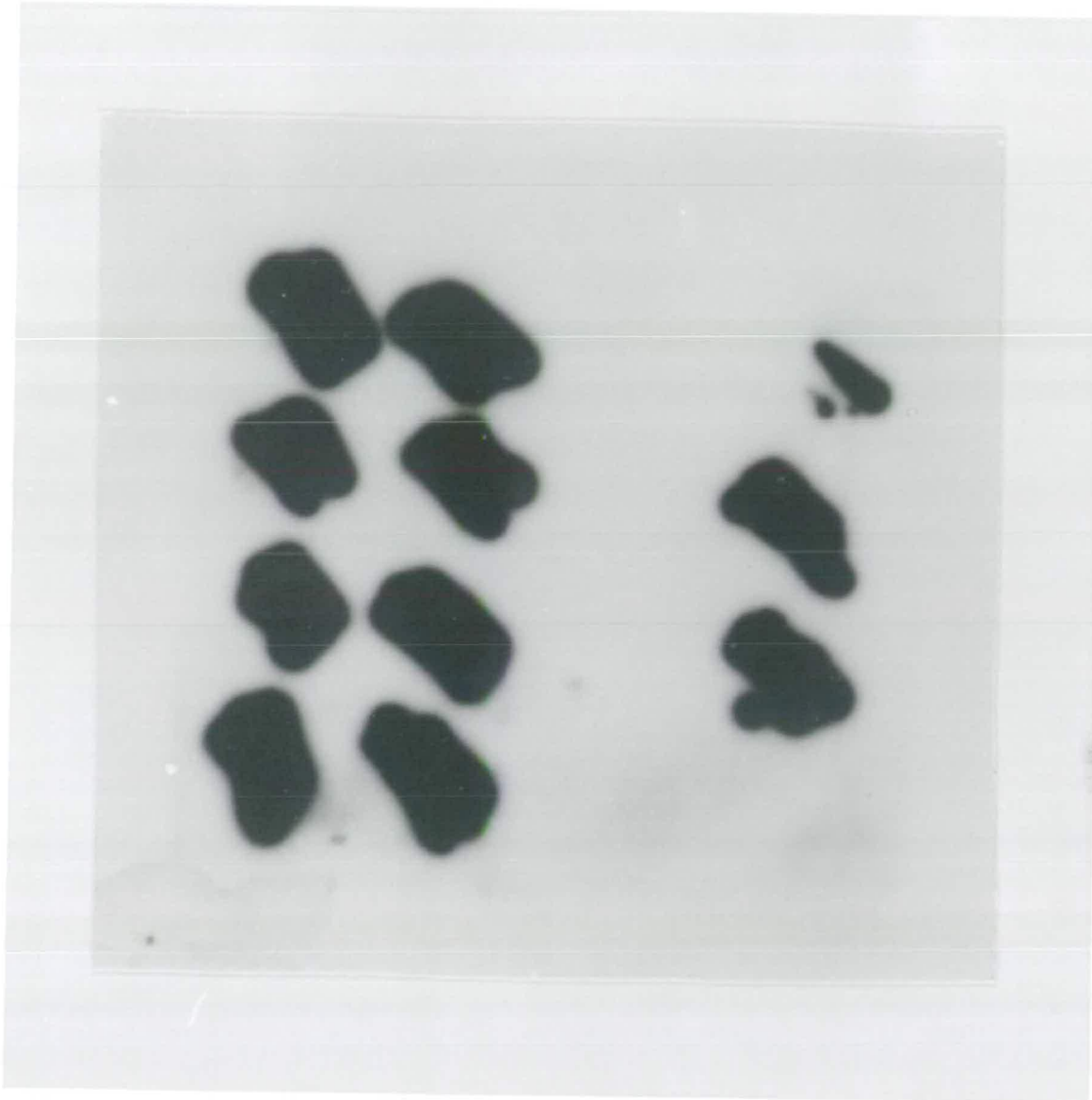
One possible problem in the transformation of pPfPRT2 from *E. coli* SH2 to *Salmonella typhimurium* KP1684, is restriction of the plasmid. To avoid such a problem, the plasmid was passaged through a r^-m^+ strain of *Salmonella* to modify according to the *Salmonella* modification pattern. Strain *S. typhimurium* LB5000 (Bullas and Ryo, 1983) was used for this purpose.

The purified plasmid from strain SH2 was transformed into the strain LB5000 [Methods], and the transformants were first selected by ampicillin resistant phenotype and later confirmed by colony hybridisation, using the original PfHPRT cDNA as the probe. 100% of the ampicillin resistant colonies were positive in the hybridisation step. Further confirmation about the structure of the plasmid was obtained by purification from one of the transformants and restriction mapping.

6.3 The modified pPfPRT2 was transformed into *S. typhimurium* KP1684.

To express the recombinant protein in strain KP1684 the modified plasmid from LB5000, was transformed into the bacterium by standard transformation protocol. The ampicillin resistant colonies were then picked and checked by colony hybridisation (Methods). The blot is shown in Figure 6.2. All of the ampicillin resistant bacteria were found to be positive when labelled HPRT cDNA was used as a probe. The plasmid was purified from one of the transformants and the structure of the plasmid was checked by restriction mapping. The transformant with the pPfPRT2 is called the *S. typhimurium*

Figure 6.2 Colony hybridization with labelled Pfhprt cDNA for the selection of pPfPRT2 transformants in *S. typhimurium* KP1684.



strain **SH4**.

6.4 The induced SH4 expressed PfHPRT protein.

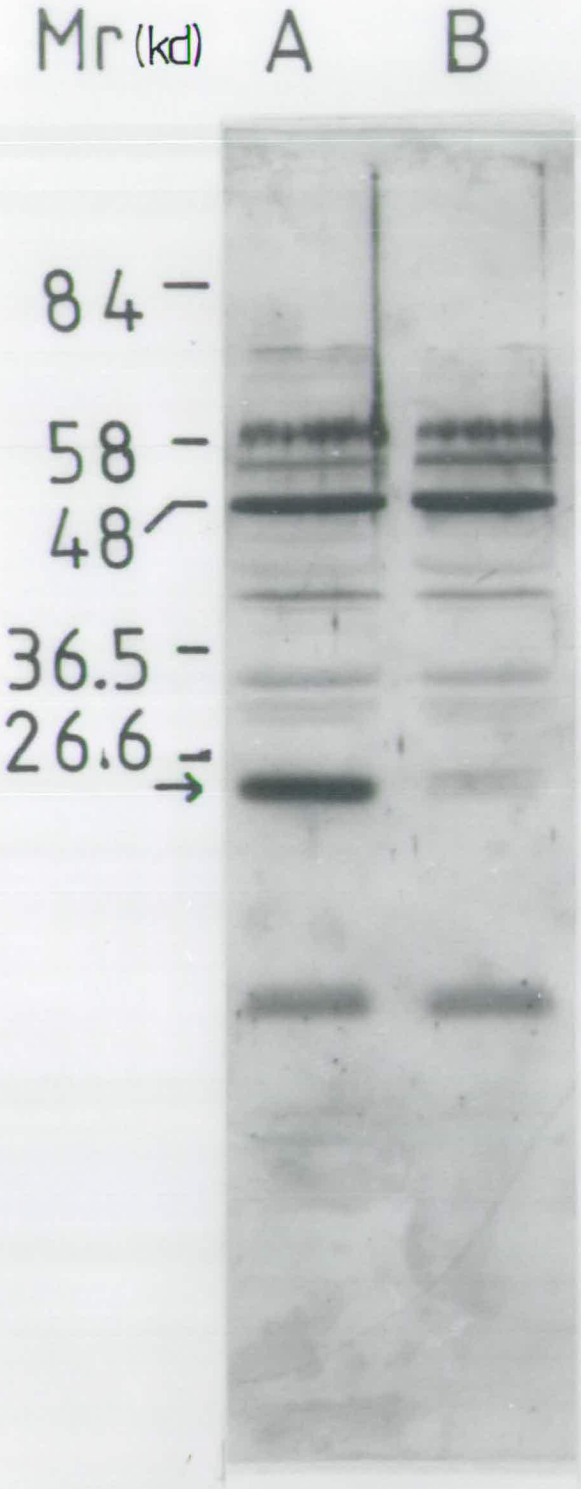
To check whether the recombinant PfHPRT does express in this Salmonella strain, the strain SH4 was grown and induced [Methods]. An uninduced culture was included as a control. Protein profiles of whole cell from both induced and uninduced SH4 were analysed by SDS-PAGE. Proteins were separated on a 12.5 % SDS-polyacrylamide gel and western blotted . The blot was then probed with PfHPRT antiserum (Chapter 4).

The result (Figure 6.3) showed that a protein of 26.5 kD is expressed in the induced SH4 (lane A) and is recognised by the anti PfHPRT serum. This protein has the same size as the protein expressed in the *E. coli* as was shown in figure 5.5 . The protein is not expressed in the uninduced SH4 (lane B). This suggests that the Plasmodium protein is successfully expressing in the *Salmonella* strain SH4 as well as in the *E. coli* SH2.

6.5 The complementation was not observed.

The result in the previous section showed that the PfHPRT is expressed in the induced SH4. And since, the protein is active in *E. coli*, it can be expected that the protein will be active in Salmonella as well. If this is true, the mutation in the hpt gene in KP1684 should be compensated by this enzyme. To test this possibility, the strain SH4 were streaked on minimal plates containing ampicillin and 30 µg/ml hypoxanthine and incubated at 42 C. A similar plate was incubated at 30 C as a control. Two other plates without hypoxanthine were also included as the control and incubated at 30 C and 42 C. After a 72 hours of incubation, no growth was observed in any of the four plates.

Figure 6.3 Expression of recombinant PfHPRT in *S. typhimurium* SH4 (KP1684/pPfPRT2). Lane A, shows the expressed recombinant protein in induced SH4 is recognized by the antiserum against PfHPRT (arrow). Lane B shows no such protein is expressed in the uninduced SH4.


Mr (kd)
A
B
84
58
48
36.5
26.6

The immediate explanation could be that, the enzyme although active in the E. coli strain but may be inactive in the *Salmonella typhimurium* strain.

6.6 The enzyme was active in in vitro assay.

Assay of HPRT in cell free extract of the induced SH4 would enable us to confirm the activity of the recombinant PfHPRT expressed in the bacterium. This is possible because the host strain KP1684 does not have any detectable HPRT activity (Houlberg and Jensen, 1983).

So to test the above explanation for the inability of the recombinant bacterium to grow on a Hypoxanthine plate, HPRT activity was measured in the extract of both induced and uninduced SH4. The uninduced and induced cell extract of the host strain KP1684 were included as the negative control. The result shown in Figure 6.4, conclusively proved that the recombinant enzyme is active in strain SH4. The figure shows that the enzyme activity increases with the addition of the cell free extract of the induced SH4. whereas there was no such detectable activity in uninduced SH4 extract and neither in the induced KP1684 extract.

6.7 A modified medium supported the growth of S. typhimurium SH4.

The result presented above argues against inactivity of the recombinant PfHPRT expressed in SH4. Then, why did the active enzyme not complement the hpt mutation in KP1684?

One explanation is suggested by the fact, that addition of hypoxanthine and other purine bases in vegetatively growing *Salmonella* cells causes severe depletion of the PRPP pool in the bacterium (Jensen, 1983). PRPP, the second substrate of the HPRT catalysed reaction is required for the biosynthesis of

