Artificial proteins as vaccines and as binding moities

Chen, Jianhua

Award date:
2001

Awarding institution:
University of Bath

Department of Biology and Biochemistry

University of Bath, UK

Artificial Proteins

as Vaccines and as Binding Moities

submitted by

Jianhua CHEN

For the degree of Doctor of Philosophy

of the University of Bath

2001

COPYRIGHT

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Department of Biology and Biochemistry

University of Bath, UK

Department of Medical Biochemistry

University Medical Center, Geneva, Switzerland

This research is under the joint supervision of

Prof. Antony REES (University of Bath)

Prof. Keith ROSE (University of Geneva)

Artificial Proteins

as Vaccines and as Binding Moities

Jianhua CHEN

From Wuhan, P.R.China

Geneva 2001

CONTENTS

A. SUMMARY

If we are to exploit more fully the potential of proteins for clinical and industrial applications (as *in vivo* diagnostic and therapeutic agents, as research tools, as components of biosensors, and perhaps later as components of nanomachines or as high-performance texile fibers, etc.) we must consider chemical modification. Over the years, some very mild and effective techniques (e.g. oxime chemistry) have been developed which permit the modification of proteins without damaging their intricate structure. In this way, part of a protein which has a desired function (as enzymatic activity, or ability to localize in tumor tissue, for example) is cut out and "pasted" together with some other structures which may themselves be proteins or may be synthetic drugs (drugs to kill tumor cells, for example) or may be other substances grafted for other purposes, such as poly(ethyleneglycol) to extend biological half-life and reduce immunogenicity, a fluorescent dye reporter group, a lipid to modulate immunological properties, or even nucleic acid for gene therapy applications. The functions of each of the several components can be preserved, and in spite of its size and complexity, the resulting product can be homogeneous, which is desirable for clinical use.

In a more drastic approach, only the minimum structural elements required for binding are preserved (or mimicked) and the rest of the macromolecule is replaced with linkers such as short poly(ethyleneglycol) chains.

The scope of the research undertaken has been to develop 'nuts and bolts' protein engineering at the protein level to build useful and complex structures through site-specific reactions: in general, oxime chemistry. In particular:

(1) I investigated the effect of peptide multiplicity on immunogenicity using a model peptide from the envelope protein gp120 of Human Immunodeficiency Virus type-1.

(2) I used cholera toxin B subunit (CTB) as a model to study the effect (structural and biological) of attaching a cascade of immunogenic peptides to the N-terminus.

(3) I increased the avidity and specificity of small peptide ligands through a cluster (multivalency) approach.

B. INTRODUCTION

1. Vaccines and Vaccination

Vaccines have been known for a long time to save a lot of lives and money since the first vaccine to prevent smallpox, an infectious disease, emerged in China in the 6th century, when scabs were taken from patients, dried, ground and then blown into the nostrils of humans [1]. However, it was not practical to vaccinate large populations until the 1870s when Pasteur made significant progress on the attenuation of the chicken cholera virus [2]. He observed that live attenuated vaccines to cowpox and smallpox consisted of a weaker form of the same organism but did not cause related diseases. Since then vaccination has had a very important impact on the prevalence of some infectious diseases. Nowadays, vaccine research is not only focusing on new initiatives for the well recognized infectious diseases but is also creating new projects on vaccines to prevent and treat autoimmune diseases and cancers [3].

Vaccines are made from antigens of pathogens. They may be natural or attenuated living organisms, intact but non-living organisms, sub-cellular fragments, toxoids, recombinant DNA, or anti-idiotype antigens. In general, the more antigens of the microbe retained in the vaccine, the better. Living

organisms tend to be more effective than killed ones [4]. The approaches to vaccination that have been tried are listed in Table 1.

Table 1: Vaccine Approaches

Types of Vaccine	**Examples**
Live attenuated or killed bacteria	BCG (Bacillus Calmette-Guérin), cholera
Live attenuated viruses	Polio, rabies
Subunit (antigen) vaccines	Tetanus toxoid, diphtheria toxoid
Conjugate vaccines	*Haemophilus influenzae*, pneumococcus
Synthetic Vaccines	Hepatitis (recombinant proteins)
Viral vectors	Clinical trials of HIV antigens in canarypox vector
DNA vaccines	Clinical trials ongoing for several infectious diseases

2. Artificial proteins as vaccines

Proteins and polypeptides function in Nature in a remarkable variety of ways, and have been used extensively in clinical and industrial applications as *in vivo* diagnostic and therapeutic agents, as research tools and as components of biosensors. Many proteins made for such applications result from recombinant DNA technology (e.g. granulocyte colony stimulating factor, G-CSF, erythropoietin, EPO). Some polypeptides are chemically synthesized (e.g.

calcitonin) and some products involve both recombinant techniques and chemistry (e.g. human insulin, made by chemical and enzymatic transformation of a precursor generated in yeast). These chemical applications have been greatly facilitated by recent advances in techniques of protein purification, characterization and synthesis [5]. Some applications have benefited from the development of solid-phase peptide synthesis pioneered by Merrifield [6] and from proteins that have been modified chemically, due to the fact that the diversity of protein behavior is sometimes influenced by even minor variations in molecular structure [7].

Synthetic peptides have been demonstrated to induce antibodies that can react with their cognate sequences in native proteins [8,9]. For this reason, a synthetic peptide can serve as an immunogen to produce passive immunoprophylaxis [8-11] or can be used to induce antipeptide antibodies useful as laboratory reagents, for example in the study of biosynthetic pathways by confirming *de novo* synthesis of proteins from recombinant DNA [9]. However, a small linear peptide may not be effective as an immunogen because of the influence of protein conformation [12, 28] and because of its lack of high molecular mass that is necessary for enhanced immunogenicity. One way to solve these problems is to create an artificial protein by coupling the antigenic peptide to protein carriers such as mucosal adhering proteins. The latter include *E. coli* heat-labile toxin B-subunit (LTB) [13], and cholera toxin B-subunit (CTB). In this way, a synthetic immunogenic peptide is attached to part of a protein carrier capable of localizing

in a specific tissue (mucosa of the gastrointestinal tract, for example) together with some other substances grafted for other purposes, especially, a reporter group. The functions of each of the several components can be preserved, and in spite of its size and complexity, the resulting product can be homogeneous, which is desirable for clinical use. Another way to enhance peptide immunogenicity is to cross-link antigenic peptides to a synthetic core in a branched (radial or comb) manner. For instance, Tam [14] in 1988 designed an amide-linked branched artificial protein, a multiple antigenic peptide (MAP) system, to increase molecular weight. This is a macromolecule containing several copies of synthetic epitopes or protein antigens that are coupled to a peptide core matrix (trivalent lysine) by peptide bond formation (see Figure 1). The polypeptide epitopes can be from the same or different immunogens. As MAPs are entirely synthetic, they are safe vaccines in respect to pathogens like human immunodeficiency or hepatitis viruses. Also, MAPs direct the immune system to a more specific response. More recently, it has been reported that MAP molecules containing 8 copies of peptide specific for sera of systemic lupus erythomatosus patients induced remarkable anti-DNA antibody titers and renal immunoglobulin deposition [15], which implies that the significant MAP immunogens may have potential for immuno-prophylaxis of additional infectious diseases. Unfortunately, with this approach it is impossible to produce homogeneous artificial proteins due to inevitable side-reactions and coupling failures, and the wanted material, when the peptide epitope is longer, cannot be purified from the many impurities present with similar structure.

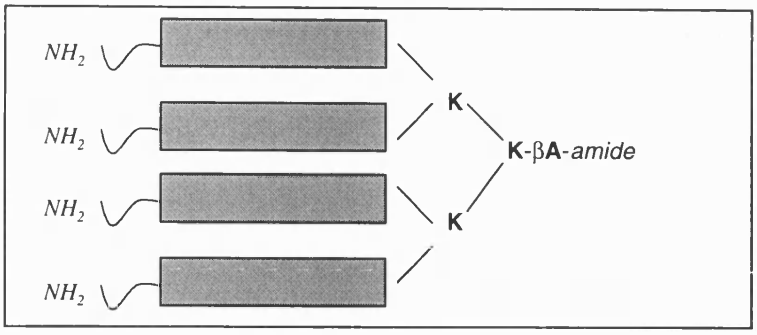


Figure 1: Schematic view of a multiple antigen peptide (MAP).

A rectangle  represents for a single synthetic antigen peptide in this thesis. Four of such polypeptides are coupled by amide bond to two lysine residues that are further linked to a lysine carrier. Amino acids are shown in one black letter code.

Over the years, site-specific conjugation, a chemical approach of ligating antigenic peptides to a protein or to a synthetic carrier, has been developed as a means of creating artificial proteins. Some mild and effective methods for N-terminal [16-20] or C-terminal [21-24] site-specific tagging have been described. One of these employs oxime chemistry that permits the modification of proteins without damaging their intricate structure [25]. In this way, part of a protein which has a desired function (an enzymatic activity, or ability to localize in tumor tissue, for example) is 'cut out' and 'pasted' together with some other structures which may themselves be proteins or may be synthetic drugs (drugs to kill tumor cells, for example) or may be other substances grafted for other purposes, such as poly(ethyleneglycol) to extend biological half-life and reduce immunogenicity, a fluorescent dye reporter group, a lipid to modulate immunological properties, or even nucleic acid for gene therapy applications. For example, the N-terminal

threonine residue of β-lactamase may be oxidized by periodate into an aldehyde function group under mild conditions and then reacted with an aminooxyacetyl-modified murine antibody Fab' to form a homogeneous enzyme-antibody Fab' conjugate with coupling efficiencies of at least 72%. In this case, the oxidation reaction is rapid and quantitative but the oximation occurs very slowly. The resulting β-lactamase/Fab' conjugate was used as a functional targeting agent [26]. Cholera Toxin B-subunit (CTB), on the other hand, is a protein that can induce a mucosal immune response and supply T helper epitopes. Its N-terminus, also threonine, can in principle undergo such oxidation and oximation to form conjugates with a cascade of immunogenic peptides [25,27,28].

3. Immunological mechanism

Synthetic peptide vaccines are made from antigens that can be classified as T cell dependent antigens (T_D) or T cell independent antigens (T_I). They stimulate specific humoral immune responses with or without T cell help, respectively. A successful vaccine should be able to: (1) induce neutralizing antibody; (2) induce immuno-tolerance related lymphokines; (3) induce cell-mediated immunity (CMI), especially cytotoxic T cells (CTL), to ensure recovery from infection; (4) induce long-lasting memory T and B cells to ensure sustained protection. B cell activation and induction of antibodies are key processes involved in vaccine-induced humoral immune response.

The main approaches to vaccination that have been tried are listed in Table 1. Among them, the goal of synthetic vaccine research is to identify the most immunogenic antigens or epitopes of microbial antigens, to synthesize these in the laboratory, and to use the synthetic antigens as vaccines. In this case, linear peptides can be synthesized as synthetic antigen vaccine based on the known sequences of microbial antigens. Sequences of the linear peptides are epitopes or even individual residues that are recognized by B or T cells or that bind to MHC (major histocompatibility complex) molecules for presentation to MHC-restricted T lymphocytes, and are identified by testing overlapping peptides and by mutational analysis. Such peptides usually are weakly immunogenic by themselves [29] and need to be cross-linked to a synthetic core in a branched (radial or comb) manner or be coupled to large proteins to induce immune response. This approach has the potential for creating at will vaccines with high potency antigenicity.

3.1 Activation of cytotoxic T cells (CTLs) [30, 31]

Peptide vaccines like other foreign antigens can initiate cell-mediated immunity (CMI), especially through cytotoxic T cells (CTLs), to ensure recovery from infection by microbial or viral agents. Vaccines are first recognized by APCs (antigen-presenting cells) in the peripheral lymphoid organs. Occupied receptors

are internalised and bound antigens are processed and presented on class I MHC molecules on the surface of APCs, which then activates the T cell receptor complex (TCR) of the T lymphocytes and initiates primary T cell responses. The activation of the naive T cells leads to proliferation of the antigen-specific clone and $CD8^+$ T cells differentiate into functional CTLs, which posses the ability to kill infected target cells by inducing target cells to undergo programmed cell death (also called apoptosis).

3.2 T cell dependent B cell activation

Vaccines made from T_D antigens, for example, sheep red blood cells (SRBC) or monomeric proteins, induce B cell responses exclusively in the presence of specific T cell help [32]. T_D antigens are recognized and processed by APCs, and then displayed in the form of class II MHC-associated peptides on the surface of APCs, which activate TCR of the helper T lymphocytes type 2 (T_H2). An additional signal that is also required for costimulation of T_H2 activation is provided either by a secreted chemical signal (e.g. *interleukin-1, IL-1*) or by the plasma-membrane-bound signaling molecule B_7 on the surface of the APCs. The latter is recognized by a co-receptor, CD_{28}, on the surface of the T_H2 cell. B lymphocytes are activated exclusively in the presence of an antigen (signal-1) followed by a second signal (signal-2). The latter is believed to be provided by the TCR of T_H2 cells [33], by costimulatory molecules (e.g. CD_{40} on B cells) and

interleukins (IL-4 and IL-5) released by T cells [31]. Lack of signal-2 will lead to B cell tolerance instead of activation.

3.3 T cell independent B cell activation

The activation mechanism of synthetic antigen vaccines made from T_I antigens is quite different from that of vaccines made from T_D antigens. These T_I antigens are potent B cell stimulators (T_I type 1, e.g. lipopolysaccharide, LPS) or repetitive antigens (T_I type 2, e.g. haptenized proteins, synthetic polymers). T_I –1 antigens such as LPS can activate polyclonal B cells by binding of its antigen determinant to the receptor on the surface of B cell, followed by binding of its mitogen structure to the corresponding receptor of B cell surface, in the absence of T cell help or APCs. T_I –2 antigens contain repetitive determinants, which are not degraded *in vivo*. They can activate B cell by cross-linking the receptor on the surface of B cells, in the presence of APCs and some residual T cell help. In addition, T_I –2 antigens, because of their organization and repetition, may induce B cell intolerance. In transgenic mice expressing the membrane form of VSV-gp as a self antigen, Bachmann et al. found no antibody production upon immunization with poorly organized VSV-gp, but the tolerance was broken and B cell responsiveness was restored when the same mice were immunized with highly organized VSV-gp particles [34, 35]. This indicates that organization itself may be an important parameter for the immune system to discriminate self-

antigens from non-self-antigens [36]. These results clearly have important consequences for the design of artificial protein vaccines.