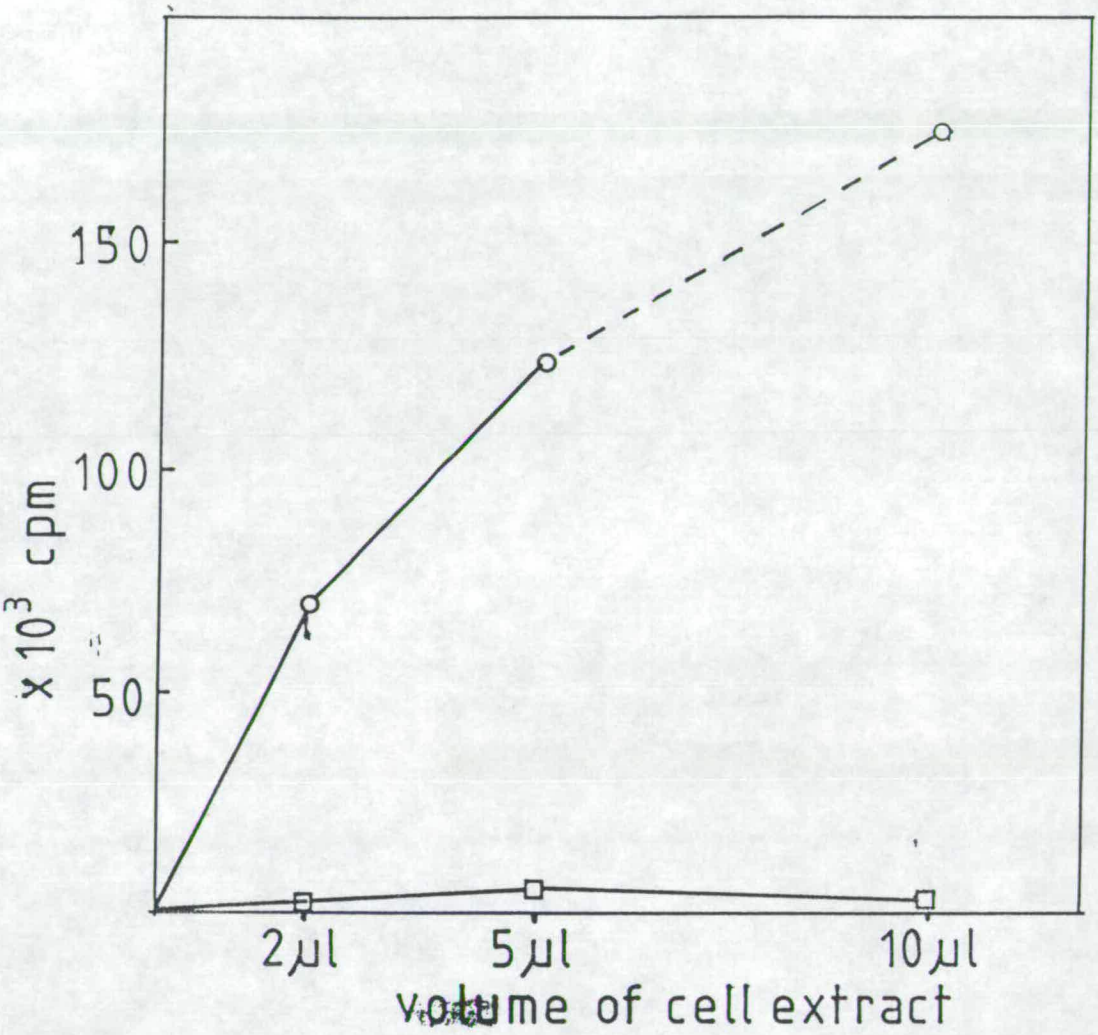


Figure 6.4 HPRT activity in induced *S. typhimurium* SH4 and KP1684. Figure shows the incorporation of the Labelled hypoxanthine into IMP by cell free extracts of the bacteria, SH4 (o—o) and KP1684 (□—□).

many other important metabolites. This includes histidine, tryptophan, pyrimidine bases, a total of 10 enzymes compete for PRPP as a substrate (Neuhard and Nygaard, 1987). Now if this is so, The overexpression of the active PfHPRT in the cell at 42 C and the abundant supply of the hypoxanthine in the medium may deplete the PRPP pool in the cell and as a result other important metabolites like histidine, tryptophan and pyrimidines can not be synthesized. To check whether this can be the reason, the minimal medium used in the previous experiment is now supplimented with each of 50 µg/ml of histidine and tryptophan and 30 µg/ml of cytidine. The bacterium was streaked on the plates and incubated as in section 6.5. This time indeed, about 2mm diameter colonies of SH4 was observed after 72 hour incubation at 42°C, in the plate supplimented with hypoxanthine. No growth was noted, either on the no hypoxanthine plate incubated at 42°C or in hypoxanthine supplimented plate incubated at 30°C. The result is shown in Table 6.2. Thus the impaired synthesis of other essential metabolites in the cells may be one of the reasons of the inability of the growth of the recombinant bacterium in the medium supplimented with only hypoxanthine.

6.8 The growth rate was enhanced by adding other amino acids.

The slow growth rate of the complemented mutant was not understood. Incubating the plates longer did not improve the colony size. One simple explanation could be that the overexpression of the active enzyme may have some effect on other important metabolic pathways. For example, during the overexpression of the PfHPRT, the cellular protein-synthesis-machinary remains mostly involved in the synthesis of the induced protein, which may cause the less availability of the ribosomes and tRNAs (specially the rare tRNAs) for biosynthesis of other essential proteins. This speculation is strengthened by the

temp.	media	KP1684	SH4
30	A	-	-
	B	-	-
42	A	-	-
	B	-	±

Table 6.2 Complementation of the *hpt* mutation in *S. typhimurium* KP1684 with PfHPRT. Strain KP1684 and SH4 was streaked on solid medium A or B and growth was recorded after 72 hours. A = minimal medium + 30 µg/ml cytidine + 50µg/ml Histidine + 50µg/ml Tryptophan, B = A + 30 µg/ml hypoxanthine. - = no growth and ± = small colony after 3 days.

observation that, during the overexpression of the fusion PfHPRT in *E. coli* other cellular protein content was decreased (Figure 5.5 lane C). This may also include the synthesis of other essential enzymes.

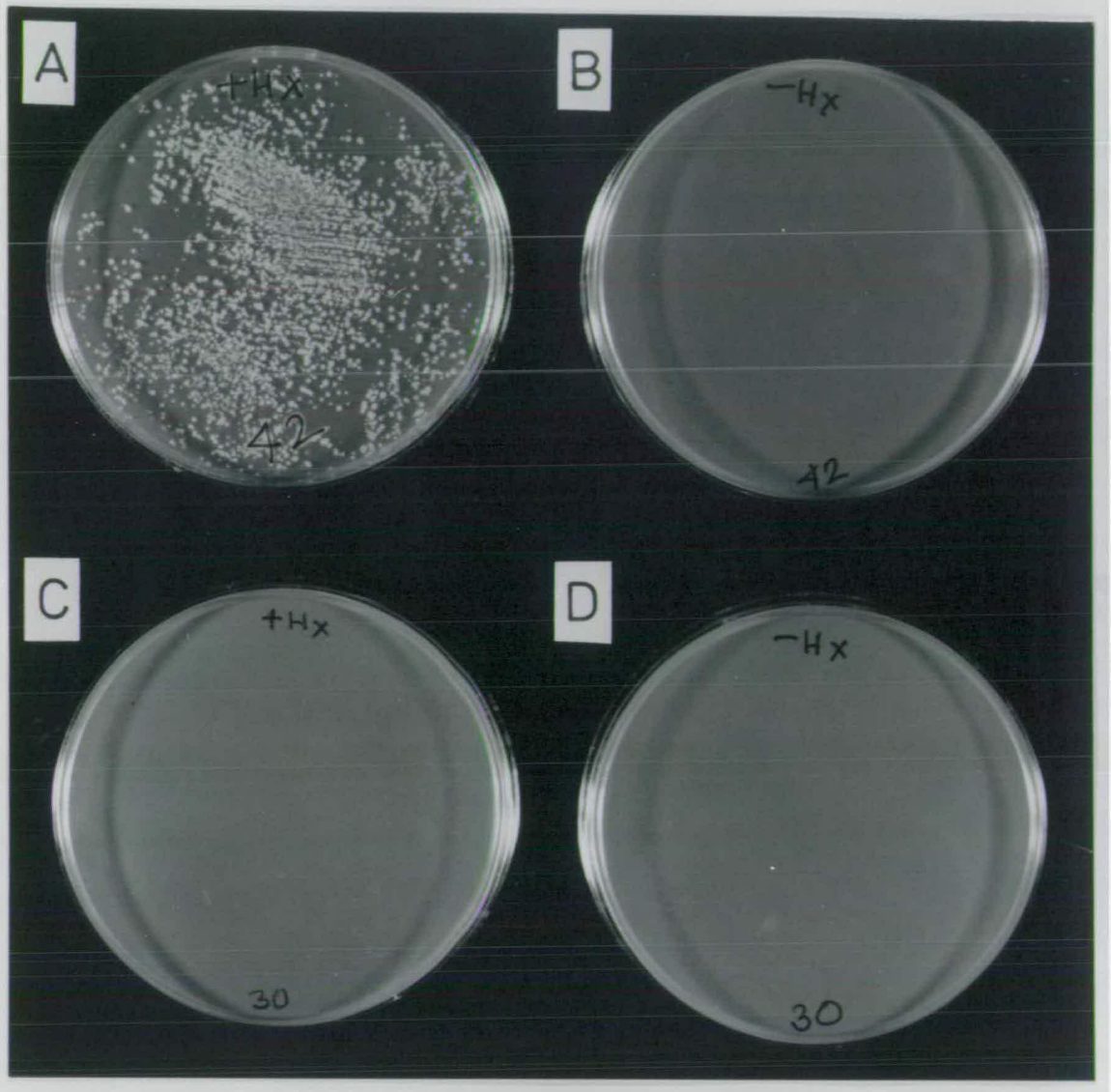
Now if this is so, alleviation of the biosynthetic pressures from the cell, at least to a certain extent, should increase the growth rate of the complemented mutant. Bearing this in mind, the minimal medium was supplimented with 1% casamino acids and cytidine as above. Casamino acids (DIFCO) are the acid hydrolysate of the pure milk protein, casein and does not contain any nucleic acid precursor and used in the media where mixture of amino acids are essential. The addition of preformed amino acids should remove the pressures from the cells to synthesize these amino acids.

When the experiment described in the previous section is repeated with the minimal medium supplimented with 1% casamino acids and cytidine a good growth was noted after 24 hrs incubation, only on the plates containing hypoxanthine and incubated at 42°C as shown in the Figure 6.5, plate A. No growth was observed in plates, supplemented with 1% casamino acid without hypoxanthine (plate C). Neither was growth observed in plates incubated at 30°C, with or without hypoxanthine (plates B and D). This result supported the explanation for the poor growth of the complemented mutant made earlier this section. But the involvement of the other mechanisms is also possible. Further study of bacterial metabolism and the effect of the recombinant protein expression on bacterial metabolism may help in designing of a better culture medium for the growth of the recombinant bacterium.

6.9 The expressed enzyme can also use xanthine and guanine as substrates.

P. falciparum HPRT has been partially purified and biochemical studies on

Figure 6.5 Complementation of the *hpt* mutation in *S. typhimurium* KP1684 by PfHPRT. Growth enhanced by improved medium. (A)growth of SH4 in minimal medium supplemented with 30 µg/ml cytidine and 1% casamino acids incubated at 42°C, (B) same as A but medium contained no hypoxanthine, (C) same as A, plates incubated at 30°C and (D) same as A except the medium contained no hypoxanthine and incubated at 30°C.


A
+Hx
42
B
−Hx
42
C
+Hx
30
D
−Hx
30

this enzyme had been reported (Queen *et al*.1988). Their study showed that the *P. falciparum* enzyme can also use both guanine and xanthine as its substrates. The property is unusual than that of the enzymes from other *Plasmodia* species.

To study whether *P. falciparum* really can use guanine and xanthine as its substrate or in other words whether the recombinant PfHPRT expressed in bacteria can fold the expressed polypeptide exactly into its native form, the induced SH4 extract was assayed for PRTase activity using guanine and xanthine as the substrates. The activities thus obtained were compared with that in the extracts of uninduced SH4 and induced KP1684. The results as shown in Figure 6.6, demonstrated that the extracts from induced KP1684 can indeed use the purines as its substrates. The extracts of the uninduced SH4 and induced KP1684 did not activities with either of these substrates. This result may suggest that *P. falciparum* HPRT is able to use guanine and xanthine as its substrates and supports the report of Queen et al (1988). This result may also suggest that the polypeptide expressed in the bacterium can fold into native form as well as in the parasite.

6.10 The recombinant enzyme uses hypoxanthine more efficiently than xanthine or guanine.

It is not quite possible to measure the Km values of an enzyme for its substrates or Ki values for the inhibitors without purifying the enzyme to homogeneity at least to a certain extent. This is because the possible degradation of the product by other enzymes will make it difficult to measure the absolute amount of the product formed. However, in SH4 the enzyme expression rate is much higher than the other enzymes and the extract gave linear rate of product formation up to a certain extent (1 µg total protein), it

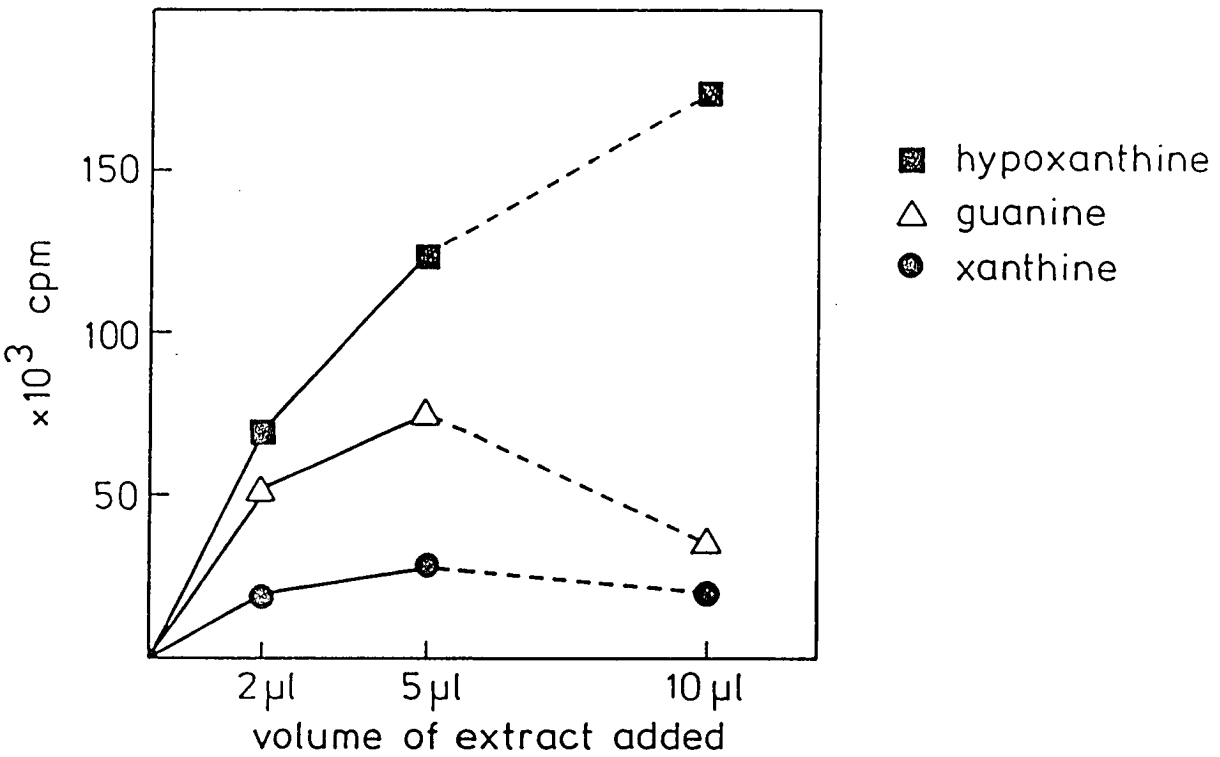


Figure 6.6 Utilization of guanine and xanthine as substrates by recombinant PfHPRT expressed induced SH4. The incorporation of the radiolabelled purines into corresponding nucleotides with respect to the volume of the induced SH4 extract added.

may be possible to measure the specific activity, allowing a degree of error. This could enable us to compare the affinity of these substrates to the enzyme. The specific activities were measured for the three substrates and expressed as the ng product formed per 10 minutes per mg of the total protein, the result is shown in Table 6.3. The table shows that the specific activity of the enzyme is about 11 times more with hypoxanthine than with xanthine as the substrate, whereas it is about 4 times higher than that with guanine. It may suggest that the efficiency of the enzyme to use hypoxanthine as substrate is highest and that is to xanthine is lowest putting guanine in between.

6.11 The enzyme activities are inhibited by 6-thioguanine.

As it is not possible to measure the Km values for Hypoxanthine, guanine and xanthine with unpurified enzyme, a different strategy was adopted to confirm that the enzyme has different affinity for these substrates. The specific activities of the enzyme was measured with the three substrates in presence of the competitive inhibitor, 6-thioguanine. The reason for choosing 6-thioguanine is its higher Ki value to the enzyme (7 μM) than that for the hypoxanthine and guanine (0.46 μM and 0.3 μM respectively) and lower than that for xanthine (23 μM). The idea was if the bacterially expressed protein shows any affinity difference the extent of inhibition of the activity by the competitive inhibitor would be different. The reaction mixture contained 100 μM purines high above the reported Kms and 1 mM 6-thioguanine. The reaction and measurement of product formation was carried out according to the method described for earlier experiments. The extent of inhibition was defined by the percent of the activity showed with the presence of 6-thioguanine compared to that of without it. The result is shown in Table 6.3.

It may be concluded from the result that the affinities of hypoxanthine and

enzyme source	purine substrate	estimated specific activity nmole/10 min/mg	% activity in presence of 1 mM 6-thioguanine
KP1684	hypoxanthine	ND	-
KP1684/ pJLA503	hypoxanthine	ND	-
SH4	hypoxanthine	60.8 (± 4.6)	76
	xanthine	5.79 (± 1.2)	2.5
	guanine	15.8 (± 1.9)	75

Table 6.3 Properties of *P. falciparum* HPRT expressed in *S. typhimurium* SH4. Under our conditions, the parent strain KP1684, has no detectable PRTase activities (methods). The table shows the estimated specific activities with hypoxanthine, xanthine and guanine. Specific activities shown are average of at least three determinations. Standard deviations appear in brackets. Inhibition of enzyme activities with 6-thioguanine was measured by adding the analogue to appropriate reaction mixtures (final concentration 1 mM).

guanine are higher than the inhibitor, so they win in the competition in binding to the enzyme active site, as a result the inhibitions of activities were not very efficient for these substrates, only about 25%. In contrast, the inhibition of the XPRT activity was immense, about 95%. This may indicate that the affinity of the enzyme to xanthine is very low. This result also indicates that the affinity of the enzyme to hypoxanthine and guanine is comparable.

Such results also suggest that the Plasmodium enzyme is efficiently folded into the native form in the bacteria, as reflected by the fact by retaining activity for all three substrates with similar affinity difference pattern as described for the native enzyme (Queen *et al.*, 1988)).

6.12 Strain SH4 can not use xanthine or guanine as its purine source.

The obvious consequence of the above result which showed that the expressed protein can use xanthine and guanine as its substrates, is that the strain should use either of these bases as its purine source. So, to check whether xanthine and guanine could substitute for hypoxanthine in the complementation study, either 15 µg/ml of xanthine or guanine was replaced for hypoxanthine in the medium. The plates were inoculated with SH4 and incubated overnight at 30°C and 42°C as described in section 6.8. After incubation for 72 hours no growth was observed in any of the plates containing either xanthine or guanine. But good growth was observed with hypoxanthine.

6.13 Discussion and conclusions.

As it was established in chapter 5 that *E. coli* can synthesise active full length PfHPRT, obviously an *E. coli* mutant would be the first choice for the

complementation study. Instead, A *Salmonella typhimurium* strain was used for this purpose. Search for a suitable *E. coli* mutant was fruitless. For example, an *E. coli* hpt mutant, strain Sϕ606, although does not have any HPRT or GPRT activity and mutation in *purR* blocks the de novo purine synthesis, but it could use hypoxanthine through purine nucleoside phosphorylase (*deoD*) mediated salvage pathway (see introduction). Thus, it was not possible to use *E. coli* for the study.

E. coli and *Salmonella typhimurium* are related organisms being in the same Enterobacteriaseae family. Along with several dissimilarities they share most of the common metabolic pathways (Neuhard & Nygaard, 1987). the purine metabolic pathway has been shown to be similar. And because a suitable strain KP1684 was available, it was used for the purpose.

One potential problem of using *S. typhimurium* was the possible restriction of the *E. coli* grown plasmid in this bacterium. Restriction-modification system is species specific as well as organism specific. Evidence showed that modification system in Salmonella is different than that of *E. coli*. For instance, Zinder *et al* (1952) found that the frequency of transformation of an *E. coli* donated plasmid to *S. typhimurium* LT2 is as low as 10^{-7}. Possibility of such problem would make the transformation of the *E. coli* grown pPfPRT2 in *S. typhimurium* KP1684 difficult. Such possibility was overcome by passing the plasmid through a $r^{-}m^{+}$ Salmonella strain LB5000, before any attempt was taken to transform it into KP1684. By doing so, a pPfPRT2 transformant of *S. typhimurium* KP1684 named SH4 was obtained, which expressed the parasite HPRT in high amount. The authenticity of the expressed protein was confirmed by probing the protein profile of the induced and uninduced SH4 in a western blot analysis. Such probing with the preabsorbed antiserum also recognised several other proteins in both induced

and uninduced cells. This may be due to insufficient preabsorption of the serum with *E. coli* extract. A further absorption of the immune serum with Salmonella extract could remove some of the nonspecific bands. A minor band in the uninduced cell was possibly due to some leakage in the control of the gene regulation in the bacterium.

As in the case of the *E. coli* expressed protein, the protein expressed in Salmonella was also active. Because the strain KP1684 does not have any HPRT and GPRT activity, the demonstration of such activity in this strain conclusively proved that the parasite enzyme is enzymatically active. In other words the recombinant polypeptide can fold into the active form as well as in the parasite.

The activity was characterised by the increase in the incorporation of radioactive hypoxanthine into the IMP formed. However, the increase of the incorporation was observed upto a limit. At higher amount of cell extract the estimated product formation was low. Such lower observed lower product formation by higher amount of the bacterial extract may be due to the presence of the nucleosidase enzymes present in the cell. For example, adenylosuccinate synthetase (encoded by *purA*) and IMP dehydrogenase (encoded by *guaA*) use IMP, the product of HPRT as substrate and produces succinyl-AMP and XMP respectively. Both these enzymes are active in KP1684. Moreover, it has been shown that purA expression is induced in *E. coli* when hypoxanthine is added in the medium (Neuhard and Nygaard, 1987). This may suggest that at higher amount of the extract the hypoxanthines are used up by the overexpressed HPRT and the product, IMP is removed by the above enzymes.

The enzymatic activity led to test the ability of the protein in the compensation of the *hpt*, *gpt* mutation of the strain. However, the first

attempt, where the minimal medium was supplemented with Hypoxanthine was failed. This was thought be due to the unavailability of the important metabolite PRPP, the second substrate of HPRT, for other biosynthetic purpose. Such hypothesis was tested by supplementing the medium with Histidine, tryptophan and cytidine. Biosynthesis these metabolites depend on the availability of PRPP. Such modification of the medium supported the growth of SH4 only if the medium contained hypoxanthine and the PfHPRT gene in the vector was allowed to express. Addition of casamino acids, which can lower the biosynthetic pressure from the organism to synthesize amino acids, increased the growth rate significantly. Further increase of the growth rate may be possible by understanding the metabolism of the bacterium and modifying the medium according to that. For example, biosynthesis of vitamin nicotinamide adenine dinucleotide (NAD) also need PRPP, so addition of NAD may improve the quality of the medium.

Queen *et al* (1988) reported that *P. falciparum* HPRT can use xanthine and guanine in addition to hypoxanthine. However, the enzyme from other Plasmodium species studied so far do not have this ability. For instance, *P. chabaudi* (Walter & Konigk, 1974) and *P. lophurae* (Schimandle *et al*, 1987) HPRT can use only hypoxanthine and guanine. The strain SH4 provided an excellent system to study such properties because, the bacterium does not have any HPRT ,GPRT or XPRT activity (hpt gpt phenotype). Assay of the induced SH4 extract for PRTase activities with xanthine and guanine showed that the enzyme indeed can use these two purines as its substrates.

However, the enzyme showed difference in efficiency for the utilisation of different purines. It was found that hypoxanthine is the most favoured substrate and xanthine is the least, and guanine is in between. This conclusion was came from the the result that the induced SH4 cell extracts showed 10

times higher specific activity with hypoxanthine than that with xanthine and about 4 times higher than that with guanine.

Such differences in efficiency could have two different causes. Firstly, the enzyme may have higher affinity to hypoxanthine than the other two, so it binds to the enzyme more efficiently. This possibility also suggested by Queen *et al*(1988). They measured the Km values of all these three substrates using partially purified enzyme preparation and reported the Km value of hypoxanthine is 0.46 µM and that of the xanthine is 29 µM. The value of guanine was 0.30 µM. Their result suggests that guanine is somewhat more favourable substrate than hypoxanthine. These differences in result may be due to the use of unpurified enzyme of recombinant bacterium in this work. Further studies using purified recombinant enzyme may give us the true values. The difference of the affinity to these substrates was also o an inhibition study. The competitive inhibitor, 6-thioguanine inhibited the utilisation of xanthine to about 98% whereas the utilisation of the hypoxanthine or guanine were inhibited to only about 25%, suggesting that the enzyme has more affinity to 6-thioguanine than xanthine but less than to hypoxanthine or guanine.

Secondly, it is possible that the turnover number of the enzyme for hypoxanthine is higher, in other words, the transfer of the phosphoribosyl group from PRPP to the enzyme-bound hypoxanthine is faster than to other substrates. At the saturation concentration of the substrate, like in our experimental concentration (100 µM), the difference in affinity does not play any crucial role in the reaction rate if the turnover number is the same. So, the result in this study may also suggest that the enzyme has different turnover numbers for different substrates. It is possible that both of these factors contribute to the different specificities observed.

Why should PfHPRT be able to use xanthine ? The role of purine

metabolism in the mosquito may bear upon this question. In man, Plasmodium, like other parasite, predominantly uses the most available purine, hypoxanthine (see introduction). By contrast, mosquitoes, like other terrestrial insects use purine metabolism in the excretion of nitrogen (Gilmour, 1965). This insects produce large amount purine ribotides from ammonia, the end product of amino acid metabolism (introduction). This are metabolized to xanthine which is later converted into uric acid by xanthine oxidase. This may result in high concentration of xanthine in the haemocoel. In the mosquito the parasite multiplies extensively (Pringle, 1966), needing much more purine than in man. Perhaps the parasite has evolved an HPRT able to exploit the available xanthine in the insect.

The ability of the enzyme to use xanthine and guanine suggests that the PfHPRT should also complement the gpt phenotype. This means the bacteria should use xanthine or guanine as its purine sources. However, replacement of the hypoxanthine from the medium with guanine or xanthine did not allow growth.

This result was surprising at the first instance. But study of the metabolic phenotype *purE deoD* double mutants of *S. typhimurium* may bear upon the reason. Neuhard and Nygaard (1987) described that a *purE deoD* double mutant can only grow on guanosine if the requirement of the adenosine nucleotides is satisfied by adenine. Although, guanosine can convert into GMP by guanosine kinase which subsequently can convert into IMP by GMP reductase, the basis of this phenomenon was not clear. He also demonstrated that, this lack of growth in guanosine is partially suppressed by a mutation in *gsk* gene which code for a altered guanosine kinase and complete suppression is achieved by a further mutation in genes of the de novo purine synthesis pathway before purE. These observations may suggest that the

complementation of the gpt-mutation could be achieved by the PfHPRT, if the medium would supplimented with adenine as the source of adenine nucleotides.