3.4 The generation of antibody diversity [37]

Interaction of B cell receptors (BCR) with antigens leads to the activation of a protein kinase cascade and increased intracellular Ca^{2+} concentration [38]. In the presence or absence of T cell help, B cells proliferate and differentiate into antibody-forming cells. During B cell development, various antibodies are produced from three pools of gene segments, encoding κ light chains, λ light chains, and heavy chains, respectively. In each pool, separate gene segments that code for different parts of the variable regions of light and heavy chains are brought together by site-specific recombination. To make an antibody molecule, a variable region of light-chain gene segment (V_L) is recombined with a joining gene segment (J_L) to produce a DNA sequence coding for the V region of the light chain. And a variable region of heavy-chain gene segment (V_H) is recombined with a diversity (D) and a joining (J_H) gene segment to produce a DNA sequence coding for the V region of the heavy chain. Each of the assembled gene segments is then co-transcribed with the appropriate constant (C) region sequence to produce an mRNA molecule that codes for the complete polypeptide chain. Thousands of different light chains and thousands of different heavy chains can be made by variously combining inherited gene segments that

code for V_L and V_H regions. Since the antigen-binding site is formed where V_L and V_H come together in the final antibody, the light and heavy chains can pair to form antibodies with millions of different antigen-binding sites. This number is enormously increased by the loss and gain of nucleotides at the site of gene-segment joining, as well as by somatic mutations that occur with very high frequency in the assembled V-region coding sequences following antigen stimulation.

3.5 Mucosal immunity

Many important pathogens, including respiratory microorganism such as influenza viruses and enteric microorganism such as *Vibrio cholerae* and enteropathogenic *Escherichia coli*, initiate their infectious processes at mucosal surfaces. But most vaccines are given by injection, which is not the usual route of entry of the majority of pathogens so it can be difficult to induce mucosal immunization. It is therefore important to develop a new strategy by using mucosal vaccination to achieve more effective immunization.

The mucosal immune system is a collection of lymphocytes and accessory cells in the epithelia and lamina propria of mucosal surfaces such as the gastrointestinal and respiratory tracts, and it responds to and protects against

microbes that enter the body through mucosal surfaces. The immune system of the mucosal surface is distinct in many ways from systemic ones (Table 2).

At the present time the rules of mucosal immunity are poorly understood. It is known that the mucosal immune system is capable of responding to and eliminating mucosal infections such as cholera and polio. In addition, oral or nasal administration of antigens usually results in inducing tolerance, and this ability can be explored as a therapeutic mechanism for reducing unwanted immune responses [39].

Table 2: Characteristics of systemic and mucosal immunization

Systemic (internal)	**Mucosal (external)**
IgG and IgM produced	IgA and IgG produced
Injectable inoculation	Injection not necessary
Training required to administer	Little training to administer
Systemic immunity only	Local and systemic immunity
Boosts mucosal response	Enhanced by parenteral priming
Responds to small amount of antigen	More antigen needed to induce response
Sensitive to complex microbial antigens (whole cells)	Mucosal adhesin is critical property Common mucosal immune system

A series of bacterial toxins, such as the *E. coli* heat-labile toxin, cholera toxin and pertussis toxin which are powerfully immunogenic at mucosal surfaces, have the property of binding to eukaryotic cells and are protease-resistant. It has been

found that the protein toxins from these organisms have adjuvant properties that are retained even when the parent molecule has been engineered to eliminate its toxic properties [39]. More importantly, if one vaccinates an individual by the oral route, the mucosal immune response is not restricted to the intestine but through the migration and homing of immune cells it also partly disseminates to other mucosal and glandular tissues (e.g. respiratory and urogenital tract) [40]. The ability of these proteins to stimulate immune responses is therefore of potential practical importance as adjuvants for oral or nasal vaccines.

4. Human Immunodeficiency Virus and vaccines for acquired immunodeficiency syndrome

Human Immunodeficiency Virus type-1 (HIV-1) is the main type of HIV virus which can cause the destruction of $CD4^+$ lymphocytes in the host resulting in the development of acquired immunodeficiency syndrome (AIDS) [41, 42]. This pathogenesis comes from a specific interaction between CD4, the viral receptor, and the gp120 exterior envelope glycoprotein of host CD4 positive cells. The binding then initiates a cascade of intracellular events leading to the fusion of the viral and host cell membrane that allows viral entry [43].

Infection with HIV generates an adaptive immune response that contains the virus but only rarely eliminates it. In most individuals neutralizing antibodies that mainly target envelope glycoproteins are produced but the titers are low during

progressive disease [44, 45]. Antibodies induced from hypervariable regions are type-specific [46], whereas the antibodies induced from conserved areas are group-specific [47]. Cellular responses are also directed against HIV-1-associated antigens. CTLs react with *env*, *pol*, and *gag* and regulatory gene products [48-50], and the CTL reactivity is mediated by MHC complex-restricted $CD3^+$-$CD8^+$ peripheral blood lymphocytes. Antibody-dependent cell-mediated cytotoxicity (ADCC) directed against HIV-1-infected cells has also been observed [51, 52]. This activity is initiated usually on virus-infected cells, which can express on their surfaces viral proteins that can be recognized by antibodies and then signal the presence of intracellular infection leading to the destruction of the antibody-coated cells by a natural killer cell (NK cell). Such activity in HIV-infected cells appears to reside in IgG fractions and is directed predominantly against exterior envelope epitopes gp120 and gp41. Non-T-effector cells also display phenotypic cytotoxic reactivity against HIV-1 gp120, resembling those with natural killer (NK) activity [53]. In addition, antibody responses to the core protein p24 of HIV-1 have been reported. The known immune responses to HIV are shown in Figure 2. It is not clear what host responses are important in protection against HIV-1 infection or disease, and it is also not clear whether such a protective response can be elicited at all. Answers to these questions are crucial to successful vaccine development.

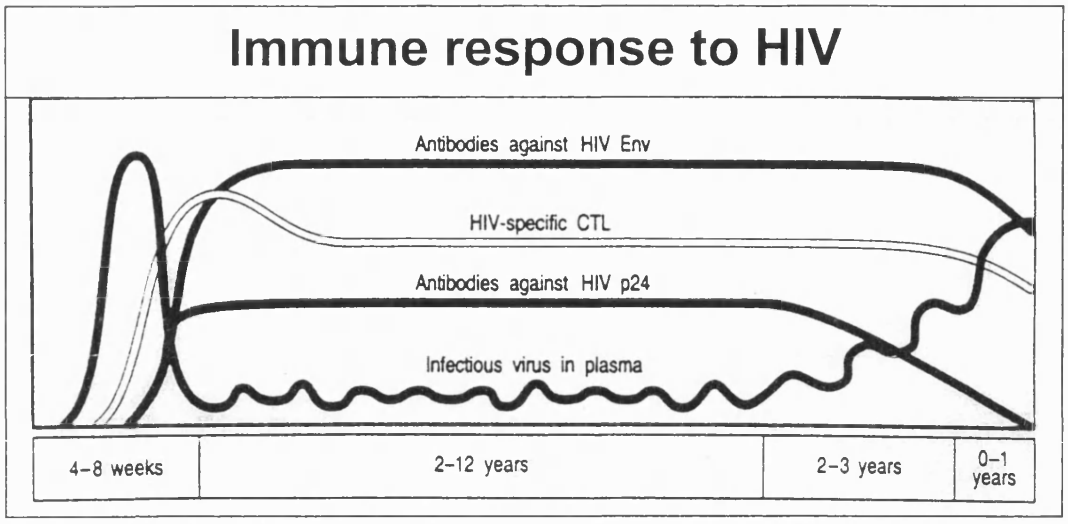


Figure 2. The immune response to HIV

Infectious virus is present at relative low levels in the peripheral blood of infected individuals during a prolonged asymptomatic phase but is replicated persistently in lymphoid tissues. During this period, CD4 T cell counts gradually decline, although antibodies and CD8 cytotoxic T cells directed against the virus remain at high levels. Two different antibody responses are shown in the figure, one to the envelope protein (env) of HIV, and one to the core protein p24. Eventually, the levels of antibody and HIV-specific cytotoxic T lymphocytes (CTLs) also decline, and there is a progressive increase of infectious HIV in the peripheral blood.

Table 3: Summary of the main hurdles in development of an AIDS vaccine

1. Identification of immunogens that induce broad and long-lasting CTL immunity.

2. Definition of structures and immunization strategies that elicit broadly neutralizing antibodies

3. Definition of immune correlates of protection in human or animal models

4. Strategies to address HIV clade and strain diversity

5. Expansion of human clinical trials:
   Clinical-grade vaccine production
   Diversity and duration of immune response
   Prioritization and analysis of candidates

The search for an effective AIDS vaccine was started over 15 years ago, but big challenges face laboratory and clinical research. Table 3 summarizes the main hurdles in the development of an HIV vaccine [54].

Firstly, a CTL response is highly desirable in an AIDS vaccine because CTLs can eliminate or reduce virus production by killing viral producer cells. However, a CTL response alone is unlikely to provide complete protection, and such a vaccine depending strictly on a CTL response may be countered by viral adaptations that allow the virus to evade detection by T cells. (For example, HIV can disrupt the major histocompatibility complex (MHC) proteins that T cells rely on to recognize foreign antigens on the cell surface [54]). Secondly, an AIDS vaccine to elicit neutralizing antibodies to the virus is also crucial, because such antibodies are dependent on memory B cells that can divide and differentiate into the antibody-producing cell upon re-exposure to antigen, thus conferring long-term protection. Both of these immune mechanisms can be manipulated in vaccine development and each has its advantages and disadvantages (Table 4).

In this thesis we speculate on immunological properties of HIV antigens displaying repetitive epitopes, which could, we propose, mimic the conformational structure of native HIV antigens due to the peptide flexibility of the Multiple Antigen Peptide (MAP). We aim to investigate the effect of peptide multiplicity on immunogenicity using a model peptide from the envelope protein gp120 of Human Immunodeficiency Virus type-1 (equivalent paper A and B) [12].

Table 4: Possible approaches to vaccine development

	Advantages	Disadvantages
CTL-eliciting vaccines	Recognition of virally infected cells	Requirement for active, long-term memory cells
	Multiple linear epitopes	Inability to recognize virus in absence of MHC
	Elimination of virus production	Down-modulation of MHC by virus
	Possible effect on latent reservoir	
Antibody-eliciting vaccines	Neutralization of virus	Inability to generate broadly neutralizing antibodies
	Ability to prevent new infection of cells	Evolution of resistant strains
	Activation of inflammatory response (complement system, neutrophiles and monocytes)	Antibodies may enhance infectivity to exacerbate infection
Replication-defective viral vaccines	Persistent antigen expression	Safety and inadvertent infection
	Induction of cell-mediated and humoral immunity	Enhanced replication during immune suppression
	Interference	Consequences of persistent immune stimulation
		Increased rates of integration

5. Cholera Toxin and its B Subunit as vaccine carriers

Cholera toxin (CT) is the primary enterotoxin produced by *Vibrio cholerae* bacteria, and is the causative agents of cholera infection. This is an AB_5 hexameric protein, 5 identical B subunits and a single A subunit. Cholera toxin A (CTA) subunit is responsible for severe fluid loss and diarrhea of infected host. Cholera toxin B subunit (CTB) is the nontoxic binding subunit of the CT holotoxin

[55] and it functions in a pentameric-form which can specifically recognize the GM1 ganglioside receptor present on the surface of mucous cells. Oral administration of CTB can produce a protective intestinal secretory response [56].

CTB was at first developed as an oral vaccine against cholera [57, 58]. This vaccine contains a mixture of B subunit and killed *V. cholerae* O1 cells, and studies showed that it is completely safe as well as strongly immunogenic and protective. Table 5 lists the functional properties of the oral cholera vaccines.

Table 5: Functional properties of the oral B subunit-O1 whole cell cholera vaccine

Safe	No adverse reactions
Immunogenic	Strong intestinal IgA immunogenicity (comparable to clinical disease)
Protective	Cholera 85% protection first 6 months 60% first 3 years

CTB is a potential carrier of mucosal vaccines. It is known that CTB, when conjugated to a poor immunogen and given orally, dramatically increases mucosal immunogenicity [59, 60]. And CTB can supply a universal T helper epitope, which then increases the systemic immunity of vaccines. Fusion of CTB to a proteolipid-protein peptide could induce an inhibition of the specific response

of the peptide [61]. Holmgren, J. et al and other workers also showed that the physical coupling of foreign antigens to CTB is a very effective way of inducing such mucosal immune response after oral vaccination [62]. In addition, Rose's group, including Chen J., also reported that the N-terminus of CTB, a threonine, could in principle undergo oxidation and oximation to form conjugates with a cascade of immunogenic peptides [28]. This also offers a possible way to generate novel mucosal vaccines that possess chemically coupled peptides and are reconstituted into a CT-like complex *in vitro*. Since the mucosal immune response from oral vaccination is not restricted to the intestine but also partly disseminates to other mucosal tissues, it then gives prospect to use CTB as a vaccine carrier against infections in the intestinal as well as in any other mucosal tissues.

In this manuscript we set up a model of CTB by engineering a peptide derived from hemaglutinin protein of influenza virus to the N-terminus (equivalent paper C). We wish to use this model to study the effect of attaching a cascade of immunogenic peptides to CTB (equivalent paper D).

6. Artificial Proteins as Targetting Moieties

Recent progress in biology has greatly benefitted from the understanding of molecular interactions such as binding activities between various receptors on

the cell surface and their specific ligands. This binding causes a conformational change in the receptor that initiates a cascade of intracellular events leading to a specific cellular response. The research on binding effects of the ligand and its specific receptor is an important means to study many biological activities including the growth, differentiation, and metabolism of the multitude of cells.