Chapter 7
Result 5

Cellular studies on native PfHPRT.

Hypoxanthine phosphoribosyl transferase is a metabolic enzyme. Since the parasite does not contain the de novo biosynthetic pathway for purine nucleotide synthesis, the enzyme plays a key role in parasite's life . The biosynthesis of DNA does not occur continuously through the parasite life cycle. However, RNA synthesis seems to occur in all stages. This suggests the necessity of the enzyme HPRT to be present in all the stages of parasite's life. The apparent presence of only one copy of the HPRT gene in the genome and the varied requirement for the enzyme in different stages (Pringle, 1966), where large amount of DNA synthesis takes place, raises the question; is the gene expression is under a certain type of regulation ? The difficulty in the purification of parasites from different stages is probably one of the major obstacles in the way of answering such a question.

Another important question to answer is about the location of the enzyme in the parasite. In other words, whether the enzyme is a soluble, cytosolic protein like the mammalian enzyme, or a membrane associated protein. The eukaryotic cell has localized many of its metabolic processes into specific membrane-bound compartments. For example, the Kreb's cycle enzymes of carbohydrate metabolism reside in mitochondria (Lehninger, 1982). In this case, acetyl coA, the end product of the Embden-Mayerhoff pathway enters into this organelle and subsequently degrades into CO_2 and H_2O. Similarly, the enzymes of photosynthesis and lysosomal proteases are present in chloroplast and lysosome respectively. Several microbodies present in the cell cytoplasm also contain different metabolic enzymes, for instance peroxysomes, glyoxysomes and glycosomes.

An indication that the PfHPRT may be associated with some membrane bound system comes from the work of Reyes *et al* (1982). They found that addition of the membrane fraction of the parasite sonicated lysate into the

reaction mixture, largely increased the conversion of hypoxanthine to AMP and GMP and suggested that maybe some of the purine salvage enzymes are associated with membranes. However, they found high activity of the HPRT in the soluble supernatant fraction of the lysate and also considered the enzyme as a cytosolic protein.

In contrast, the enzyme is a membrane-bound 'glycosomal' protein in several other intracellular parsites, Such as, *Trypanosoma brucei* (Hammond *et al.*, 1985), *T. cruzi* (Gutteridge and Davies 1982), *Leishmania* spp. (Hassan *et al.*, 1985). These parasites obtain their purine nucleotides through the salvage pathway like *Plasmodia*.

Moreover, Queen *et al*(1988) reported very low yield of the enzyme while purifying from the soluble fraction of the sonicated parasite lysate. Moreover, western blot of the parasite proteins with antiserum against PfHPRT suggested that the enzyme is quite abundant in the parasites. This information together suggests that the protein may be compartmentalised in some membrane bound organelle or a membrane associated one. However, Queen and co-author's low yield may be due to the loss of enzymatic activity during the purification process.

Finally, comparison of the predicted amino acid sequence of the protein with that of the mouse (King & Melton, 1987)) reveal that the *P. falciparum* protein contains an extra stretch of 8 amino acids in the N-terminal region and 3 amino acids in the C-terminal region, which are not present in mouse where the protein is cytosolic. This may also indicate the possible targeting of the protein into some membrane bound compartment. In this chapter, I will be presenting results of *in vivo* probing of the PfHPRT with the antiserum prepared in chapter 4 and will discuss the findings.

7.1 The anti-PfHPRT antiserum does not recognize human HPRT

One possible problem of using the *P. falciparum* anti-HPRT antibody for probing the location of the enzyme in vivo, may be the possible cross-reactivity with the homologous human enzyme. Red blood cells are rich in HPRT and amino acid sequence homology between *P. falciparum* and mouse HPRT showed that although the overall homology is low (48%) but patches of high sequence homologies are present between two enzymes. Such homology is particularly noticeable in the postulated active site (75%) (King & Melton, 1987).

To resolve this question, proteins from 20µl of uninfected human blood (O+) were analysed by western blotting using the anti-PfHPRT antiserum as a probe. Induced SH2 protein (from section 5.5) and the parasite protein were included in the experiment for comparison. The blot was then probed with preabsorbed anti-PfHPRT antiserum as described in section 5.5 . The result shown in Figure 7.1, indicates that none of the human red blood cell proteins (lane B) were recognised by the anti-PfHPRT anti serum. However the protein is recognised both in parasite (lane D) and bacterial lysate (lane B). A similar result was obtained with a higher amount of RBC proteins. This result clearly suggest that the antiserum is specific to the parasite protein and does not cross-react with the host enzyme.

One possible explanation of these results may be that, since the antibodies were raised in a rabbit, common epitopes between human and the parasite enzyme were not recognised by the rabbit's immune system as foreign epitopes

Figure 7.1 Western blot of human red blood cell proteins with the PfHPRT antiserum. Figure shows the protein profiles as recognized by the antiserum. (lane A), uninduced SH2 cell extract, (lane B), induced SH2 cell extract, (lane C), whole red blood cell extract and (lane D) Whole *P. falciparum* strain K1 extract.


A
B
C
D
kD
58
48
36
26.8
Pf HPRT

7.2 Evidence that the homologous region in the parasite and mouse enzyme did not elicited immune response in rabbit.

As mentioned before the highest homology (75%) between mammalian HPRT (mouse) and plasmodium HPRT is at the predicted active site of the enzyme (Argos *et al.*, 1983). To check whether this region elicited any immune response in the rabbit, the inhibition of the enzyme activity was measured in presence of the anti PfHPRT antiserum. The antiserum used for this study was incubated at 55 C for 1 hour. The serum did not have any detectable HPRT activity. Twenty µls of the cell free extract of the induced SH4 were mixed with 100 µls of the serum and incubated at room temperature for 10 minutes. The PRTase activities of the incubated extract were measured using 10 µls of the incubation mixture [method]. In a control experiment the antiserum was replaced by BSA (10 mg/ml). The result is shown in Figure 7.2 and indicates that the enzyme activities with all three substrates (panel 1,2,3) are not inhibited by the antibody at all, and appear if anything to be stimulated. This indicates that the antiserum does not contain any antibody against the active site of the protein, otherwise the bound antibody would restrict the access of the substrate to the active site.

An increase in activity was observed for all the substrates in presence of the antiserum. This may be due to the binding of other antibodies, at sites other than the active sites which may have increased the affinity of the enzyme to its substrates by changing the structure of the enzyme.

7.3 Confirmation of the specificity of the antiserum.

It was discussed in section 7.1 that none of the proteins in red blood cell were recognised by the polyclonal serum. To confirm this result and to get

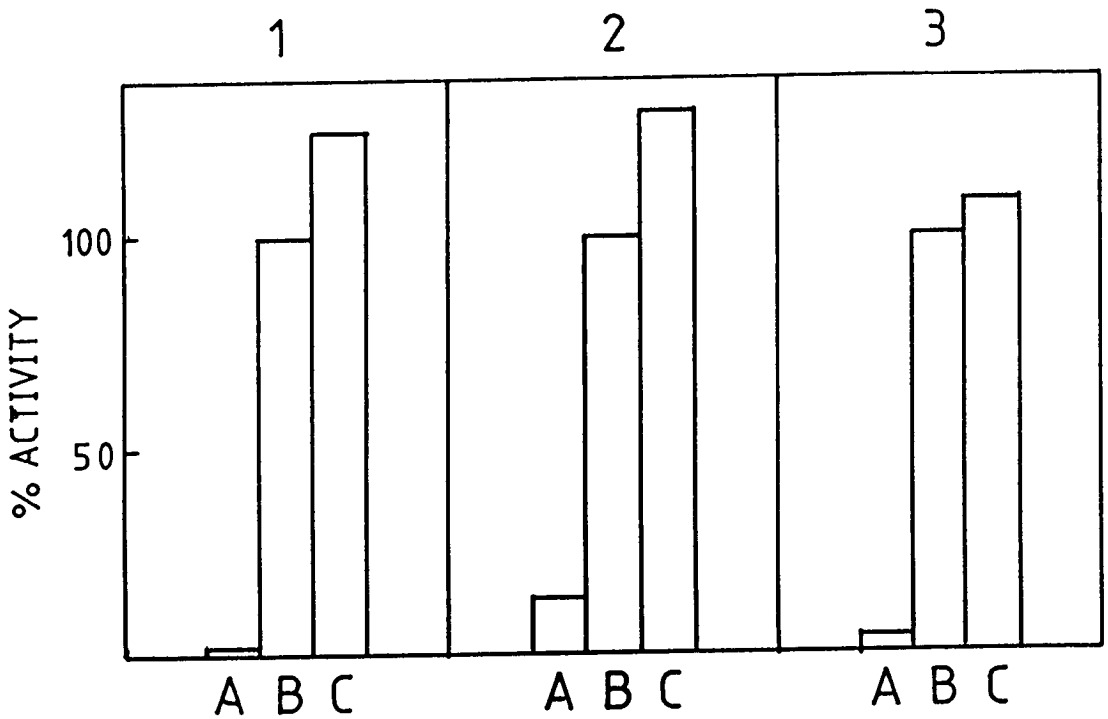


Figure 7.2 Enhancement of PRT activities with xanthine, hypoxanthine and guanine of the recombinant PfHPRT by antibodies raised in rabbit. Data is expressed as the percentage of the activities without any antiserum added (bar B). Bar C, shows the activities in presence of the antiserum (see text). bar A, shows the PRTase activities present in the antiserum. Panels 1, 2 and 3 show the activity measurements with xanthine, hypoxanthine and guanine respectively.

some direct evidence that the antiserum is parasite-specific, indirect immunofluorescence assay (IFA) was carried out using the antiserum preabsorbed with bacterial extract at a final dilution of 1:50 and 1:100 [method]. The result is shown in Figure 7.3.

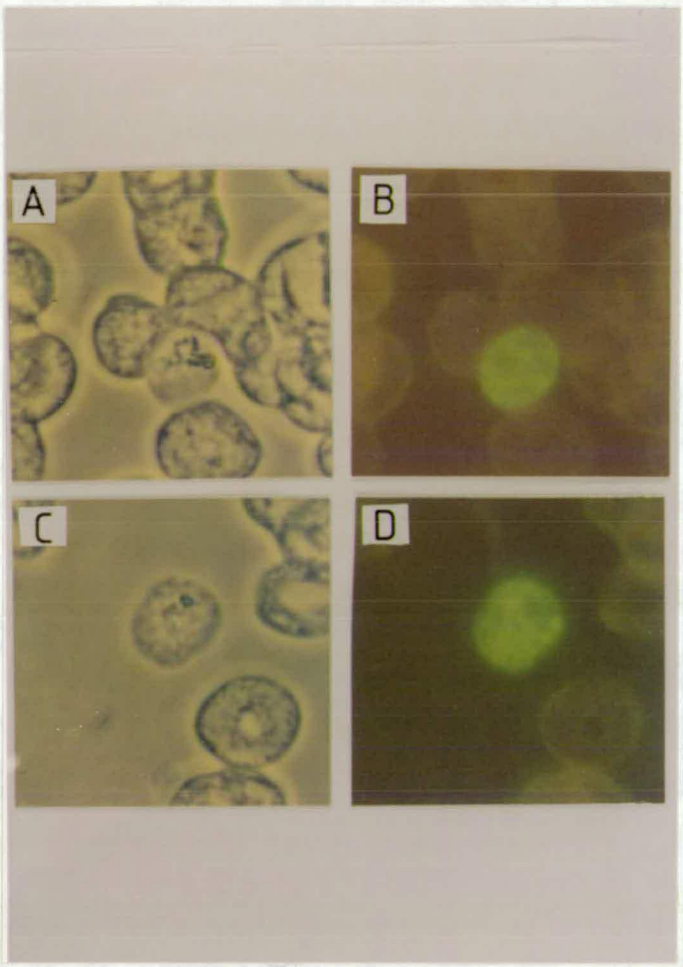
It is clear from the result that only parasitized cells (pigmented in the phase contrast, frame A/C), gave fluorescence with the antiserum (frame B/D). Whereas the uninfected red blood cells remained completely unstained. This result confirmed that the anti-PFHPRT antiserum prepared in rabbits using the recombinant protein is parasite specific and does not recognize the human HPRT.

7.4 IFA pattern of P. falciparum infected red blood cells probed with anti-PfHPRT antiserum.

Indirect immunofluorescence (IFA) has been used successfully to study the distribution of antigens in malaria parasites (Mcbride, *et al.*,1982, Hall *et al.*, 1983) . These studies demonstrated that IFA with several antibodies against different antigens showed characteristic patterns, which correlated with the location of the antigens. For instance, antibody against p190, a merozoite surface antigen gives a "raspberry" pattern with the mature schizont stage (Hall *et al.*, 1983), whereas antibody against lactate dehydrogenase, which is a cytoplasmic protein gives a spherical dot pattern for the same stage of the parasite (Simmons et al., 1985).

However, although the result in the last section confirmed that the polyclonal serum against PfHPRT is specific to the parasite protein and does not cross-react with the host HPRT, it did not indicate clearly the location of the protein in the parasite or in the infected red blood cells. In other words, the IFA on infected red blood cells with antiPfHPRT antibodies did not show any

Figure 7.3 Confirmation of the specificity of the AntiPfHPRT serum. (B) and (D) show the specific binding of the PfHPRT antibodies to parasitized red blood cells only (pigmented in the phase contrast field, A and C).

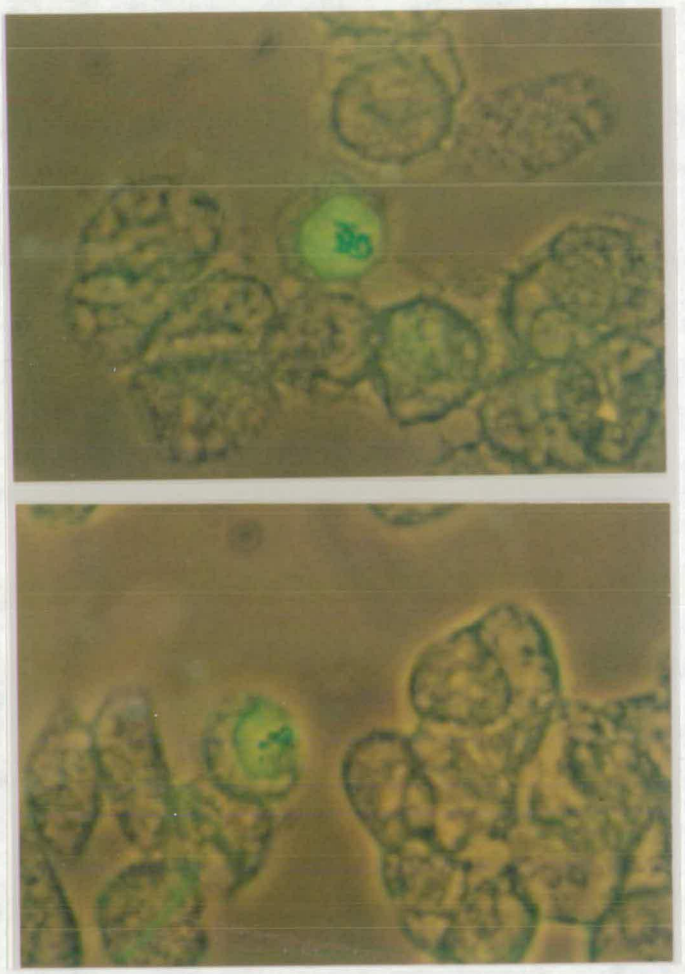

A
B
C
D

characteristic fluorescence pattern for individual parasites, rather a more or less homogeneous staining of the whole infected RBC was seen. Such a pattern can be explained in two ways. Firstly, perhaps at the schizont stage, growing parasites fill the intra erythrocyte space completely and the enzyme is a cytoplasmic one like its mammalian counterpart. This may result in the homogeneous staining of the infected schizont stage of the infected RBC. Alternatively, the enzyme could secrete into the extra-parasitic spaces in the RBC. Thus, antibodies binding to extraparasitic spaces, may mask the individual parasite's profile resulting in the homogeneous staining. IFA on unfixed slides did not show antibody binding to the infected RBC membrane(result not shown), suggesting that the protein is not exported to the surface of the RBC.

Both the above possible interpretations could apply to the schizont IFA pattern fig 7.3. However, earlier stages of the intraerythrocytic growth cycle also gave interesting patterns. The early trophozoite stage (Figure 7.4), shows a pattern suggesting that at the early stage of the parasite's life, the protein is found in a particular membrane-bound vesicular region in the infected RBC. This vesicle could be the parasitophorous vacuole or the growing parasite itself. Since, there is no evidence in favour of either of these possibilities, it is difficult to make any definite conclusion about the nature of the vesicles at this moment.

However, it is clear from the pattern that within the vesicles the fluorescence is homogeneous- there is no sign of differential staining inside it. This may suggest that if the vesicle is the growing parasite itself, the protein is cytoplasmic. On the other hand, if the vesicle is the parasitophorous vacuole, perhaps the protein secretes into the vacuolar space, and is seen in the IFA as a stained sack-like space, filled with the protein. In this case, the pattern also may suggest that the protein does not pass through the parasitophorous

Figure 7.4 IFA pattern of infected red blood cells at a early stage of infection by the PfHPRT antiserum. The uninfected cells did not show any fluorescence.



vacuol membrane (PPVM).

Confirmation of the specific IFA pattern of earlier stages of the intraerythrocytic stages can be obtained from Figure 7.5, which shows that the size of the vesicle is related to the stages of the parasite. If the vesicle is the parasitophorous vacuole, then the result may suggest that the vacuole grows bigger as the parasite passes through the stages and that the PfHPRT releases into the parasitophorous vacuolar space. on the other hand it could be the parasite growing inside the RBC. Since there is no other supporting evidence for either of the explanations, these suggestions are not conclusive.

7.5 Immunogold electron microscopy reveals that PfHPRT is localized in electron dense granules.

Because of the poor resolution of indirect immunofluorescence assay, the two different explanations of IFA pattern can not be resolved. In such a case immunogold electron microscopy on thin sections of purified merozoites and infected RBCs could throw some light on the problem. The preabsorbed antiserum was sent to professor M. Aikawa in Cleveland, USA and the immunogold micrographs obtained with the antibody on thin sections of purified merozoites are shown in Figure 7.6. Figure 7.7 shows that of an infected RBC probed with same antibody. Both the figures suggest that the protein is associated with electron dense granules (Torii et al., 1989) in the cytoplasm. Very little labelling in the cytosol indicates the absence of the protein in this space. Control experiments with preimmune serum and a antiserum against a nonrelevant fusion protein also gave negative results, emphasizing the specificity of the immunogold pattern with the antibodies against PfHPRT.

Such location is expected for a secretory protein. In eukaryotes, secretory

Figure 7.5 IFA pattern of infected red blood cells by the PfHPRT antiserum at different growth stages of the intraerythrocytic parasite.

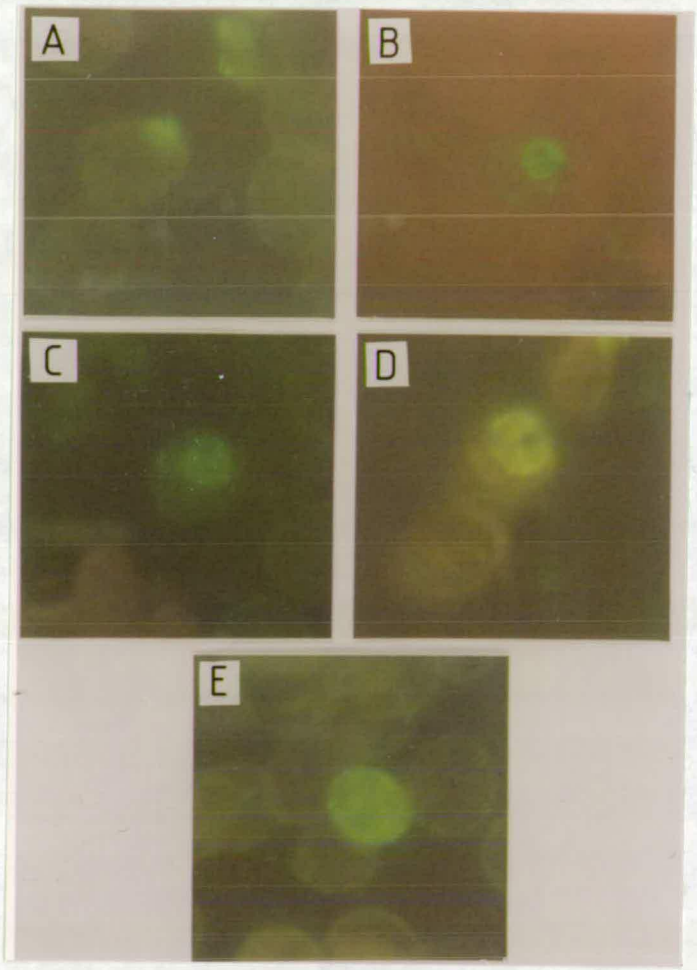

A
B
C
D
E

Figure 7.6 Immunogold electronmicrograph of mature merozoite sections with the PfHPRT antiserum. Overall enlargement is 41000x. The parasite enzyme appears to be present in some vesicular structure. (Note: This experiment was kindly done by Professor M. Aikawa. cleavland, USA.).

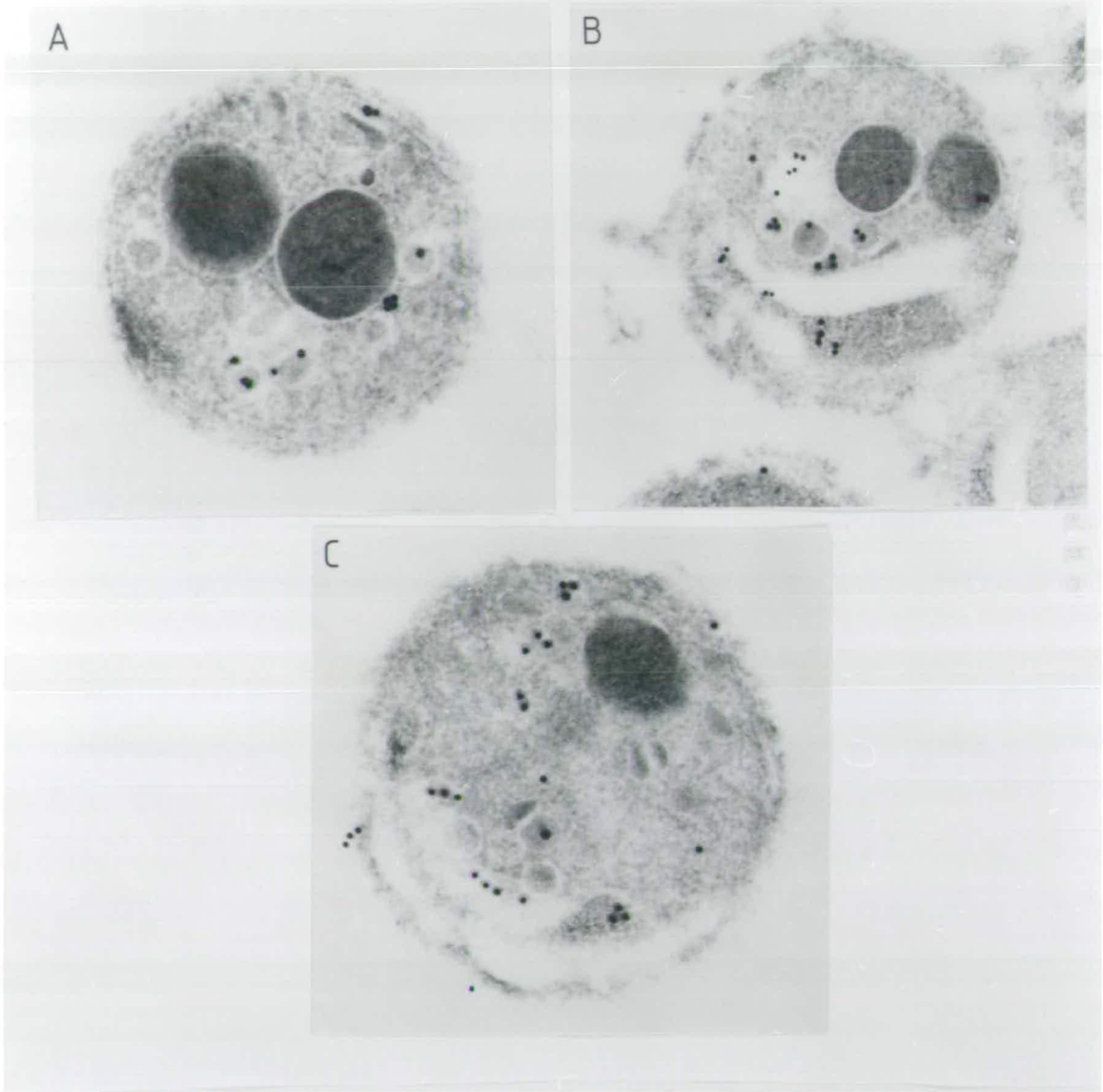
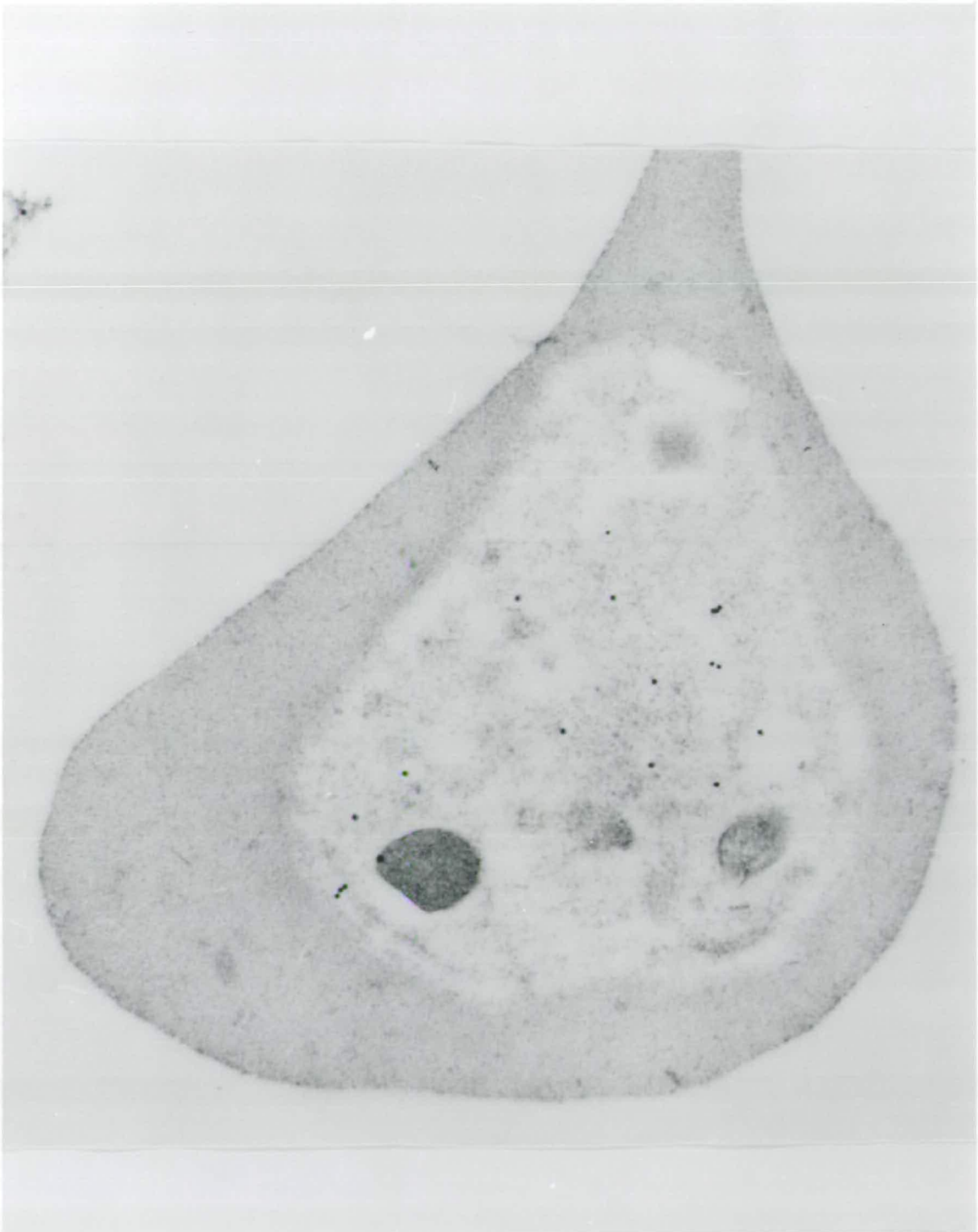