In the study of binding activities, the peptide ligands are often identified using a powerful technique "phage display random peptide library". As these peptides do not represent the natural ligand of the receptor but rather mimic it, they are called *mimitopes* which can either be displayed as a linear or, if flanked by two cysteine residues, as a circular peptide, and the latter may also mimic conformational structure. Briefly, a DNA fragment expressing the ligands is first inserted into a minor coat protein gene of filamentous phage creating a fusion protein that is incorporated into the virion (named "fusion phage"). The fusion phage that displays billions of different short peptides is then screened for tight binding to a receptor or antibody [63, 64]. The selected peptides can be reproduced as a synthetic or recombinant peptide outside the context of the phage particle.

However, the obtained ligands are usually of low-affinity (micromolar range). [65, 66] This may be due to the high degree of conformational freedom and small number of contact residues within a short peptide molecule. But, if several copies of the peptide molecule with low-affinity binding sites are present in a single multimeric molecule, avidities (and bioactivities) can be greatly enhanced over

the affinity or activity of monomeric peptide. In nature, there are many examples of molecules with low-affinity binding sites, yet capable of high avidity interactions with their targets due to multivalent binding. For example, IgM produced during the primary immune response is pentameric and this confers high avidity toward repetitive antigenic determinants present on the surface of bacteria or viruses [67]. Such enhancement (10^5-fold) occurred with the peptabody (see Figure 3) [68], a protein produced through recombinant DNA techniques. In this protein, the low affinity of polypeptide derived from phage library is compensated by its pentameric structure resulting in a high avidity toward their targets. Recently, Rose K [69] also designed such a molecule named as "chemobody" (see Figure 4). This is a synthetic molecule displaying multiple copies (at least two) of a peptide subunit capable of binding non-covalently to a complementary structure, thus mimicking a major feature of the antibody molecule. The amino acid sequence of the binding peptide was identified using phage library techniques and the subunits themselves were assembled using oxime chemistry. To be able to bind through two or more subunits simultaneously, an appropriate spacer polyethyleneglycol (PEG) chain and a linker were also introduced, which are flexible, amphiphilic, non-immunogenic, and unsusceptible to proteases. And some reporter group such as FITC (fluorescein isothiocyanate) or Biotin can also be introduced into the linker moiety of the Chemobody much more easily than into the Peptabody. Thus the Chemobody possesses more flexibility and it is more convenient for biological use.

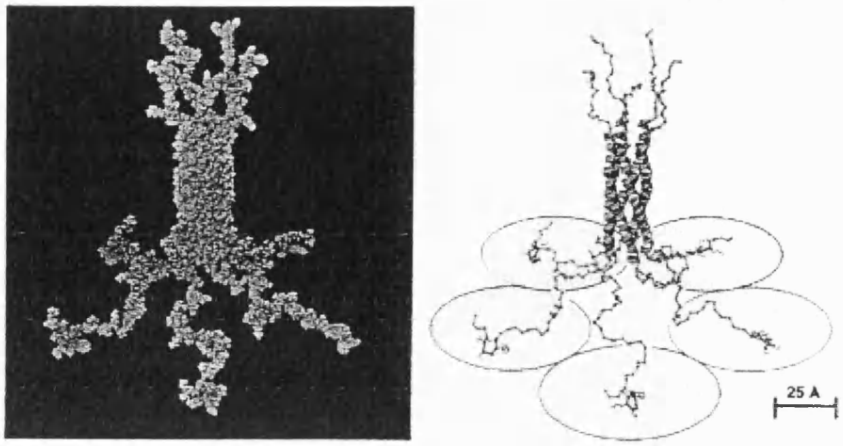


Figure 3: Space-filling (Left) and ribbon (Right) representations of a model of the three-dimensional structure of Pab-S. Binding peptides are in red. The upper part of the structure shows six histidine residues at each C terminus. One chain within the pentameric molecule is highlighted in blue in the space-filling representation. Five shaded circles (radius of 40 Å) under the ribbon structure schematically denote receptor molecules. The ribbon representation was generated with the program MOLSCRIPT.

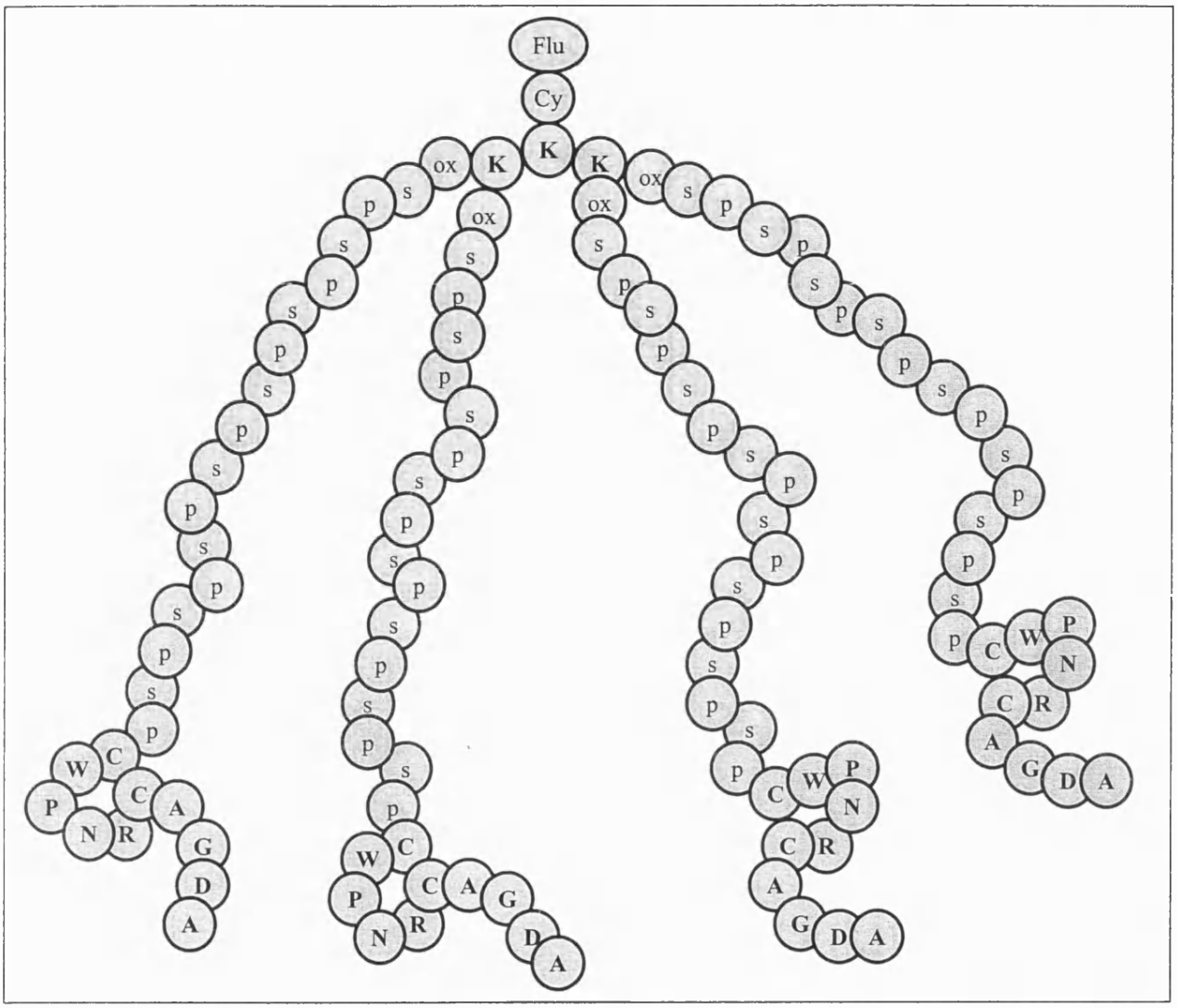


Figure 4: Profile of structure of a tetrameric chemobody

Structure of a tetrameric chemobody displaying four copies of the phage derived peptide SVWRWLPYDKYE. Flu, acetaminofluorescein; Cy, cysteamine linker; ox, oxime linker; s, $-COCH_2CH_2CO-$; p, $-NH-CH_2CH_2CH_2-(OCH_2\ CH_2)_3-CH_2-NH-$.

We have created in our laboratory a model chemobody [69] that displayed 2 or 4 copies of synthetic erythropoietin mimetic peptide together with flexible polyethyleneglycol (PEG) linkers. In this manuscript, we wish to apply this strategy to design and synthesize a set of multimeric molecules that display binding peptides to insulin-secreting cells, and to exploit the enhanced avidity through multivalent binding.

References

[1] Hume E.H. (1940) Chinese way in medicine *Hopkins press* (*Baltimore*)

[2] Pasteur L. (1880) De l'atténuation du virus du choléra des poules. *Cr. Acad. Sci. Paris* 91:673

[3] Ivinson A.J. (1998) Why vaccines? *Nature Medicine* 4: 474

[4] Playfair J., (1998) Vaccination *Immunology 5th edition* (Roitt, I., Brostoff J. and Male D. eds) pp263-7. Mosby International Limited, London, UK

[5] Dent A.H. and Aslam M. (1998), Coupled protein reagents and their applications. *Bioconjugation* (Aslam M and Dent AH, eds.), pp. 2-49, Macmillan Reference Ltd, London, UK

[6] Merrifield R.B. (1963) Solid phase peptide synthesis I, The synthesis of a tetrapeptide. *J.Am.Chem.Soc.* 85, 2149-54

[7] Dent A.H. and Aslam M. (1998), The functional chemistry of proteins and protein coupling. *Bioconjugation* (Aslam M and Dent AH, eds.), pp. 51-100, Macmillan Reference Ltd, London, UK

[8] Brown F. (1996) Peptide vaccines: Dream or reality. *Peptides:chemistry, structure and biology* (Kaumaya PTP and Hodges R, eds.), pp. 869-71, Mayflower Scientific Ltd., Kingswinford, UK

[9] Lerner R.A. (1982) Tapping the immunological repertoire to produce antibodies of predetermined specificity. *Nature* (London) 299, 593-6

[10] Bittle J.L., Houghten R.A., Alexander H., et al. (1982) Protection against foot-and-mouth disease by immunization with a chemically synthesized peptide predicted from the viral nucleotide sequence. *Nature* (London) 298, 30-3

[11] DiMarchi R., Brooke G., Gale C., et al. (1986) Protection of cattle against foot-and-mouth disease by a synthetic peptide. *Science* 232, 639-41

[12] Kelker H.C., Schlesinger D. and Valentine F.T. (1994) Immunogenic and antigenic properties of an HIV-1 gp120-derived multiple chain peptide. *J Immun* 152, 4139-48

[13] Tamura S., Asanuma H., Tomita T., et al. (1994) Escherichia coli heat-labile enterotoxin B subunits supplemented with a trace amount of the holotoxin as an adjuvant for nasal influenza vaccine. *Vaccine* 12, 1083-9

[14] Tam J.P. (1988) Synthetic peptide vaccine design: synthesis and properties of a high-density multiple antigenic peptide system. *Proc Natl Acad Sci USA* 85, 5409-13

[15] Putterman C. and Diamond B. (1998) Immunization with a peptide surrogate for double-stranded DNA (dsDNA) induces autoantibody production and renal immunoglobulin deposition. *J Exp Med* 188(1), 29-38

[16] Drijfhout J.W., Bloemhoff W., Poolman J.T., et al. (1990) Solid-phase synthesis and applications of N-(S-acetylmercaptoacetyl) peptides. *Anal Biochem* 187, 349-54

[17] Wetzel R., Halualani R., Stults J.T., et al. (1990) A general method for highly selective cross-linking of unprotected polypeptides via pH-controlled modification of N-terminal alpha-amino groups. *Bioconjug Chem* 1, 114-22

[18] Geoghegan K.F. and Stroh J.G. (1992) Site-directed conjugation of nonpeptide groups to peptides and proteins via periodate oxidation of a 2-amino alcohol: Application to modification at N-terminal serine. *Bioconjug Chem* 3, 138-46

[19] Rose K. (1994) Facile synthesis of artificial proteins *J Am Chem Soc* 116, 30-3

[20] Zhang L., Tam J.P. (1996) Thiazolidine formation as a general and site-specific conjugation method for synthetic peptides and proteins *Anal Biochem* 233, 87-93

[21] Schwarz A., Wandrey C., Bayer E.A., et al. (1990) Enzymatic C-terminal biotinylatin of proteins *Methods Enzymol* 184,160-2

[22] Rose K., Jones R.M.L., Sundaram G. and Offord RE (1989) Attachment of linker groups to carboxyl termini using enzyme-assisted reverse proteolysis. *Peptides in 1988* (Jung G and Bayer E, eds.), pp. 274-6, Walter de Gruyter, Berlin, Germany

[23] Tam J.P., Rao C., Liu C.F., et al. (1995) Specificity and formation of unusual amino acids of an amide ligation strategy for unprotected peptides. *Int J Pept Protein Res* 45, 209-16

[24] Muir T.W., Sondhi D. and Cole P.A. (1998) Expressed protein ligation: a general method for protein engineering. *Proc Natl Acad Sci USA* 95, 6705-10

[25] Rose K., Zeng W., Regamey P.O., et al. (1996) Natural peptides as building blocks for the synthesis of large protein-like molecules with hydrazone and oxime linkages. *Bioconj Chem* 7, 552-6

[26] Mikolajczyk S.D., Meyer D.L., Starling J.J., et al. (1994) High yield, site-specific coupling of N-terminally modified beta-lactamase to a proteolytically derived single-sulfhydryl murine Fab'. *Bioconjug Chem* 5, 636-46

[27] Rose K., Zeng W., Dragovic M., et al. (1996) Polyoxime artificial proteins as vaccines. *Peptides:chemistry, structure and biology* (Kaumaya PTP and Hodges R, eds.), pp. 767-9, Mayflower Scientific Ltd., Kingswinford, UK

[28] Rose K., Chen J., Dragovic M., et al. (1999) A new cyclization reaction at the amino terminus of peptides and proteins. *Bioconjug Chem* 10,1038-43

[29] Janeway, C.A. Jr, (1999) Manipulation of the immune response: Synthetic peptides of protective antigens can elicit protective immunity. *Immuno Biology 4th edition* (Austin P. and Lawrence E. eds) pp568-9. Elsevier Science Ltd, London, UK

[30] Albert B. (1994) The immune system: cytotoxic T cells *Molecular biology of the cell 3rd edition* (Robertson M. eds) pp1235-8. Garland Publishing, Inc., New York

[31] Albert B. (1994) The immune system: Helper T cells and T cell activation *Molecular biology of the cell 3rd edition* (Robertson M. eds) pp1238-46. Garland Publishing, Inc., New York

[32] Parker D.C. (1993) T cell-dependent B cell activation. *Annu Rev Immunol* 11:331

[33] Cohn M. and Langman R.E. (1990) The protection: the unit of humoral immunity selected by evolution *Immunol Rev* 115:11

[34] Bachmann M.F., Rohrer U.H. and Kundig T.M. et al. (1993) The influence of antigen organisation on B cell responsiveness. *Science* 262:1448.