A
B
C

Figure 7.7 Immunogold electronmicrograph of parasitized red blood cell with the PfHPRT antiserum. No labelling with the PfHPRT antibodies was observed in red cell cytoplasm. Overall enlargement is 41000X. (NOTE: This experiment was kindly done by Professor M. Aikawa, Cleaveland, USA.)



proteins are synthesised by membrane bound ribosomes and the newly synthesized protein translocates into the RER matrix. After any essential modification the protein transports into the Golgi apparatus which subsequently gives rise to electron dense secretory granules. These granules then migrate to the plasma membrane and fused with it by a mechanism called reverse pinocytosis. Examples of such proteins include insulin and peptide hormones.

If PfHPRT is a secretory protein, similar events might prevent the identification of the protein in the cytosol but would permit their recognition in the electron dense granules. This mechanism needs the protein to be secreted out of the parasite cell, i.e. into the parasitophorous vacuole membrane. IFA pattern with the antibodies suggests the presence of the protein in the PPV, but surprisingly, electron micrograph of infected RBC section (Figure 7.7) did not show any specific labelling in the parasitophorous vacuole spaces. However, we have not analysed enough infected RBC micrographs to be able to suggest confidently that no protein is present in the PPV. Moreover, it should be remembered at this point, that immunogold electron microscopy is not as sensitive as the indirect immunofluorescence microscopy. Perhaps only the vesicular proteins are recognised in the electron micrographs because of the high concentration of the protein in these vesicles. The proteins in the PPV may not be concentrated enough to be detected by the latter technique. On the other hand, IFA being more sensitive may recognise the protein in the PPV.

The other explanation of the IFA pattern which stated that the whole parasite is recognised, requires PfHPRT to be a cytosolic protein. Well perhaps it is, and for some reason the protein tends to concentrate in intracellular vesicles. This may cause a higher concentration of the protein in these vesicles compared to the cytosol and so immunogold microscopy would only recognise the protein in these vesicles where the proteins are concentrated

enough for their detection. The cytosolic proteins would not be recognised due to their lower concentration.

What is the significance of vesicle bound cytosolic HPRT ? In mammalia, HPRT is a soluble cytosolic protein (Melton, 1987). However, the enzyme in some other parasites like, Trypanosoma (Hammond *et al.*, 1985; Gutteridge and Davies, 1982), and Leishmania (Hassan *et al.*, 1985) was found to be associated with the glycosome, a membrane bound organelle only reported in these parasites (Opperdoes, 1987) and thought to be of the same origin as the peroxysome or glyoxysome of higher eukaryotes . The matrix of these microbodies is electron dense and their major function is reported to be anaerobic glycolysis in these organisms the first nine enzymes of the glycolytic pathway are found to be concentrated in these microbodies of the parasites (Opperdoes, 1988). Glycosomes are found to be very abundant in the blood stream stage of *Trypanosoma brucei* about 240 per cell. Another important feature of glycosomes is their rapid turnover; a turnover time of less than 2 hours has been reported. The fate of these rapidly degenerating vesicles is not known. This lack of information is partly due to the lack of any probe to identify this microbody *in vivo*. Glycosomes have not been seen in *Plasmodium* . If the electron-dense vesicles seen in the electron micrograph are the glycosome-like microbodies, this work is reporting for the first time the presence of these in *Plasmodia*. And if so, the antibody against PfHPRT could be a probe to identify the microbodies of the parasite *in vivo* or *in vitro*.

Hart *et al* (1987) showed that the mRNAs of glycosomal proteins translate on free polysomes and reported the sequence of events involved in the synthesis of glycosomal enzymes in the cytosol and their subsequent translocation into the organelle. Studies with glyceraldehyde phosphate dehydrogenase, aldolase and glycerol-3-phosphate dehydrogenase of T. brucei.

These authors also showed that the enzymes are first detected in the cytosole and after a few minutes, appeared in glycosome enriched fractions. If this is the case for PfHPRT, it may explain the reason for the lower concentration of the protein in the cytosole.

7.6 Scoring PfHPRT as a glycosomal and secretory protein.

The secretory proteins known to date contain a leader peptide at the N-terminal of the protein which directs the synthesizing polysome to the rough endoplasmic reticulum Although there is evidence of particular consensus sequences for prokaryotic secretory proteins, sequences in eukaryotic secretory proteins seem more heterogeneous. Comparison of the predicted primary structure of PfHPRT protein with that of the mammalian protein reveal that there is an extension of 8 amino acids present in the parasite protein. However, the amino acid sequence of this leader peptide does not match with any known sequence of signal peptides.

The signal peptides in both prokaryotes and eukaryotes are most commonly cleaved off after the translocation of the protein across the targeted membrane. Comparison of the recombinant protein and native parasite protein did not suggest any obvious cleavage modification in the native protein (section 5.6).

One consensus feature of the glycosomal proteins is that they are not processed . The sizes of *in vitro* translated glycosomal glyceraldehyde phosphate dehydrogenase (GAPDH) and glycerol phosphate dehydrogenase (GPDH) and those of the native proteins was found to be same (Hart *et al.*, 1987). This feature fits with that of the PfHPRT.

One more feature in which the glycosomal proteins differ, is that they all have a relative molecular mass larger (between 1 - 5 kD) than their cytosolic

homologues (Opperdoes, 1988). Sequence homology analysis showed that all these proteins contain insertions and extensions in their amino acid sequences. For example, comparison of the twelve GAPDH sequences showed 5 unique insertions in the glycosomal enzymes (Misset *et al.*, 1987), and also analysis of the phosphoglucokinase (PGK) sequence revealed a 20 amino acid extension at the c- terminus of the protein (Osinga *et al.*, 1985). No such generalization can be made for secretory proteins. Comparison of the PfHPRT with mouse HPRT showed that the parasite enzyme has 231 amino acids whereas the mammalian enzyme has only 218 amino acids. Moreover, bestfit alignment of the two sequences suggested that there is an insertion of three amino acids and a three amino acid extension at the C-terminus of the parasite protein. These may suggest the glycosomal protein property of PfHPRT. In addition to these, studies with aldolase, triose phosphate isomerase (TIM) and GAPDH sequences led to the suggestion that these proteins have a significantly higher (as much as +13) overall charge than the cytoplasmic proteins (-1) (Wierenga *et al.*, 1987). The overall charge of PfHPRT protein is +1, whereas mouse and human HPRT are -1 and -2 respectively. Figure 7.8 shows the substitution of the neutral amino acids in mouse sequence into charged amino acids in PfHPRT. It is also noticeable that two positively charged amino acids are present in the inserted tripeptide and are among the three extra amino acids present in the C-terminus (one is a hydrophobic, Leucine and the other two are serine and threonine, both hydroxyl-containing amino acids). These features may be of importance, because Swinkels *et al* (1988) found that the C-terminal extension region of *Crithidial* phosphoglucokinase (PGK), a glycosomal enzyme, is positively charged and is rich in hydrophobic and hydroxyl containing amino acids and suggested that the C-terminal extension is responsible for the different topogenesis compared to the cytoplasmic enzyme.

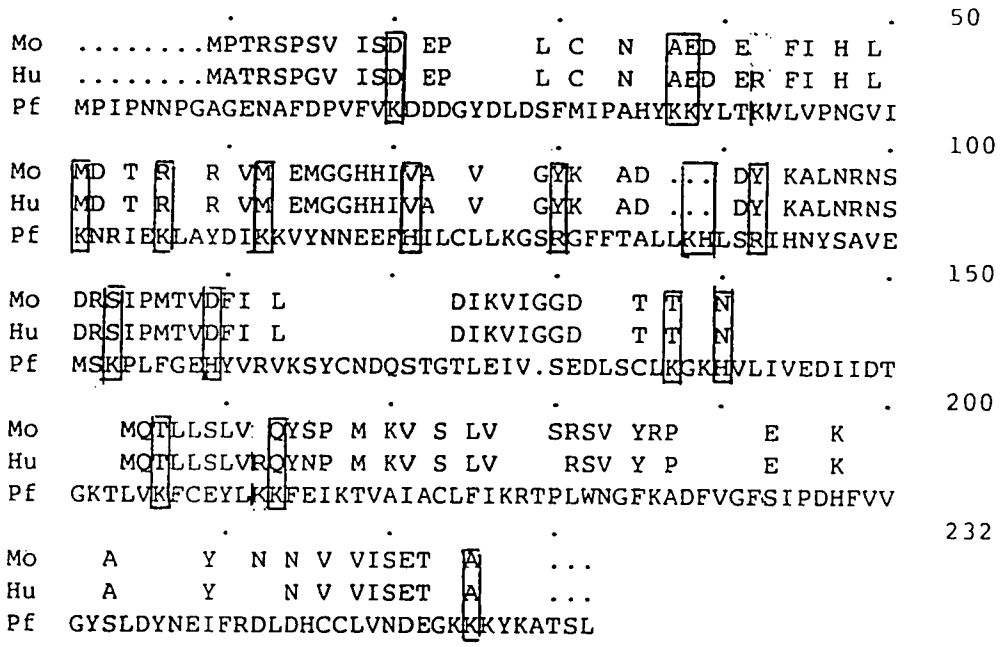


Figure 7.8 Comparison of mouse, human and P. falciparum HPRT protein sequences. In mouse and human sequences only the amino acids which are different from the parasite sequence are shown. Dots show the position of deletions or insertions of amino acids in the corresponding sequence. Substitution of the neutral and acidic amino acids in mouse and human proteins to basic amino acids in the parasite protein are boxed. Such substitutions may contribute to the overall positive charge of the parasite protein.

In contrast, the evidence against the PfHPRT being a membrane bound protein is that in sonicated lysate of *P. falciparum*, the protein is found in the supernatant fraction, indicating the protein is present in the cytoplasm, as is the mammalian protein. But, Torii *et al* (1989) recently, reported that the content of the dense granules are released into the parasitophorous vacuol during the invasion process.

Finally, analysis of the electron micrographs showed that the size of the PfHPRT containing vesicle are approximately 0.20 µm, a comparable size with the Trypanosomal glycosomes (0.25 µm) (Opperdoes, 1987)).

7.7 Is PfHPRT transported into the RBC cytoplasm?

There is evidence that some proteins which are transported to the infected RBC membrane, first release into the parasitophorous vacuol and then traffic through the RBC cytoplasm to RBC membrane as membrane-bound vesicles (e.g. the so-called 5.1 antigen, Simmons *et al.*, 1987). Vesicle bound cellular components may release by budding out. In such cases, small vesicular bodies are seen in the infected RBC cytoplasm when probed with antibodies against those antigens. Detail of such proteins have been discussed by Howard (1988). In this article he proposed two different mechanisms for trafficing particular proteins from parasite to RBC membrane. If PfHPRT releases into the PPV, it should be present is such vesicles.

Cellular proteins which are targeted to particular membrane bound structures may also bud out to form particular vesicles. For example, the proteins in rough endoplasmic reticulum (RER) when transported into the Golgi complex lumen or during formation of secretory vesicles, the lumen buds out from the RER or golgi complex respectively. So if PfHPRT is present in a membrane bound vesicle, the question arises, whether similar events occur in

the case of PfHPRT.

To study such possibilities, the IFA slides were reexamined. The observations are presented in Figure 7.9. It is seen in the IFA slides that that antibody also recognised small structures in the infected RBC cytoplasm. Such structures are not seen in uninfected cells. Reexamination of some of the previously taken photographs also showed these structures. These observations may indicate that the protein does transport into the RBC cytoplasm enclosed by some membrane. The number of such structures is variable. In some cases it was about 4-5 per cell, whereas in others no structure was seen at all. The higher number of such structure did not correlate with the later stages of the intraerythrocytic development. Another observation of interest is that such structures are normally seen near the host cell membrane.

7.8 In P. falciparum HPRT is present in all developmental stages.

To study whether HPRT expression is regulated in different stages of parasite development, the protein from various stages was probed with the antiPfHPRT antibody. Acetone-fixed slides of gametocytes and gametes were made with *in vitro* cultured *P. falciparum* strain 3D7 or HB3. Sporozoite slides were made using pooled salivary glands of infected mosquitoes and fixed with acetone. IFA was carried out by the same method as described in method section using preabsorbed antibody. Figure 7.10 shows the characteristic IFA patterns as seen with the PfHPRT antibody on each stage of parasite used. The general observation is that the protein is present in all the stages of parasite. And no clear indication of quantitative difference was observed. However, sporozoite stage showed most intense pattern. This may simply be because the sporozoites are an extracellular stage of the parasite and so, the antibodies

Figure 7.9 IFA pattern of infected red blood cell cytoplasm. Possible evidence of presence of PfHPRT in the RBC cytoplasm.

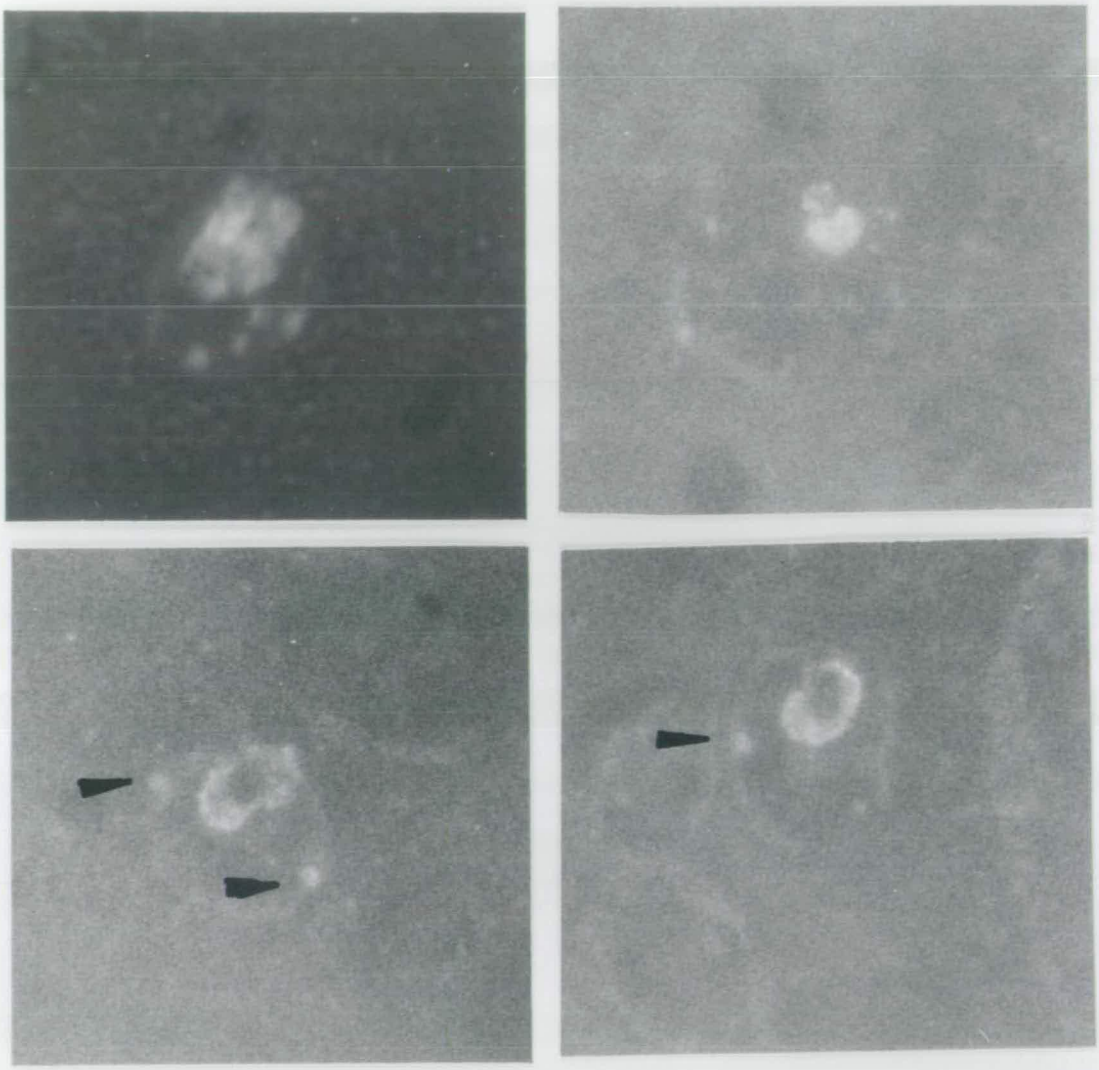
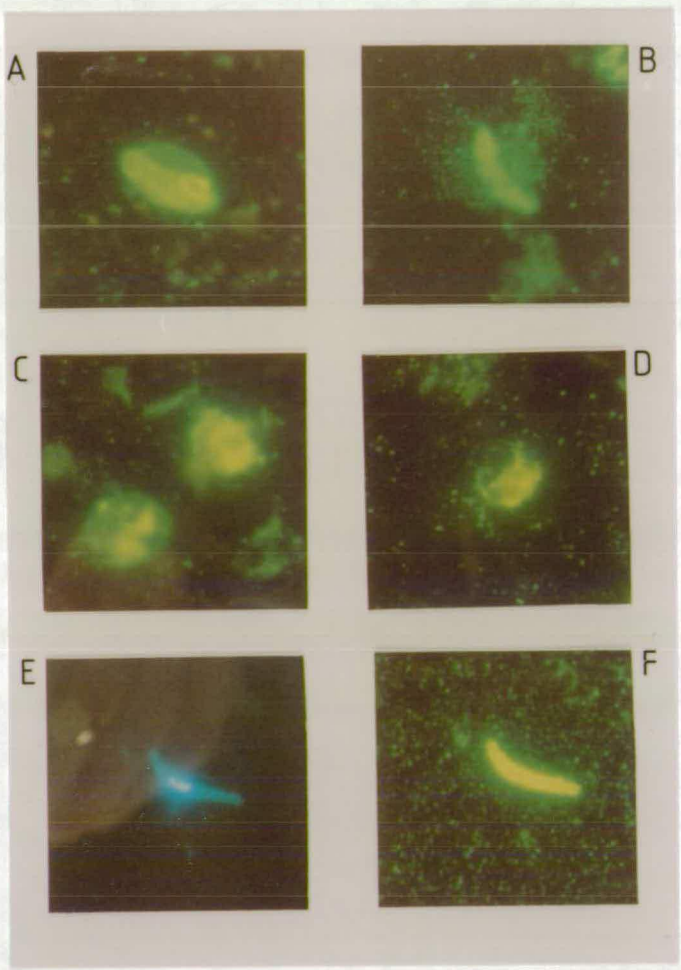


Figure 7.10 IFA pattern of the parasite with the PfHPRT antiserum at different stages of the parasitic growth. (A) and (B) Gametocytes, (C) and (D) gametes and (F) sporozoite stages. (E)shows the same frame as (F) but stained with DAPI for nucleus.


A
B
C
D
E
F

are more accessible to the proteins in this stage than in the intracellular stages. However, the possibility of higher enzyme production can not be ruled out.

The pattern of IFA in these stages are also remarkable. In contrast to the pattern seen for earlier asexual blood stages, in the gametocyte stages although the whole parasitized cell was recognised, two distinct zones of different intensities were observed. The most intense was the crescent part of the gametocyte and a lighter zone was observed at the middle was observed of the crescent.

There was a large number was observed of granular bodies present in the surrounding area of some gametocytes and gametes in acetone fixed slides. Initially, these granules were thought to be an experimental artefact. However, the reproducibility of this feature suggested that these unknown granular bodies may be of parasite origin and recognised by the antiHPRT antibodies. Similar granular patterns have also been observed with antibody against other parasite proteins (Richard Carter, personal communication).

7.9 Antibodies against PfHPRT are not present in immune human sera.

The result in section 7.7 indicated that in the cytoplasm of *P. falciparum* -infected RBC, the enzyme HPRT may be present in vesicular structures. The significance of this is not clear. It was suggested that after invasion, some parasite antigens are transported into the host cell membrane e.g.. Knob associated Histidine Rich Protein (HRP) (Howard, 1988). But the IFA pattern with the PfHPRT antibody did not label the protein on the host cell membrane and so the latter theory is unlikely. Another possibility is that the protein is secreted into the host serum along with other possible secretory proteins.

To study such a possibility, an attempt was made to detect antibodies against PfHPRT in immune human sera. The idea was, if the protein secretes into the serum, it must elicit immune response in the human body. Pooled sera of four immune individuals from Nigeria was used as the primary antibody in western blot analysis. These sera were previously shown to have antibodies against *P. falciparum* antigen p190 and 5.1 . The induced SH2 whole cell protein was separated on a 12% SDS-polyacrylamide gel and western blotted. Pooled human sera was used as the primary antibody with a final dilution of 1 in 50 . The result is shown in Figure 7.11 indicates that although the serum recognised other E. coli proteins, the serum did not contain any antibodies against PfHPRT.

This result may indicate that the protein is not secreted into the serum or indeed it may not be a secretory protein at all.

7.10 In the sporozoite, the enzyme may secrete into the intrapellicular spaces.

In the IFA studies with different stages of the parasite (fig. 7.10), the sporozoite was the most intensely stained stage. However, the interesting observation was that most of the fluorescence was seen in the peripheral region of the parasite (Figure 7.12 and 7.10), although the protein was not found to be membrane associated as shown by the immunogold electron micrograph. So, what is the explanation of such a pattern ?

The following feature may bear upon the answer. In section 7.5 it was discussed that the enzyme or some degradative part of it may secrete into the parasitophorous vacuole. The sporozoite is not an intracellular stage of the parasite, rather it is a free living organism in the mosquito salivary gland . Ultrastructural examination has revealed that the sporozoites pellicle is composed of three distinct membranes as illustrated in figure 7.14, one

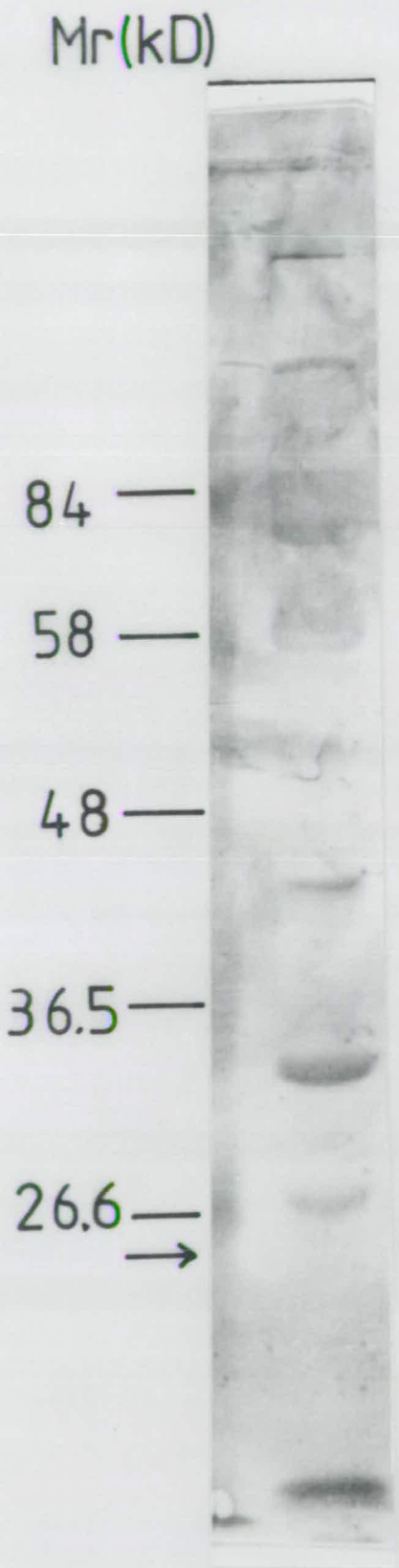

Mr(kD)
84
58
48
36.5
26.6

Figure 7.12 IFA pattern of the sporozoite stage of the parasite from mosquito salivary gland with the PfHPRT antiserum. Frame (A) shows a detail and close look of the pattern seen in figure 7.10. (B) shows the same frame with DAPI stain.