[35] Zinkernagel R.M., Cooper S., Chambers J., et al. (1990) Virus-induced autoantibody response to a transgenic viral antigen *Nature* 345: 68

[36] Bachmann M.F., Hengartner F.H. and Zinkernagel R.M. (1995) T helper cell-independent neutralizing B cell response against vesicular stomatitis virus: role of antigen patterns in B cell induction? *Eur J Immunol* 25:3445

[37] Alberts B., Bray D. and Lewis J. et al. (1994) The generation of antibody diversity *Molecular Biology of the Cell* 3rd edition (Alberts, B., D. Bray, J. Lewis, et al. eds) pp.1221-7. Garland Publishing, Inc. New York

[38] Cambier, J.C., Pleiman C.M. and Clark M.R. (1994) Signal transduction by the B cell antign receptor and its coreceptors *Annu Rev Immunol* 12:457.

[39] Janeway, C.A. Jr, (1999) Manipulation of the immune response: The route of vaccination is an important determinant of success. *Immuno Biology 4th edition* (Austin P. and Lawrence E. eds) pp570. Elsevier Science Ltd, London, UK

[40] McDermott R.P. and Elson C.O. (1991) Mucosal immunology: I. Basic principles. *Gastroenterology Clinics of North America* W.B. Saunders, Philadelphia

[41] Barre-Sinoussi F., Chermann J.C., Rey F., et al. (1983) Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science* 220, 868-71

[42] Gallo R.C., Salahuddin S.Z., Popovic M., et al. (1984) Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS *Science* 224, 500-3

[43] Kwong P.D., Wyatt R., Robinson J., et al. (1998) Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. *Nature* 393, 648-59

[44] Groopman J.E., Benz P.M., Ferriani R., et al. (1987) Characterization of serum neutralization response to the human immunodeficiency virus (HIV). *AIDS Res Hum Retroviruses* 3(1):71-85

[45] Ho D.D., Sarngadharan M.G., Hirsch M.S., et al. (1987) Human immunodeficiency virus neutralizing antibodies recognize several conserved domains on the envelope glycoproteins. *J Virol* 61(6):2024-8

[46] Rusche J.R., Javaherian K., McDanal C., et al. (1988) Antibodies that inhibit fusion of human immunodeficiency virus-infected cells bind a 24-amino acid sequence of the viral envelope, gp120. *Proc Natl Acad Sci U S A* 85(9):3198-202

[47] Ho D.D., Kaplan J.C., Rackauskas I.E., et al. (1988) Second conserved domain of gp120 is important for HIV infectivity and antibody neutralization. *Science* 239(4843):1021-3

[48] Plata F., Autran B., Martins L.P., et al. (1987) AIDS virus-specific cytotoxic T lymphocytes in lung disorders. *Nature* 328(6128):348-51

[49] Walker B.D., Chakrabarti S., Moss B., et al. (1987) HIV-specific cytotoxic T lymphocytes in seropositive individuals *Nature* 328(6128):345-8

[50] Walker B.D., Flexner C., Paradis T.J., et al. (1988) HIV-1 reverse transcriptase is a target for cytotoxic T lymphocytes in infected individuals. *Science* 240(4848):64-6

[51] Lyerly H.K., Matthews T.J., Langlois A.J., et al. (1987) Human T-cell lymphotropic virus IIIB glycoprotein (gp120) bound to CD4 determinants on normal lymphocytes and expressed by infected cells serves as target for immune attack. *Proc Natl Acad Sci U S A* 84(13):4601-5

[52] Rook A.H., Lane H.C., Folks T., et al. (1987) Sera from HTLV-III/LAV antibody-positive individuals mediate antibody-dependent cellular cytotoxicity against HTLV-III/LAV-infected T cells. *J Immunol* 138(4):1064-7

[53] Weinhold K.J., Lyerly H.K., Matthews T.J., et al. (1988) Cellular anti-GP120 cytolytic reactivities in HIV-1 seropositive individuals *Lancet* 1(8591):902-5

[54] Nabel G.J. (2001) Challenges and opportunities for development of an AIDS vaccine *Nature* 410(6831):1002-7

[55] Sun J.B., Holmgren J. and Czerkinsky C. (1994) Cholera toxin B subunit: an efficient transmucosal carrier-delivery system for induction of peripheral immunological tolerance. *Proc Natl Acad Sci U S A* 91, 10795-9

[56] Svennerholm A., Lange S., and Holmgren J. (1978) Correlation between intestinal synthesis of specific immunoglobulin A and protection against experimental cholera in mice *Infect Immun* 21(1):1-6

[57] Holmgren J., Svennerholm A.M., Jertborn M., et al. (1992) An oral B subunit: whole cell vaccine against cholera. *Vaccine* 10(13):911-4

[58] Quiding M., Nordstrom I., Kilander A., et al. (1991) Intestinal immune responses in humans. Oral cholera vaccination induces strong intestinal antibody responses and interferon-gamma production and evokes local immunological memory *J Clin Invest* 88(1):143-8

[59] Rask C., Fredriksson M., Lindblad M., et al. (2000) Mucosal and systemic antibody responses after peroral or intranasal immunization: effects of conjugation to enterotoxin B subunits and/or of co-administration with free toxin as adjuvant. *APMIS* 108(3):178-86

[60] McKenzie S.J. and Halsey J.F. (1984) Cholera toxin B subunit as a carrier protein to stimulate a mucosal immune response *J Immunol* 133(4):1818-24

[61] Yuki Y., Byun Y., Fujita M., et al. (2001) Production of a recombinant hybrid molecule of cholera toxin-B-subunit and proteolipid-protein-peptide for the treatment of experimental encephalomyelitis. *Biotechnol Bioeng* 74(1):62-9

[62] Holmgren J., Czerkinsky C., Lycke N., et al. (1994) Strategies for the induction of immune responses at mucosal surfaces making use of cholera toxin B subunit as immunogen, carrier, and adjuvant. *Am J Trop Med Hyg* 50(5 Suppl):42-54

[63] Smith, G.P. (1985) Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface *Science* 228(4705):1315-7

[64] Winter G., Griffiths A.D., Hawkins RE, et al. (1994) Making antibodies by phage display technology. *Annu Rev Immunol* 12:433-55

[65] Scott J.K. and Smith G.P. (1990) Searching for peptide ligands with an epitope library *Science* 249(4967):386-90

[66] Renschler M.F., Bhatt R.R., Dower W.J., et al. (1994) Synthetic peptide ligands of the antigen binding receptor induce programmed cell death in a human B-cell lymphoma. *Proc Natl Acad Sci U S A* 91(9):3623-7

[67] Roitt, I. M. (1991) Essential Immunology (Oxford/Blackwell, London), pp. 65-84

[68] Alexey V., Terskikh, Jean-Marc Le Doussal, et al. (1997) "Peptabody": A new type of high avidity binding protein *Proc. Natl. Acad. Sci. USA* 94,1663-8

[69] Rose K. and Vizzavona J. (1999) Stepwise solid-phase synthesis of polyamides as linkers *J. Am. Chem. Soc.* 121,7034-8

C. DISSERTATION EQUIVALENTS

Dissertation Equivalent A

J. Chen, H. Kelker, K. Rose

Improved syntheses of Multiple Antigen Peptides and polyoximes

Dissertation Equivalent B

J. Chen, H. Kelker, K. Rose

Artificial proteins as candidate AIDS vaccines

Dissertation Equivalent C

K. Rose, J. Chen, M. Dragovic, W. Zeng, D. Jeannerat, P. Kamalaprija, U. Burger.

New cyclization reaction at the amino terminus of peptides and proteins.

Bioconjug Chem 1999 : 6(10), 1038-43

Dissertation Equivalent D

J. Chen, W. Zeng, K. Rose

Enhanced binding properties of cholera toxin B subunit when conjugated with a polyoxime

Dissertation Equivalent E

J. Chen, C. Sauser, K. Rose, C. Bonny

Artificial Proteins as Targeting Moieties of Insulin-Secreting Cells

Dissertation Equivalent A

J. Chen, H. Kelker, K. Rose

Improved syntheses of Multiple Antigen Peptides and polyoximes

Experimental design and execution was performed by Jianhua Chen, except where indicated (Edman degradation performed by a commercial organisation).

Improved syntheses of Multiple Antigen Peptides and polyoximes

Jianhua Chen[1], Hanna Kelker[2], Keith Rose[1]

[1] Department of Medical Biochemistry, University Medical Center, Geneva, Switzerland;

[2] Department of Medicine, New York University Medical Center, NY 10016.

Correspondence and reprint requests: Prof. Keith Rose
Department of Medical Biochemistry
University Medical Center
1211 Geneva 4, Switzerland

Tel. +41 22 702 5523
Fax. +41 22 702 5502
Email. Keith.Rose@medicine.unige.ch

Summary

A multiple antigen peptide (MAP) system consisting of antigenic peptides linked by amide bonds to a synthetic core can elicit enhanced immunogenecity. However, it is difficult to prepare MAPs possessing long polypeptide chains in homogeneous form. We describe the synthesis of octa-branched MAPs using direct Boc-chemistry. Using a peptide from HIV-1, three different MAP constructs were made by stopping the synthesis at different stages. Warming the reaction mixture was essential for three critical couplings. The MAP with the shortest peptide sequence was characterized by MALDI-TOF mass spectrometry. Edman degradation of the MAPs possessing intermediate and full-length peptides showed that the correct sequence was present but it was not possible to obtain a mass spectrum by MALDI or electrospray technique. An octaoxime was made by site-specific conjugation of 8 pre-purified peptides to a synthetic radial polylysine core. Results showed that it was homogeneous on RP-HPLC and characterizable by ESI-MS. Attempts to use thioether chemistry failed, producing up to a 7-branched product but no octamer. Factors influencing the syntheses of MAPs, oximes and thioether constructs are discussed.

Introduction

Synthetic peptides can induce antibodies that are useful in vaccination strategies or as laboratory reagents, for example, in the study of biosynthetic pathways by confirming *de novo* synthesis of proteins from recombinant DNA [1]. However, a small linear peptide may not be effective as an immunogen because of the influence of protein conformation [2] or because of its lack of high molecular mass that appears necessary for enhanced immunogenicity. One way to enhance peptide immunogenicity is to attach antigenic peptides to a synthetic core in a branched (radial or comb) manner. Tam [3] in 1988 described an amide-linked branched artificial protein to increase molecular weight. In this approach a trifunctional amino acid, Boc-Lys(Boc), is used to create a multiple antigenic peptide (MAP) system. Many useful results have been obtained with this approach but, unfortunately, it is rather difficult to produce homogeneous artificial proteins with many (e.g. 8) branch points and long polypeptides due to inevitable side-reactions and coupling failures. It is difficult to purify the wanted material from the many impurities present with similar structure and physico-chemical properties.

Over the years, site-specific conjugation, an effective chemical approach of ligating antigenic peptides to a protein or a synthetic carrier under mild conditions [4-12], has been developed as a means of creating artificial proteins. For example, N-terminal Serine residues in an octavalent core can be oxidized by periodate into aldehyde functional groups under mild conditions, and then aminooxyacetyl polypeptides can be reacted with each aldehyde group to give a homogeneous octaoxime [13]. An alternative chemoselective ligation method involves thioether chemistry [14-15]. While disulphide formation and non-specific alkylations are possible side-reactions, some useful immunogens have been made by the thioether approach.

Human Immunodefienciency Virus type-1 (HIV-1) is the main type of HIV virus which can cause the destruction of $CD4^+$ lymphocytes in the host resulting in the development of acquired immunodeficiency syndrome (AIDS) [16-17]. gp120 is the envelope glycoprotein spiked on the virion surface which mediates HIV entry into the target cell, a critical step of its pathogenesis. In previous work, a peptide was synthesized by mimicking certain epitope residues (419-439) of gp120, which was expected to induce an antibody to disrupt the entry of HIV, thus interfering with its pathogenesis [18]. However, the antibody induced by peptide monomer was found not to recognize the native conformation of gp120. Surprisingly, an octamer synthesized directly as an amide-linked MAP was able to induce the desired neutralizing antibodies, but the preparation was heterogeneous and unpurifiable, being the result of over two hundred coupling reactions.

In this article we compare the syntheses of octa-branched immunogens, based on the gp120 (419-439) peptide, using three approaches: direct amide bond formation, and chemo-selective ligation involving either thioether or oxime chemistry.

Methods

Materials

Unless otherwise specified, all solvents and reagents were obtained from Fluka, Buchs, Switzerland, were of analytical or higher grade and were used without further purification. All amino acids were purchased from Peptide Institute Inc. Japan. Resins were from Applied Biosystems, USA; Novabiochem, Switzerland, or Bachem, Switzerland. Water was repurified using a Milli-Q system (Millipore, Inc.).

RP-HPLC

Analytical RP-HPLC was performed using a column 250 x 4 mm i.d. packed with Nucleosil 300-A 5 μm C_8 particles at a flow rate of 0.6 ml/min and effluent was monitored at 214nm. Semi-preparative peptide purification was carried out using a C_8 column (250 x 10 mm i.d. Nucleosil 300-A 5 μm particle size) at a flow rate of 4 ml/min monitoring at 214 nm, and preparative purification using a C_{18} column (210 x 25 mm i.d. Novapak 6 μm particle size) at 20 ml/min monitoring at 229 nm. Solvents used in RP-HPLC were as follows: A, 0.1% TFA (1 g TFA in 1 liter H_2O); B, 0.1% TFA in 90% acetonitrile (1 g TFA mixed with 100 ml H_2O and then brought to1 liter with acetonitrile). Generally, the conditions used in the analytical work were 5 min isocratic at 100% A followed by a linear gradient 2% B/min to 100% B, and in the preparative work a shallower linear gradient (usually 0.5% B/min) was used. Components were collected manually at the detector exit, evaporated at room temperature, frozen and then recovered by lyophilization.

Mass Spectrometry

Electrospray ionization mass spectrometry (ESI-MS) was performed in positive ion mode on a Trio 2000 machine or a Platform II instrument (both from Micromass, Manchester, England). Samples were introduced either at 2 µl/min (Trio) in an acidified solvent acetonitrile/water/AcOH (49.5 : 49.5 : 1) or at 10 µl/min (Platform) in solvent acetonitrile/water/formic acid (49.9 : 49.9 : 0.2). MALDI-TOF mass spectrometry was performed in linear mode using sinapinic acid as matrix on a Voyager Elite machine (PE Biosystems) equipped with delayed extraction. External calibration was performed on the electrospray machines by using a solution of horse heart apomyoglobin, and on the MALDI-TOF machine using the mixture of peptides supplied by the manufacturer.

Protein sequencing

Unpurifiable MAP-2 and MAP-3 were sent to Eurosequence (Nijenborgh 4 – NL 9747 AG Groningen – The Netherlands) for protein sequence analysis by Edman degradation.

Syntheses of MAP constructs

Three MAP (multiple antigenic peptide) constructs were designed as before [18], and their sequences are as follows:

MAP-1: $(VGKAMYAPPIGGG)_8K_4K_2K$-βA-OH

MAP-2: $(INMWQEVGKAMYAPPIGGG)_8K_4K_2K$-βA-OH

MAP-3: $(RIKQIINMWQEVGKAMYAPPIGGG)_8K_4K_2K$-βA-OH

They were synthesized stepwise on Boc-βAla-PAM resin using Boc-chemistry. The syntheses were monitored by ninhydrin test, and re-couplings were

performed on some amino acid residues in order to achieve apparently quantitative acylation. Three amino acid residues, Tyr(435), Glu(429), and Arg(419) required heating to between 40°C-60°C to couple quantitatively as monitored by the ninhydrin test. All of the MAP products were cleaved from the resin with HF, purified by semi-preparative HPLC and characterized by MALDI-TOF mass spectrometry.

Syntheses of a polylysine core and linear peptides

The core structure used in the project was $S_8K_4K_2K$-βA-OH. Synthesis was performed manually on 0.2 mmol of Boc-βAla-PAM resin (substitution 0.88 mmol/g) using Boc-chemistry. Three cycles of Boc deprotection and couplings of Boc-Lys(Boc) formed the polylysine core and were followed by attachment of Boc-Ser(Bzl) to the branched core with 8 mmol Boc-Ser(Bzl)-OSu in 8 ml DMSO and 2 ml N-methylmorpholine.

The antigenic peptide sequence RIKQIINMWQEVGKAMYAPPI*GGGK* from gp120 envelope protein of HIV-1 [18] was synthesized by Boc chemistry on methylbenzhydrylamine resin (0.2 mmol MBHA resin, PE ABI division) using an automated solid-phase synthesizer (model ABI 430A, Applied Biosystems Inc.). Italic type denotes linker residues not present in the corresponding protein sequences. Side-chain protection was Arg(Tos), Asn(xan), Trp(CHO), Glu(OcHx), Tyr(2-BrZ), Lys(ClZ), C-terminal Lys(Fmoc). After chain elongation by Boc chemistry, side-chain protection (Fmoc) at the C-terminal Lys was removed with 20% piperidine in DMF. At this stage, an aminooxyacetyl (AOA) group was attached to the Lys residue with Boc-AOA-Osu (5-fold excess over the substitution of the resin in 5 ml DMSO, 1 ml N-methylmorpholine) to form an HIV1-derived aminooxyacetyl peptide.

All of the peptides were cleaved from the resin with HF (0°C for 1 h in the presence of 5% p-cresol), precipitated with cold ether, extracted with 50%

acetonitrile, filtered and lyophilized. The crude peptides were purified by preparative HPLC and characterized by ESI-MS.

Periodate oxidation

3.75 μmol of core was dissolved in 23.5 ml imidazole buffer (50 mM, pH 6.95, chloride counter ion) and 1.3 ml of methionine (200 mM) was added. Then 625 μl of $NaIO_4$ (0.2 M in water) was added to start the oxidation. After 5 min the reaction was quenched by adding 500 μl of ethylene glycol (500 mM in water). The product was purified by preparative HPLC and characterized by mass spectrometry.

Oximation

The aldehyde obtained by oxidation was reacted with the HIV-1 derived aminooxyacetyl peptide, H-RIKQIINMWQEVGKAMYAPPI*GGGK*($COCH_2ONH_2$)-NH_2 to form an octa-oxime (see Figure 1). 4.8 μmol of aminooxyacetyl peptide was dissolved in about 50 μl of acetonitrile and 1.2 ml of NaOAc buffer (acetic acid 0.57 ml in 100 ml of water, sodium acetate 0.82 g dissolved in 100 ml of water, mixed part of both until pH 4.0). 0.3 μmol of oxidized core was dissolved in 100 μl NaOAc buffer and added to the above solution, followed by addition of acetic acid to a final concentration of 4%. After 24 h reaction at room temperature, the product was purified by semi-preparative RP-HPLC and characterized by mass spectrometry.

Results and Discussion

Characterization of MAP constructs of HIV-1

A MAP (multiple antigenic peptide) construct with 8 identical peptides (residues 419-439, RIKQIINMWQEVGKAMYAPPI, from the gp120 glycoprotein of HIV-1) was able to induce antibodies recognizing conformational determinants on the gp120 envelope protein which were not recognized by the antibodies induced by monomer envelope-derived peptide [18]. The MAP constructs were referred to as MCP (multiple chain peptides) in that paper but we will use the more conventional term here. We repeated the synthesis of the MAP construct using an octavalent branched lysine core, and removed aliquots of resin at 3 different stages: Val(430), Ile(424) and Arg(419). Three different MAP constructs were thus obtained: with 8 identical short-length peptides VGKAMYAPPI (MAP-1), with 8 identical medium-length peptides INMWQEVGKAMYAPPI (MAP-2), and with 8 identical full-length peptides RIKQIINMWQEVGKAMYAPPI (MAP-3). During the synthesis, some amino acid residues such as Tyr(435), Glu(429), and Arg(419) were found difficult to couple at room temperature even after multiple attempts, even with very activated coupling species, and had to be warmed to 40 degrees or even 50-60 degrees. The peptide resins were cleaved and the crude products purified on semi-preparative-HPLC. MAP-1 (see figure 2) was successfully characterized by MALDI-TOF mass spectrometry (M + $H^{\oplus}$ found 10587, calculated 10581), but MAP-2 and MAP-3 (see figure 2) failed to give interpretable spectra which was consistent with previous work [17]. Results of 7 cycles of Edman degradation of MAP-2 and MAP-3 showed that the expected amino acid sequence was present. Initial yields of the main sequence were approximately 50 pmol for MAP-2, 100 pmol for MAP-3: MAP-2, Ile-Asn-Met-Trp-Gln-Glu-Val; MAP-3, Arg-Ile-Lys-Gln-Ile-Ile-Asn. Initial yields of minor sequences: (approximately 1 – 5 pmol for both) were: MAP-2, (Ile?/Thr?)-(Pro/Met)-(Gln/Trp)-(Gln/Phe/Glu?)-/Gly/Ala/Val)-(Val/Gly/Leu)-(Lys/Gly); MAP-3, (Ile)-(Lys/Pro/Gln)-

(Gln)-(Ile)-Asn)-(Asn/Met)-(Met/Asp). The failure to produce homogeneous synthetic MAP-2 and MAP-3 constructs led us to explore alternative strategies for the synthesis of an octameric immunogen.

Thioether constructs

In order to obtain homogeneous multimeric immunogens, we employed site-specific conjugation, by which pre-purified peptides can be ligated to a synthetic polylysine core. We investigated alkylation of a bromoacetylated core with a C-terminal Cys peptide. While the reaction was very clean when analyzed by HPLC, Mass Spectrum showed that only seven peptides became attached to the octa-bromoacetyl core in spite of many attempts with large excesses of Cys peptide.

Polyoxime constructs of HIV-1 gp120 (419-439)

Oxime formation requires an aminoxyacetyl group and a carbonyl group, generally an aldehyde [7]. The latter is easily generated from N-terminal or side chain-linked Ser, Thr, or carbohydrates [6, 7, 19], and can then react with the aminooxyacetyl-containing species.

Oxime bond formation involves the specific reaction between an aminooxy group and a carbonyl group (aldehyde or ketone) and explusion of a water molecule in aqueous medium under acidic conditions. Oximation is very slow at neutral pH but proceeds close to completion at acidic pH over a period of hours [13]. Using oxime chemistry, it was possible to assemble multimeric constructs that possessed eight copies of the HIV1-derived peptide described by Kelker et al [18]. Because the peptide subunit was purified and characterized prior to assembly, it was possible to characterize the final product by mass spectrometry.

The octameric construct was built on a radial polylysine core containing a Ser residue at the tip of each of 8 branches. Eight Ser residues were periodate-oxidized and then reacted with HIV1-derived peptide in aminooxy form to yield the octaoxime shown in Figure 1. The octaoxime was purified on reverse phase HPLC and characterized by ESI-MS (Figure 3). Relative molecular mass found 24156, theoretical 24157. Some evidence was found of components with higher masses [+73, +156, +(156+73)]. These components, which coeluted on HPLC with the desired product, were probably due to acylation of the Boc-$NHOCH_2CO$-nitrogen with a second Boc-$NHOCH_2CO$- group during synthesis of the HIV1 peptide (calculation, +73 for NH_2OCH_2CO replacing H) and of incomplete removal of the tosyl group (calculation, +154). We now use less strong acylation conditions (1 equivalent instead of five) which avoid the +73 problem.

Comparison of syntheses of MAPs and Octaoxime

The syntheses of MAP constructs were difficult and the products were heterogeneous. This is inevitable since over 200 reactions were involved without the opportunity to purify any intermediates. It is rather difficult to purify the final product from the many impurities present with similar properties. Warming to 40-60 degrees when coupling difficulties were encountered dramatically improved the synthesis. Indeed, when coupling Tyr435, Glu429 and Arg419, warming was essential. The standard Kaiser ninhydrin test involves heating, and we found through use of this test that coupling of these three residues did not occur at room temperature and did not improve upon repeated attempts using HBTU, HATU or BOP activation protocols and extended coupling times at room temperature (22°C). Warming the coupling reactions to 60°C, 60°C and 40°C, residues 435, 429 and 419, respectively, led to quantitative coupling within 30 minutes as determined by ninhydrin reaction at 100°C. Warming is thus a simple and effective method to improve the synthesis of MAPs, but if repeated too many may lead to racemization or to degradation of the product [20].

Octaoxime was built up by conjugation of pre-purified HIV-1 derived aminooxyacetyl-peptides to the radial octalysine core possesing a carbonyl group at the tip of 8 branches. Octaoxime is thus comparable in structure with MAP-3. But, in contrast with the MAP octamers, octaoxime can be prepared in characterizable form because there are only 8 coupling reactions involved in the conjugation and any coupling failures lead to materials which lack 1 or 2 whole peptides and are thus easily separable by RP-HPLC. A comparison between MAPs and Octaoxime is shown in the table.

Conclusion

Three MAP constructs and an octaoxime were obtained by using improved Boc-chemistry and oxime site-specific conjugation, respectively, with a model peptide derived from HIV-1. Only the MAP with a short peptide sequences could be characterized by mass sepectrometry. MAPs with intermediate and full-length peptides were characterized by Edman degradation. Warming to 40-60 degrees when coupling critical residues improved the synthesis. It was not possible to prepare an octameric construct using thioether chemistry: the maximum number of peptide chains that became attached to the core was seven. An octaoxime was prepared by site-specific conjugation of 8 identical peptides to a synthetic radial octalysine core and was characterized by mass spectrometry. The octaMAP constructs and the octaoxime are being tested as immunogens in mice.

Acknowledgements

We thank the Swiss National Science Foundation, the University of Geneva and NIH for financial support.