outermembrane and two in
that perhaps the protein
parasite pellicle, but is una
result in the accumulation
may give rise to the typica
may support this postulatic
by the presence of a nucl
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the shedded coat or oute
contain some of the re
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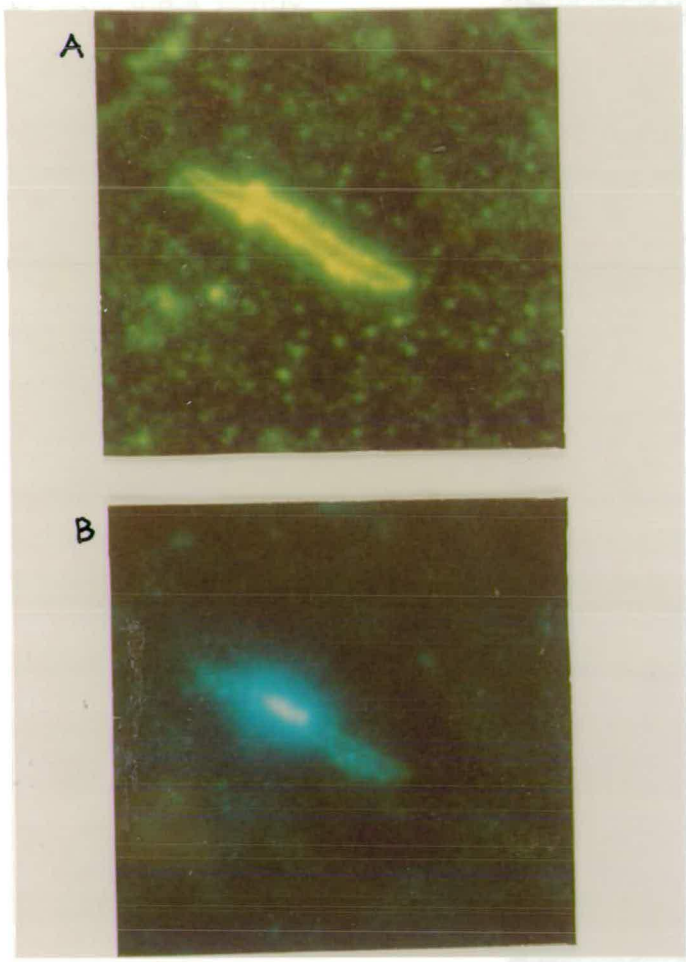
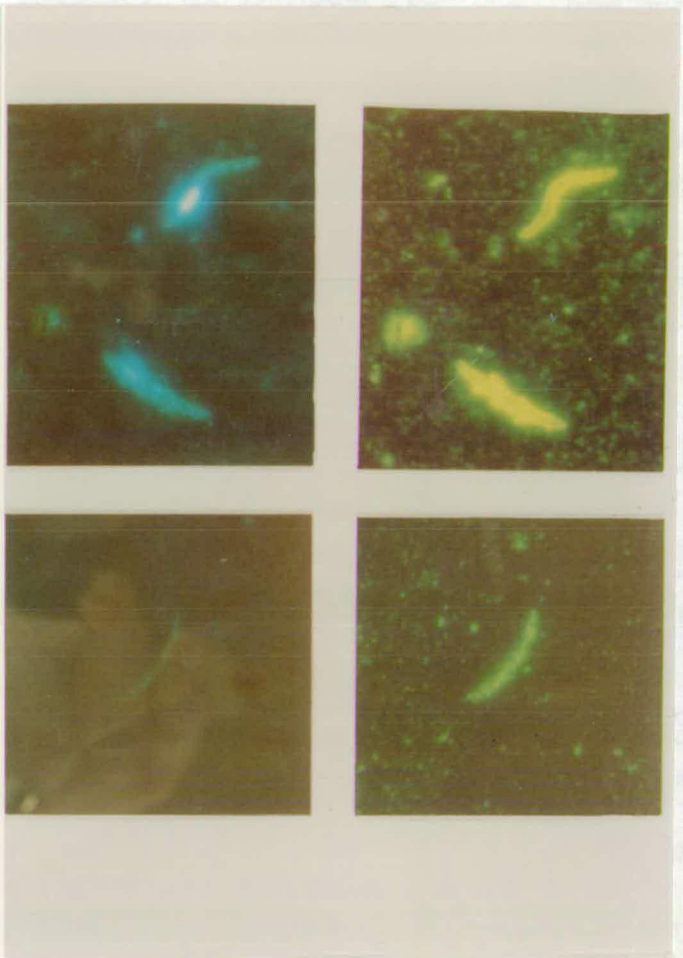
suggest that the protein
Figure 7.14 illustrates the al



outermembrane and two inner membranes (Aikawa, 1980). It can be postulated that perhaps the protein is secreted through the inner membranes of the parasite pellicle, but is unable to pass through the outer membrane. This may result in the accumulation of the enzyme in the intra pellicular spaces which may give rise to the typical IFA pattern. Another interesting observation which may support this postulation is that along with live sporozoites, characterised by the presence of a nucleus with DAPI-staining, there are also some faintly fluorescing sporozoite ghost lacking a nucleus (Figure 7.13). These are perhaps the shedded coat or outer membranes of the sporozoites, which may still contain some of the residual proteins. This shedding is a common characteristic of plasmodium sporozoites. The faint fluorescence may also suggest that the protein was not bound to the membrane of the parasite. Figure 7.14 illustrates the above explanation of the sporozoite IFA pattern.

Figure 7.13 Unusual IFA pattern of sporozoite stage of the parasite with PfHPRT antiserum. (A) and (C) show the DAPI staining of the same frame as shown in IFA frames (B) and (D). Note the absence of nucleus and faint fluorescence compared to the normal nucleus containing parasites (Fig. 7.12).



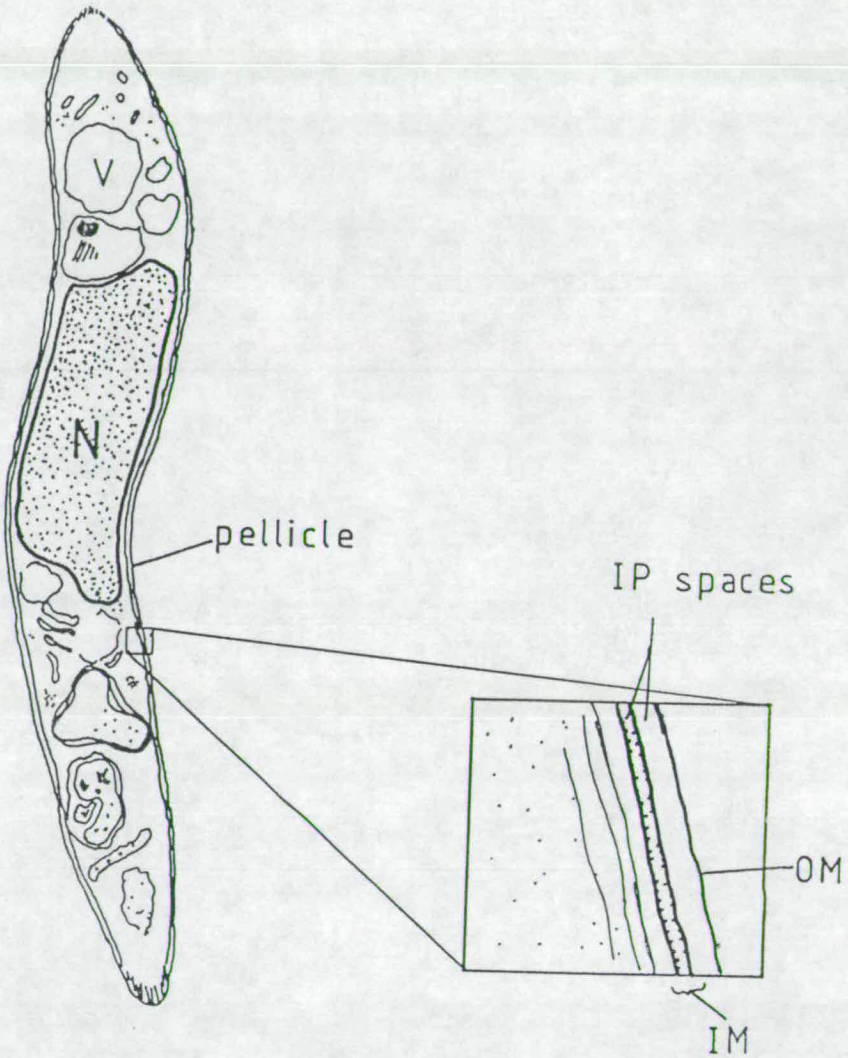


Figure 7.14 Cartoon explaining the sporozoite IFA pattern with PfHPRT antiserum as seen in figure 7.12. OM = Outer membrane, IM = Inner membrane and IP = Intrapellicular.

Chapter 8

Concluding remarks and future directives.

Largely because of the complexity of the problem of vaccine development against malaria, chemotherapy seems to be the only hope in hand at least in the immediate future. A considerable amount of work on the parasite biochemistry and elucidation of the mechanisms of action for several drugs have revealed that the parasites metabolic pathways, specially those are different from the host could be the target for successful antimalarial drugs. Study of the mechanism of drug resistance also emphasized this fact.

Purine metabolism in Plasmodia is one such target, because unlike its host the parasite cannot synthesize purine nucleotides, but rather scavanges preformed purine from its host by the salvage pathway. Hypoxanthine was found to be the most favourable form of purine for the parasite and the enzyme, hypoxanthine phosphoribosyltranferase which utilizes the purine, is the key enzyme of the pathway and is important for the parasite's life. Hence, the enzyme is a promising target for drug design against malaria.

Difficulty in the large scale culture of the parasite *in vitro* and extraordinary lability of the enzyme during purification steps, are major obstacles in the way of the study of the enzyme at the molecular level.

To overcome the afore-mentioned problems, this work has described the successful use of molecular biological and genetic engineering approaches to the study of the enzyme. Initially, expression of PfHPRT in *E. coli* as a β-galactosidase fusion protein facilitated the rapid purification of protein for making antibody against the parasite enzyme. This antibodies were powerful tool in the study of the cellular biology of the enzyme. Probing the enzyme *in vivo* with these antibodies revealed that :

1. The enzyme is expressed in the parasite at all the stages of the parasite's life, without any noticeable difference in the expression level.

2. The enzyme is concentrated in some vesicular bodies, whose identity is

yet to be revealed.

3. in the sporozoite stage, the enzyme is possibly released into the intrapellicular spaces.

However, much remains to be done in this area. The significance of the intracellular localization must be elucidated and whether this is an exploitable feature must be studied. Moreover, the regulation of the gene expression has to understood clearly in order to find the exact time at which the parasite is most vulnerable to the drug attack.

The enzyme was subsequently expressed directly in *E. coli*. The expressed full length enzyme was active and provides a new method of obtaining large amounts of parasite enzyme without growing the parasite. Thanks to molecular biology, it will now be possible to obtain large amounts of PfHPRT for molecular study, in order to understand the structure-activity relationship of the enzyme which may provide an insight into the design of an effective drug against the parasite. It must be pointed out at this stage that this is the first Plasmodial enzyme expressed in *E. coli* in an active form. The future work in this area may involve the purification of large amounts of the enzyme, for crystal preparation for X-ray crystallography. The antibodies against the enzyme, prepared at the initial stage of the work, may facilitate the purification stage greatly. Moreover it is now possible to use site-directed mutagenesis to map the active site of the enzyme. A combination of active site mapping and 3-D structure from x-ray crystallographic data will reveal the architechture of the substrate binding site of the enzyme. This will help in the designing of an inhibitor of the enzyme.

The activity of the *E. coli*-expressed enzyme enabled the compensation of a bacterial *hpt*$^-$ mutant. The compensated strain of *S. typhimurium* can only grow on hypoxanthine plates if the recombinant PfHPRT is active. This strain

SH4 can now be used to screen large number of putative antimalarial drug which might act against the parasite HPRT . This can be done in a simple and inexpensive way.

Since the *S. typhimurium hpt* mutant strain did not have any endogenous HPRT activity, the estimation of the HPRT activity in the induced bacterium elucidated several interesting properties of the parasite enzyme. For instance, it was possible to confirm the finding that unlike any other Plasmodia studied so far, *P. falciparum* HPRT can use xanthine as its purine source. Furthermore, specific activity measurements and competitive inhibition studies revealed that hypoxanthine is the most favourable purine. Further studies in this field may reveal the affinity of the enzyme to different purine analogues which, I believe will help to design an effective antimalarial drug, which the whole world is looking forward to.

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