References

[1] Lerner RA (1982) Tapping the immunological repertoire to produce antibodies of predetermined specificity. *Nature* 299(5884):593-6

[2] Kaumaya PTP, Kobs-Conrad S, and DiGeorge AM (1994) Synthetic peptide vaccines : Misconceptions and problems, strategies and prospects. Innovation and perspectives in solid phase synthesis (Editor : Roger Epton) pp279-92

[3] Tam JP (1988) Synthetic peptide vaccine design: synthesis and properties of a high-density multiple antigenic peptide system. *Proc Natl Acad Sci USA* 85, 5409-13

[4] Drijfhout JW, Bloemhoff W, Poolman JT, et al. (1990) Solid-phase synthesis and applications of N-(S-acetylmercaptoacetyl) peptides. *Anal Biochem* 187, 349-54

[5] Wetzel R, Halualani R, Stults JT, et al. (1990) A general method for highly selective cross-linking of unprotected polypeptides via pH-controlled modification of N-terminal alpha-amino groups. *Bioconjug Chem* 1, 114-22

[6] Geoghegan KF and Stroh JG (1992) Site-directed conjugation of nonpeptide groups to peptides and proteins via periodate oxidation of a 2-amino alcohol. Application to modification at N-terminal serine *Bioconjug Chem* 3, 138-46

[7] Rose K (1994) Facile synthesis of artificial proteins. *J Am Chem Soc* 116, 30-3

[8] Zhang L, Tam JP (1996) Thiazolidine formation as a general and site-specific conjugation method for synthetic peptides and proteins *Anal Biochem* 233, 87-93

[9] Schwartz A, Wandrey C, Bayer EA, et al. (1990) *Methods Enzymol* 184,160-2

[10] Rose K, Jones RML, Sundaram G and Offord RE (1989) Attachment of linker groups to carboxyl termini using enzyme-assisted reverse proteolysis. *Peptides in 1988* (Jung G and Bayer E, eds.), pp. 274-6, Walter de Gruyter, Berlin, Germany

[11] Tam JP, Rao C, Liu CF, et al. (1995) Specificity and formation of unusual amino acids of an amide ligation strategy for unprotected peptides. *Int J Pept Protein Res* 45, 209-16

[12] Muir TW, Sondhi D, Cole PA (1998) Expressed protein ligation: a general method for protein engineering. *Proc Natl Acad Sci USA* 95, 6705-10

[13] Rose K, Zeng W, Regamey P.O., et al. (1996) Natural peptides as building blocks for the synthesis of large protein-like molecules with hydrazone and oxime linkages. *Bioconj Chem* 7, 552-6

[14] Tam JP, and Spetzler JC (1997) Multiple Antigen Peptide System *Methods Enzymol* 289, 612-36

[15] Lu YA, Clavijo P, Galantino M, et al. (1991) Chemically unambiguous peptide immunogen: preparation, orientation and antigenicity of purified peptide conjugated to the multiple antigen peptide system. *Mol Immunol* 28(6):623-30

[16] Barre-Sinoussi F, Chermann JC, Rey F, et al. (1983) Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science* 220, 868-71

[17] Gallo RC, Salahuddin SZ, Popovic M, et al. (1984) Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS *Science* 224, 500-3

[18] Kelker HC, Schlesinger D and Valentine FT (1994) Immunogenic and antigenic properties of an HIV-1 gp120-derived multiple chain peptide. *J Immun* 152, 4139-48

[19] Tam JP and Spetzler JC (1995) Chemoselective approaches to the preparation of peptide dendrimers and branched artificial proteins using unprotected peptides as building blocks. *Biomed Pept Proteins Nucleic Acids* 1, 123-32

[20] Albericio F and Carpino LA (1997) Coupling reagents and activation *Methods Enzymol* 289, 104-26

Figure Legends

Figure 1. Reaction scheme showing periodate oxidation of radial-branched octaaldehyde and oxime formation with aminooxyacetyl-peptide, RIKQIINMWQEVGKAMYAPPIGGGK(AOA)-amide, from HIV-1 gp120. The branching Lys residues of the core are acylated on both alpha and epsilon amino groups and are shown (together with βAla and Ser residues) unconventionally with their carboxyl groups to the left.

Figure 2. (A) MALDI Mass spectrum of the MAP-1: (VGKAMYAPPI*GGG*)$_8$k$_4$k$_2$ka-OH (calculated 10581, found: 10587); (B) Protein sequence analyses of MAP-2: (INMWQEVGKAMYAPPI*GGG*)$_8$k$_4$k$_2$ka-OH and MAP-3: (RIKQIINMWQEVGKAMYAPPI*GGG*)$_8$k$_4$k$_2$ka-OH.

Figure 3. Electrospray ionization mass spectrum of the Octaoxime constructed on a radial core (calculate mass, 24157, found, 24156), see Figure 1 for structure.

Table: Comparison between OctaMAPs and Octaoxime

	OctaMAPs	**Octaoxime**
Synthesis	Required warming	Room temperature
Reaction mixture	Resin bound	Oximation gelled after a few minutes
Resolution on RP-HPLC	MAP-1, purifiable MAP-2, unpurifiable MAP-3, unpurifiable	purifiable
Mass spectrometry	None characterizale by ESI-MS, MAP-1 characterized by MALDI-TOF, MAP-2 and MAP-3 uncharacterizale by MS	Characterized by ESI-MS
Solubility	Soluble in water	Soluble in a drop of CH3CN + water + 5% acetic acid
Stability	Relative stable (amide bonds)	Stable in solution at pH 2.1-7.0, room temperature for >65h, and in powder at -20ºC for a long period

Figure 1

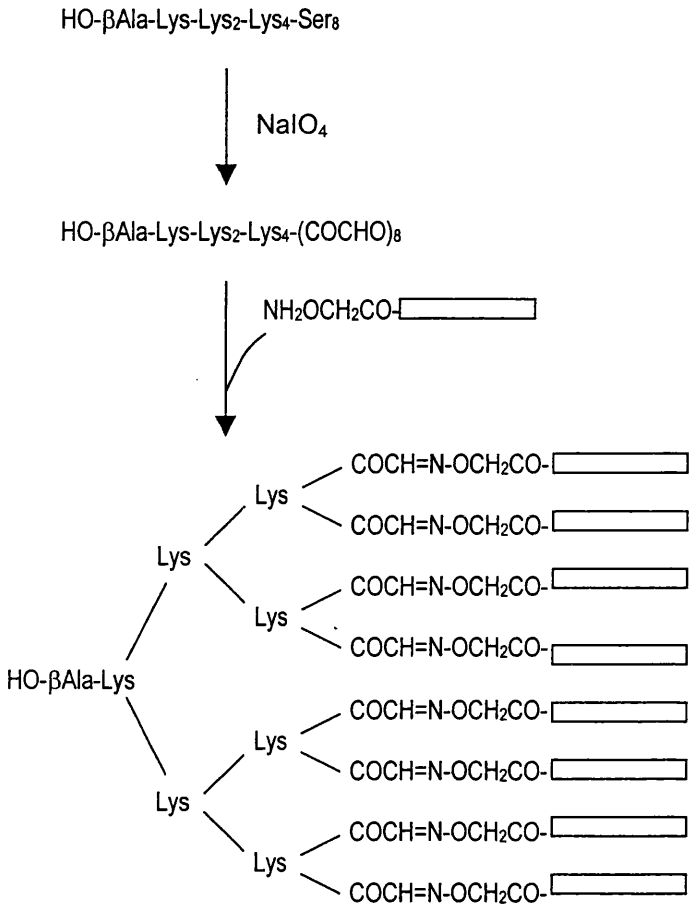
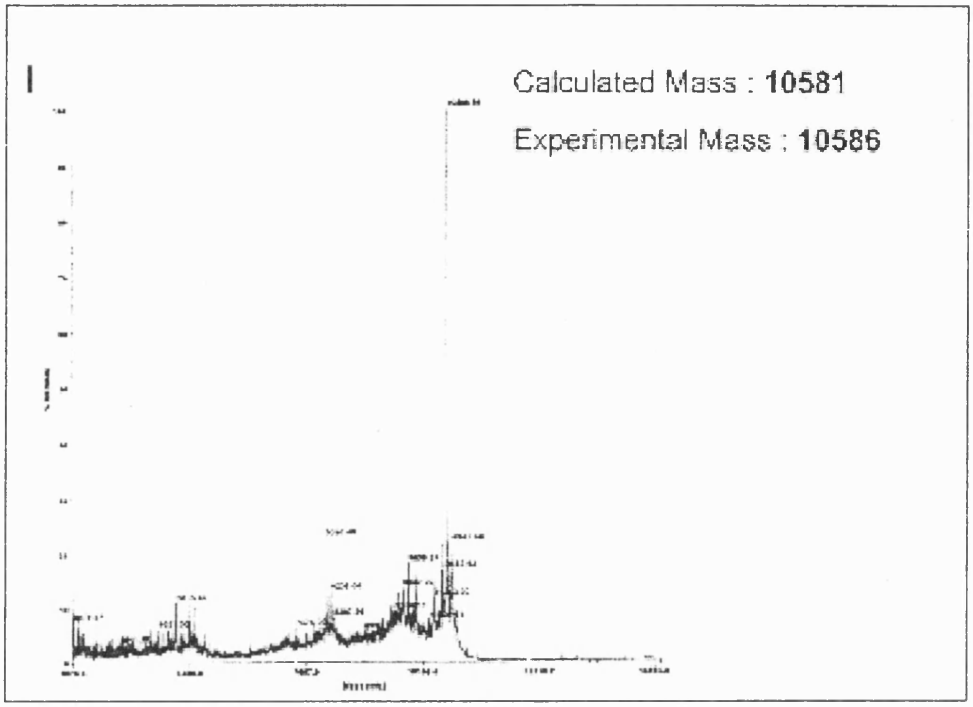

HO-βAla-Lys-Lys2-Lys4-Ser8
NaIO4
HO-βAla-Lys-Lys2-Lys4-(COCHO)8
NH2OCH2CO-
HO-βAla-Lys
Lys
Lys
Lys
Lys
Lys
COCH=N-OCH2CO-
COCH=N-OCH2CO-
COCH=N-OCH2CO-
COCH=N-OCH2CO-
COCH=N-OCH2CO-
COCH=N-OCH2CO-
COCH=N-OCH2CO-
COCH=N-OCH2CO-

Figure 2

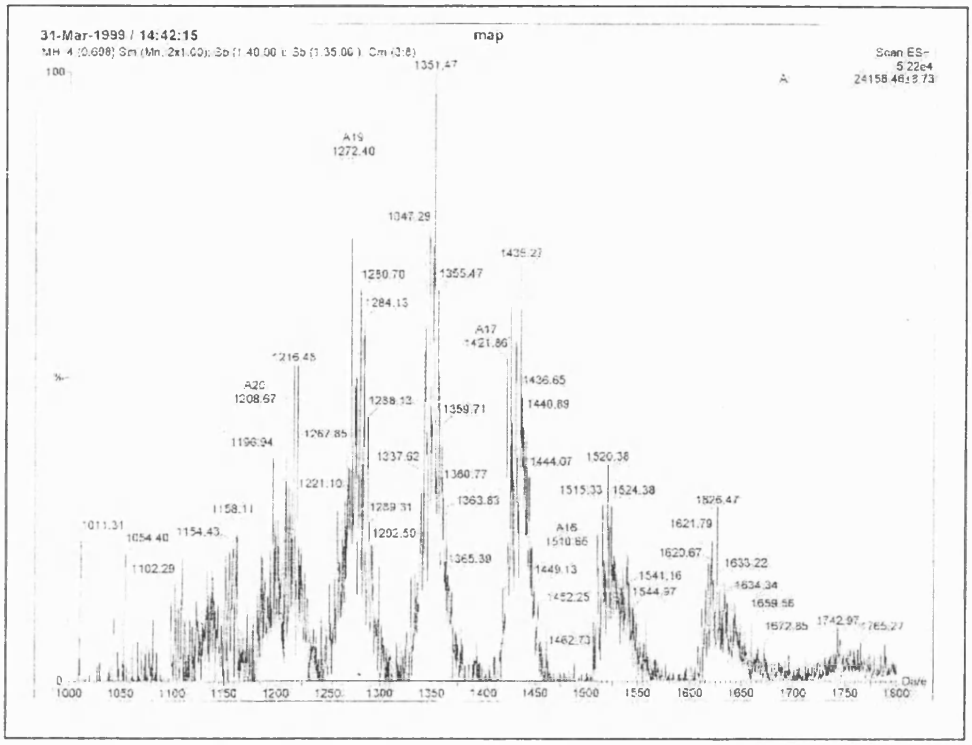
(A) **MAP-1**



(B)

MAP-2	**MAP-3**
Main Sequence Position 1 5 7 Amino Acid Ile-Asn-Met-Trp-Gln-Glu-Val Initial Yield: appr. 50 pmol Minor Signals Position 1 Amino Acid: (Asn ?/Thr ?)-(Pro/Met)-(Gln/Trp)-(Gln/Phe/Glu ?)- 5 7 (Gly/Ala/Val)-(Val/Gly/Leu)-(Lys/Gly) Initial Yield: appr. 1-5 pmol	Main Sequence Position 1 5 7 Amino Acid Arg-Ile-Lys-Gln-Ile-Ile-Asn Initial Yield: appr. 100 pmol Minor Signal Position 1 5 7 Amino Acid: (Ile)-(Lys/Pro/Gln)-(Gln)-(Ile)-(Asn)-(Asn/Met)-(Met/Asp) Initial Yield: appr. 1-5 pmol

Figure 3


31-Mar-1999 / 14:42:15
map
Scan ES-
5.22e4
100
%
A19
1272.40
1351.47
1047.29
1435.27
1250.70
1355.47
1284.13
A17
1421.86
1216.45
A20
1208.67
1436.65
1440.89
1288.13
1359.71
1267.85
1196.94
1237.92
1444.07
1520.38
1360.77
1221.10
1515.33
1524.38
1363.83
1158.11
1289.31
1826.47
1011.31
1054.40
1154.43
1202.50
A16
1510.86
1621.79
1620.67
1365.39
1102.29
1449.13
1633.22
1541.16
1544.97
1452.25
1634.34
1658.56
1572.85
1742.97
1765.27
1462.73
0
1000
1050
1100
1150
1200
1250
1300
1350
1400
1450
1500
1550
1600
1650
1700
1750
1800
Da/e

Dissertation Equivalent B

J. Chen, H. Kelker, K. Rose
Artificial proteins as candidate AIDS vaccines

Synthetic approach and execution by Jianhua Chen, and bioassay studies by

Hanna Kelker

Artificial proteins as candidate AIDS vaccines

Jianhua Chen[1], Hanna Kelker[2], Keith Rose[1]

[1] Department of Medical Biochemistry, University Medical Center, Geneva, Switzerland;

[2] Department of Medicine, New York University Medical Center, NY 10016.

Correspondence and reprint requests: Prof. Keith Rose
Department of Medical Biochemistry
University Medical Center
1211 Geneva 4, Switzerland

Tel. +41 22 702 5523
Fax. +41 22 702 5502
Email. Keith.Rose@medicine.unige.ch

Summary

An octamer MAP (multiple antigen peptide) consisting of HIV-1 gp120-derived peptides linked by amide bonds to a synthetic core is known to elicit enhanced immunogenecity to native gp120. However, it was heterogeneous and could not be characterized by mass spectrometry. In this study we prepared a set of multimeric constructs (comb-design and tree-design) employing HIV1-derived peptides as building blocks through oxime chemistry. They were tested for their antigenicity and immunogenicity in mice. Antibodies elicited by three polyoximes (the octa- and tetra-oxime comb and tetraoxime tree configuration) are capable of recognizing the native envelope protein gp120 of the virus, whereas antibodies raised against the monomeric peptide and other polyoximes cannot. Our results indicate that model octameric constructions from gp120 of HIV-1 can be made in pure form in good yield using oxime chemistry but not by direct synthesis as a MAP, and that our model constructs increase the immunogenicity.

Introduction

HIV-1 is the main type of HIV virus which can cause the destruction of $CD4^+$ lymphocytes in the host resulting in the development of acquired immunodeficiency syndrome (AIDS) [1, 2]. Many efforts have been made to search for an effective AIDS vaccine over the last 15 years, some are on-going, and for example, live, attenuated viruses and DNA vaccines are being tested in animal models. But big challenges still face this area of laboratory and clinical research.

Synthetic peptides are known to act as immunogens [3-6]. By mimicking certain epitope residues (419-439) of gp120, the envelope glycoprotein spiked on the virion surface which mediates HIV entry into cells, a peptide might induce an antibody that can be expected to disrupt the entry of HIV, thus interfering with its pathogenesis [7-12]. However, antibodies induced by the peptide monomer were found not to recognize the native conformation of gp120. This may be because of the influence of protein conformation [7] and because of its lack of high molecular mass that is necessary for enhanced immunogenicity. One way to solve these problems is to cross-link antigenic peptides to a synthetic core in a branched manner as Tam [13] described, whereby multiple copies of antigenic peptides are amide-linked to a lysine based core to create a multiple antigen peptide (MAP) system. An octamer made using this strategy and employing epitope residues (419-439) of gp120 was able to induce the desired neutralizing antibodies, where the octamer was also referred to as Multiple Chain Peptides (MCP) [7]. Unfortunately, the preparation was inhomogeneous and unpurifiable, being the result of over a hundred coupling reactions.

Over the years, site-specific conjugation, a chemical approach of ligating antigenic peptides to a protein or to a synthetic carrier, has been developed as a means of creating artificial proteins. One such method employs oxime chemistry

that permits the mild and effective modification of proteins without damaging their intricate structure [14]. In this way, multiple copies of pre-pruified immunogenic peptides can be linked to a polylysine core in pure form in good yield, thus providing homogeneous materials for clinic use.

In this article, we describe the construction a series of tetra- and octamers of either radial or comb design to investigate the effect of peptide multiplicity on immunogenicity using a model peptide from the envelope protein gp120 of HIV type-1.

Materials and Methods

1. Materials

Unless otherwise specified, all solvents and reagents were obtained from Fluka, Buchs, Switzerland, were of analytical or higher grade and were used without further purification. All amino acids were purchased from Peptide Institute Inc., Japan. The resins were from Applied Biosystems, USA; Novabiochem, Switzerland, or Bachem, Switzerland. Water was repurified using a Milli-Q system (Millipore, Inc.).

2. RP-HPLC

Analytical RP-HPLC was performed using a column 250 x 4 mm i.d. packed with Nucleosil 300-A 5 μm C_8 particles at a flow rate of 0.6 ml/min and effluent was monitored at 214nm. Semi-preparative peptide purification was carried out using a C_8 column (250 x 10 mm i.d. Nucleosil 300-A 5 μm particle size) at a flow rate of 4 ml/min monitoring at 214 nm, and preparative purification using a C_{18} column (210 x 25 mm i.d. Novapak 6 μm particle size) at 20 ml/min monitoring at 229 nm. Solvents used in RP-HPLC were as follows: A, 0.1% TFA (1 g TFA in 1 litre H_2O); B, 0.1% TFA in 90% acetonitrile (1 g TFA mixed with 100 ml H_2O and then brought to1 litre with acetonitrile). Generally, the conditions used in analytical work were 5 min isocratic at 100% A followed by a linear gradient 2% B/min to 100% B, and in preparative work a shallower linear gradient (usually 0.5% B/min) was used. Components were collected manually at the detector exit, partially evaporated at room temperature, frozen and then recovered by lyophilization.

3. Mass Spectrometry

Electrospray ionization mass spectrometry (ESI-MS) was performed in positive ion mode on a Trio 2000 machine or a Platform II instrument (both from Micromass, Manchester, England). Samples were introduced either at 2 μl/min (Trio) in an acidified solvent acetonitrile/water/AcOH (49.5 : 49.5 : 1) or at 10 μl/min (Platform) in solvent acetonitrile/water/formic acid (49.9 : 49.9 : 0.2). MALDI-TOF mass spectrometry was performed in linear mode using sinapinic acid as matrix on a Voyager Elite machine (Perkin Elmer) equipped with delayed extraction. External calibration was performed on the electrospray machines using a solution of horse heart apomyoglobin, and on the MALDI-TOF machine using the mixture of peptides supplied by the manufacturer.

4. Synthesis of linear peptides

The antigenic peptide sequences from gp120 envelope protein of HIV-1 [12] appear in Table 1.

Italic type denotes linker residues not present in the corresponding protein sequences. Side-chain protection of Boc-protected amino acid on HIV-1 peptide was Arg(Tos), Asn(xan), Trp(CHO), Glu(OcHx), Tyr(BrZ), Lys(2-ClZ), C-terminal Lys(Fmoc), C-terminal Cys(4-MeBzl). All the syntheses were performed using Boc chemistry on 0.4 mmol of methylbenzhydrylamine resin (MBHA resin, Perkin Elmer ABI division) using an automated solid-phase synthesizer (model ABI 430A, Applied Biosystems Inc.). For peptides 1, 3 and 4, the Fmoc group at the C-terminal Lys was removed with 20% piperidine in DMF. Boc-aminooxyacetyl (AOA) was attached to the Lys residue with an activated mixture solution (Boc-AOA-OSu, 5-fold excess over the substitution of the resin in 5 ml DMSO, 1 ml N-methylmorpholine) to form aminooxyacetyl HIV1-derived peptide. On peptides 3 and 4, Boc-AOA group was attached to the C-terminal as above. The resin and all other side-protection groups were cleaved with HF (0°C for 1 h in the

presence of 5% p-cresol). The peptides were precipitated with cold ether, extracted with 50% acetonitrile, filtered and lyophilized. The crude peptides were purified by preparative HPLC and characterized by ESI-MS.

5. Synthesis of core structures

The core structures used in the project are shown in Table 2.

The synthesis of cores 1 and 2 were started with Fmoc-Lys(Boc) and Fmoc-Lys(Mtt), respectively, on 0.2 mmol MBHA resin (substitution, 0.63 mmol/g). On core 1, two cycles of Fmoc deprotection (20% piperidine in DMF) and couplings (TBTU/HOBt/DIEA activation) with Fmoc-Lys(Fmoc) were then performed to provide the polylysine core structure. The side-chain protection of C-terminal Lys was removed by TFA, and a Boc-AOA group was attached to the Lys residue as above. On core 2, Boc-Ser(Bzl) was attached as above to the free amino group formed upon deprotection of the Fmoc group of Fmoc-Lys(Mtt) residue on the resin. The protecting group Mtt was removed with 1%TFA in DCM, followed by a coupling of a succinic group. This group was activated by CDI (carbonyl diimidazole) solution, and a PEG-based diamine $NH_2CH_2CH_2CH_2(OCH_2CH_2)_3CH_2NH_2$ was attached with a solution consisting of 4 ml 'PEG' in 2 ml DMF and 2 ml HOBt (0.5 M). Then two cycles of Fmoc-Lys(Fmoc) were coupled to the free amino group of the PEG to form the polylysine core as above. Finally, Fmoc protection was removed from the branches of the cores 1 and 2, and bromoacetyl groups were attached to the branched core with an activated mixture solution (2 mmol of bromoacetic acid in 2 ml DCM and 1 mmol of DIC).

The syntheses of cores 3 and 4 were performed manually on 0.2 mmol Boc-βAla-PAM resin (substitution 0.88 mmol/g) using Boc-chemistry. Three cycles of Boc deprotection and couplings of Boc-Lys(Boc) formed the polylysine core. At this stage, three cycles of Boc-deprotection and couplings of Boc-Gly were performed

on core 4. Boc-Ser(Bzl) was then attached to both branched cores with an activated mixture solution (8 mmol Boc-Ser(Bzl)-OSu in 8 ml DMSO and 2 ml N-methylmorpholine).

Cores 5 and 6 were synthesized manually on 0.2 mmol Boc-Gly-PAM resin. After Boc deprotection, 7 and 3 cycles, respectively, of coupling with Fmoc-Lys(Boc) and deprotection were performed by Fmoc-chemistry to form the linear polylysine core, followed by two cycles of Fmoc-Gly. A Boc-Gly was attached after Fmoc deprotection of the last cycle. The side chain protection of Lys core and protection of N-terminal Gly were removed with TFA, and Boc-Ser(Bzl) was attached as for core 3.

Core 7 was synthesized similarly to core 5 on 0.2 mmol Boc-Gly-PAM resin, but Fmoc-Lys(Mtt) instead of Fmoc-Lys(Boc) was used in the 4^{th} cycle. After the coupling cycle of Boc-Gly, the Mtt group was removed with 1% TFA in DCM through the reaction column and the removal was spectrophotometrically monitored at 470 nm, followed by attachment of Fmoc-Ser(Bzl)-OH to the side chain of 4^{th} Lys by using Fmoc chemistry. Boc deprotection was performed on the side chain of the rest of the Lys residues and on the N-terminal Gly, and then the amino groups were acetylated for 1 hour with acetic anhydride (14 mmol) and DIEA (28 mmol). Fmoc group on the Ser residue was removed prior to final cleavage and deprotection.

All of the core peptides were cleaved from the resin with HF, purified by preparative HPLC and characterized by ESI-MS.

6. Alkylation of HIV peptide by cores 1 and 2

To 15 mg of C-terminal Cys HIV peptide was added 50 µl of acetonitrile and 100 µl of water (peptide not fully soluble). Fresh solutions of cores 1 and 2,

respectively, were prepared (0.9 mM in 0.1M sodium phosphate buffer, pH 7.0). Then the alkylation was started by adding 1ml of core 1 or 2 to the HIV peptide suspension: the peptide dissolved completely. After 40 min, the product was purified on semi-preparative RP-HPLC, lyophilized, and characterized.

7. General Procedure of Periodate Oxidation

Cores 2-7 underwent periodate oxidation. In general, 3.75 μmol (based on Ser content) of core was dissolved in 23.5 ml imidazole buffer (50 mM, pH 6.95, chloride counter ion) and 1.3 ml of methionine (200 mM) was added. Then 625 μl of $NaIO_4$ (0.2 M in water) was added to start oxidation. The reaction was allowed to proceed for 5 min and was then quenched by adding 500 μl of ethylene glycol (500 mM in water). The product was purified on preparative HPLC and characterized by mass spectrometry.

8. Oximation

All of the aldehydes generated from oxidation were reacted with corresponding aminooxyacetyl peptides to form an oxime bond (see Figure 1).

On Figure 2, for tetraoxime tree/comb and octaoxime tree/comb/I/II solutions of the aminooxyacetyl peptide (4 mM in NaOAc buffer) and the oxidized core (1 mM in NaOAc buffer) were prepared. Oxime formation was initiated by mixing 300 μl of core (0.3 μmol core, 2.4 μmol aldehyde groups) with 1.2 ml of peptide (4.8 μmol) derivative. After 24 h at room temperature, the product was isolated by semi-preparative RP-HPLC and characterized by mass spectrometry.

For '4+4' oxime, aminooxyacetyl core 1 (0.2 μmol) with attached HIV-peptide was dissolved in 40 μl of acetonitrile and 160 μl of NaOAc buffer (acetic acid 0.57 ml in 100 ml of water, sodium acetate 0.82 g dissolved in 100 ml of water, mixed

part of both until pH 4.0). An equal amount of oxidized core 2 with HIV-peptide attached was dissolved in 19 μl acetonitrile and 77 μl of NaOAc buffer. Both solutions were mixed, followed by addition of acetic acid to a final concentration of 4%. After 24-h reaction at room temperature, the product was purified by semi-preparative RP-HPLC and characterized by mass spectrometry.

9. Bioassay

4 BALB/c mice were immunized in each case with 50 μg of multimer peptides above emulsified in Complete Freund's Adjuvant (CFA). Animals were boosted 3 – 4 times at average 3 weeks intervals (days as indicated in Tables) with 25 μg of the same peptides emulsified with Incomplete Freund's Adjuvant (IFA). Blood was collected 1 week after each boost for antibody titer determination, and the antibody titers reported here were the mean value of results from 4 antisera determined by ELISA assay.

Results and Discussion

A convenient approach to the synthesis of peptide multimers involves oxime chemistry. Oxime formation requires an aminoxyacetyl group and a ketone or an aldehyde. The latter is easily generated from N-terminal or side chain-linked Ser, Thr, or carbohydrates [15-17], and can then react with the aminooxyacetyl-containing species. Oxime chemistry was thus introduced in the study to assist design and synthesis of multimeric immunogens. Pre-purified polypeptides possessing, say, an aminooxyacetyl group are condensed with a core molecule that carries multiple copies of, say, an aldehyde group, although of course the polarity may be inverted: aldehyde peptides condensed with an aminooxyacetyl core [16].

1. Synthesis of aminooxyacetyl-containing peptides

The aminooxyacetyl (NH_2OCH_2CO- or AOA-) peptides we used in this study are synthetic antigenic peptides, either from envelope glycoprotein gp120 of HIV-1 or from Influenza hemagglutinin protein. The AOA group exists in commercial aminooxyacetic acid that was protected with Boc group then reacted with dicyclohexycarbodiimide (DCC) and N-hydroxysuccinimide to form Boc-AOA-OSu, which is a convenient acylation reagent.

2. Generation of carbonyl groups (oxidation)

Oxidation by periodate of 1,2-aminoalcohol (Ser, Thr), or of the 1,2-diols of carbohydrate groups of glycoproteins is the most convenient method by which a carbonyl group can be introduced into an unprotected peptide or protein [18]. Such an oxidation rapidly (< 5 min) generates at pH 7.0 an aldehyde, which can then react with a 1,2-aminothiol to form a thiazolidine [18] or aminooxyacetyl group to form an oxime [16]. Methionine was used for protection against

sulphoxide formation. After oxidation of N-terminal Ser, product was isolated by HPLC. For example, the peak arrowed in Figure 4 was identified by retention time and mass spectrum as the aldehyde of core-3.

3. Multimeric constructions employing HIV1-derived peptides as building blocks

Oxime bond formation involves the specific reaction between an aminooxy group and a carbonyl group (aldehyde or ketone) and expulsion of a water molecule. In water, the oximation is very slow at neutral pH but proceeds close to completion at acidic pH over a period of hours [14]. Several oxime constructs that possess multiple copies of a single oligopeptide were designed and conjugated in this way. Using oxime chemistry, or a combination of oxime and thioether chemistry, it was possible to assemble multimeric constructs that possessed four or eight copies of the HIV1-derived peptide described by Kelker et al [7]. Because the peptide subunit was purified and characterized prior to assembly, it was possible to characterize products by mass spectrometry. The structures of the multimeric constructs are shown in Figure 2.

The first construct (tetraoxime tree, Figure 2) was built on a radial polylysine core containing a Ser residue at the tip of each of 4 branches. These Ser residues were periodate-oxidized and then reacted with HIV1-derived peptide in aminooxy form to yield a tetramerized product (tetraoxime). HPLC and ESI-MS analysis revealed the desired product (Figure 5): Mr found 12059, Mr theoretical 12059. It was stable at acidic and neutral pH over 24 h.

Octaxime tree (see Figure 2) was formed in a similar way to the tetraoxime tree, but possessed 8 copies of HIV1-derived peptide connected to the lysine core through their C-termini. Thus Octaxime tree is comparable in structure with the MAP described by Kelker, but, in contrast with the MAP, Octaxime tree can be characterized by mass spectrometry (Figure 7). Relative molecular mass found

24156, theoretical 24157. Some evidence was found of components with higher masses [+73, +156, +(156+73)]. These components, which coeluted on HPLC with the desired product, were probably due to acylation of the Boc-$NHOCH_2CO$-nitrogen with a second Boc-$NHOCH_2CO$- group during synthesis of the HIV1 peptide (calculation, +73 for NH_2OCH_2CO replacing H) and of incomplete removal of the tosyl group (calculation, +154). We now use less strong acylation conditions (1 equivalent instead of five) and thereby avoid the +73 component.

Tetraoxime comb (see Figure 2) was assembled on a linear polylysine core by conjugating HIV1-derived peptides at the tip of each of 3 side chains and the N-terminus using periodate-oxidation and oximation in a similar manner to the tetraoxime tree (characterization, see Figure 6). Evidence of components possessing extra -$NHOCH_2CO$- groups (+ 73 amu) was obtained.

Octaxime comb (see Figure 2) was formed in a similar way to the tetraoxime comb, but possessed 8 copies of HIV1-derived peptide connected to the lysine core through their C-termini (characterization, see Figure 8). Evidence of components possessing extra -$NHOCH_2CO$- groups (+ 73 amu) was obtained.

Octaoxime I and Octaoxime II (see Figure 2) were formed in a similar way to the octaoxime tree with some differences. In octaoxime II, the oxime bonds were in between a three-Glycine spacer and the N-terminal residue, Arginine, of the HIV1-derived peptide, in contrast with octaoxime tree in which oxime bonds were in between polylysine residues and a three-Glycine spacer. In octaoxime I, the oxime bonds were in the same position as octaoxime tree, but the HIV1-derived peptide was conjugated in a reverse way to the both alpha and epsilon position of Lys residues of the core in comparison with octaoxime tree. HPLC and MALDI analysis revealed the desired product (Figure 9, 10). Relative molecular mass of octaoxime I: found 23174, theoretical 23138. The found mass was +36 higher in this product than that expected. Besides main products, some minor signals were present in the MALDI mass spectrum which may be due to loss of 1 or more

conjugated peptides: it is known that oxime bonds are susceptible to cleavage during MALDI analysis [19]. Relative molecular mass of octaoxime II: found, 24600, theoretical 24543. The mass of found compound was +57 higher than the desired. Signals corresponding to loss of 1 or more conjugated peptides were also present.

Preliminary biological results (immunogenicity assays performed in mice at New York University by Kelker) showed that the tetraoxime, unlike the tetraMAP of the previous study, was capable of inducing the desired antibody response: antiserum recognized native gp-120 but not denatured gp-120, and neutralized viral infectivity. However, the titres were lower than those obtained in the previous study (data not shown) with the uncharacterizable octaMAP. This led us to attempt to connect two copies of a tetramer through a short linker, our '4+4' construct (see Figure 2), while awaiting bioassay of the tetraoxime comb, octaoximes tree and comb, etc.

The two tetramers of the '4 + 4' construction were linked by an oxime bond. Each tetramer was a radial polylysine core possessing a copy of HIV1-derived peptide at the tip of each of 4 branches, which was conjugated through a thioether bond arising from a specific reaction between bromoacetyl and thiol groups. One of the tetramers had a Ser residue on the core and this was oxidized by periodate to generate an aldehyde, which was then reacted with an aminooxy group on the core of the other tetramer to form the oxime-linked '4 + 4' construct. The oximation in this case proceeded essentially to completion and the product was characterized by HPLC and ESI-MS (Figure 11). The relative molecular mass found was 23871, close to the calculated mass 23876. No evidence of an extra $NHOCH_2CO$ group (+73 amu) was found, which is not surprising since the 8 copies of HIV-1 peptide employed had not undergone acylation with Boc-AOA-OSu.

A control oxime was also constructed which consisted of a linear polylysine core but only a HIV-derived peptide conjugated. The relative molecular mass found was 4334, similar to the calculated mass 4334. Evidence of components possessing extra -$NHOCH_2CO$- groups (+ 73 amu) was obtained

3. Bioassay

In our previous study, an octamer MAP (also refered as to MCP), when injected into mice, was able to induce high titers of antibodies against native gp120 of HIV type 1 (Table 3). This MCP, however, cannot be purified and characterized precisely due to unavoidable synthesis failure, and thus cannot be envisaged for clinical use. In this study, we adopted site-specific conjugation using oxime chemistry to synthesize a series of tetramers or octamers that were comparable to MCP in structures. Analyses of these octamers showed they were purifiable and characterizable. And they were then injected into mice for the assessment of immunogenicity.

ELISA assays (Table 4) revealed that 2 of these multimers: octaoxime comb and tetraoxime comb, can induce high titers of antibodies recognizing native gp120 although the titers were 2 fold less than that from MCP at 107 days after injection. Another multimer, tetraoxime tree design can also elicit antibodies against native gp120 but the titers were lower, 12 fold less than that from MCP and 6 fold less than that from octaoxime comb and tetraoxime designs, the latter is, however, different in another experiment (Table 6). This may be due to different types of gp120 protein (type LAV and MN) used in the two different experiments. The other multimer designs: '4+4' oxime, octaoxime I and II, octaoxime tree design, were all demonstrated not capable of inducing correct antibodies against native gp120 (Table 5 and 6). It is not clear why the different copies of multimers and different designs elicit different antibodies against native gp120, which might be due to the conformations of these multimers. Octaoxime comb, tetraoxime comb and tetraoxime tree designs may possess a

conformation similar to the selected epitope of native gp120 and thus induce antibodies to recognize the protein. Antibodies elicited from octaoxime comb, tetraoxime tree and MCP can only recognize native gp120 and not the denatured protein whereas antibodies raised against monomer peptide mainly recognize denatured gp120 (Table 7), which further confirmed these multimers may display a similar conformation to the epitope of native gp120. Since these multimers are comparable in covalent structures and immunological properties to the MCP but in contrast are purifiable and characterizable, they are promising candidates for AIDS vaccine development.

Conclusion

Model octameric constructions employing HIV1-derived peptides as building blocks can be made in pure form in good yield using oxime chemistry but not by direct synthesis (MAP) nor by thioalkylation. Antibodies elicited by three polyoximes of the 419-439 sequence: tetraoxime tree- and comb-design, and octaoxime comb-design, are capable of neutralising the virus in high titres in mice. These polyoximes are comparable to previous octamer MAP (or MCP) in covalent structures as well as in immunogenicity.

References

[1] Barre-Sinoussi F, Chermann JC, Rey F, et al. (1983) Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science* 220, 868-71

[2] Gallo RC, Salahuddin SZ, Popovic M, et al. (1984) Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. *Science* 224, 500-3

[3] Brown F (1996) Peptide vaccines: Dream or reality. *Peptides:chemistry, structure and biology* (Kaumaya PTP and Hodges R, eds.), pp. 869-71, Mayflower Scientific Ltd., Kingswinford, UK

[4] Lerner RA (1982) Tapping the immunological repertoire to produce antibodies of predetermined specificity. *Nature* (London) 299, 593-6

[5] Bittle JL, Houghten RA, Alexander H, et al. (1982) Protection against foot-and-mouth disease by immunization with a chemically synthesized peptide predicted from the viral nucleotide sequence. *Nature* (London) 298, 30-3

[6] DiMarchi R, Brooke G, Gale C, et al. (1986) Protection of cattle against foot-and-mouth disease by a synthetic peptide. *Science* 232, 639-41

[7] Kelker HC, Schlesinger D and Valentine FT (1994) Immunogenic and antigenic properties of an HIV-1 gp120-derived multiple chain peptide. *J Immun* 152, 4139-48

[8] Kwong PD, Wyatt R, Robinson J, et al. (1998) Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. *Nature* 393, 648-59

[9] Wyatt R, Kwong PD, Desjardins E, et al. (1998) The antigenic structure of the HIV gp120 envelope glycoprotein. *Nature* 393, 705-11

[10] Lasky LA, Nakamura G, Smith DH, et al. (1987) Delineation of a region of the human immunodeficiency virus type 1 gp120 glycoprotein critical for interaction with the CD4 receptor. *Cell* 50, 975-85

[11] Olshevsky U, Helseth E, Furman C, et al. (1990) Identification of individual human immunodeficiency virus type 1 gp120 amino acids important for CD4 receptor binding. *J Virol* 64, 5701-7

[12] Cordonnier A, Riviere Y, Montagnier L, et al. (1989) Effects of mutations in hyperconserved regions of the extracellular glycoprotein of human immunodeficiency virus type 1 on receptor binding. *J Virol* 63, 4464-8

[13] Tam JP (1988) Synthetic peptide vaccine design: synthesis and properties of a high-density multiple antigenic peptide system. *Proc Natl Acad Sci USA* 85, 5409-13

[14] Rose K, Zeng W, Regamey P.O., et al. (1996) Natural peptides as building blocks for the synthesis of large protein-like molecules with hydrazone and oxime linkages. *Bioconj Chem* 7, 552-6

[15] Geoghegan KF and Stroh JG (1992) Site-directed conjugation of nonpeptide groups to peptides and proteins via periodate oxidation of a 2-amino alcohol. Application to modification at N-terminal serine. *Bioconjug Chem* 3, 138-46

[16] Rose K (1994) Facile synthesis of artificial proteins *J Am Chem Soc* 116, 30-3

[17] Schwartz A, Wandrey C, Bayer EA, et al. (1990) *Methods Enzymol* 184,160-2

[18] Tam JP and Spetzler JC (1995) Chemoselective approaches to the preparation of peptide dendrimers and branched artificial proteins using unprotected peptides as building blocks. *Biomed Pept Proteins Nucleic Acids* 1, 123-32

[19] Nardin EH, Calvo-Calle JM, Oliveira GA (1998) Plasmodium falciparum polyoximes: highly immunogenic synthetic vaccines constructed by chemoselective ligation of repeat B-cell epitopes and a universal T-cell epitope of CS protein *Vaccine* 16(6), 590-600

Tables and Figures

Table 1: Synthetic peptide sequences from residues 419-439 of gp120.

Table 2: Core structures used in the study.

Table 3. MCP with eight peptide chains elicits antibodies recognizing envelope glycoprotein.

Table 4. Tetraoxime tree and comb design or octaoxime comb design elicit antibodies recognizing gp120.

Table 5. MCP but not "4+4" oxime nor octaoxime I nor octaoxime II elicit antibodies recognizing gp120.
C57Bl/10 mice were immunized with 25 μg of peptide constructs listed in Table, emusified in CFA. Mice were boosted 3 times (at 3 week intervals) with peptide emulsified in IFA. Mice were bled at ~2 weeks after the boosts and the antibody titers were determined by ELISA with antigens listed in Table.

Table 6. Tetraoxime tree and octaoxime comb elicit high titers of antibodies recognizing gp120

Table 7. Polyoxime constructs elicit antibodies that recognize conformational determinant(s) in gp120.
Antisera to the antigens listed were raised in BALB/c mice and their recognition of the "native" or denatured gp120 was determined by ELISA. ELISA wells were coated with rGp120 MN at 1 μg/ml in PBS either: maintained at 4°C ("native" control), or denatured by heating at 95°C for 5 minutes with 1 mM DTT, 0.01% SDS.

Figure 1. A general scheme for site-specific modification of peptides and proteins through orthogonal coupling of an aminooxyacetyl group and an aldehyde to form an oxime. (a) oxidation of N-terminal Ser (R=H) or Thr ($R=CH_3$) with periodate. (b) reaction of an aminooxy compound with an aldehyde to form an oxime.

Figure 2. Structure schemes representing polyoxime (radial- or comb-branched) using a model of peptide, RIKQIINMWQEVGKAMYAPPI, from HIV-1 gp120. They are modified as seen in Table 1: ■ is peptide 1; ▨ is peptide 2; ▥ is peptide 3; ▩ is peptide 4. The branching Lys residues of the cores are acylated on both alpha and epsilon amino groups with corresponding peptides and are shown unconventionally with their carboxyl groups to the left for Tetraoxime tree and Octaoxime tree / I / II, and also for '4+4' oxime. The

structure of the C-terminal Cys peptide in '4+4' oxime has been simplified after alkylation. Figure 2A shows a control-oxime for polyoxime.

Figure 3. Structure of the control-oxime (comb-branched) using peptide RIKQIINMWQEVGKAMYAPPI from HIV-1 gp120. The peptide is modified as seen in Figure 2. Only the side chain of Lysine in the middle site has the peptide attached. The N-terminal of Glycine and the side chain of 6 other Lysine residues are blocked with $-COCH_3$.

Figure 4. (A) RP-HPLC analysis of periodate oxidation of core-3 to yield a radial-branched octa-aldehyde; (B) ESI Mass spectrum of the octaaldehyde (calculated 1434.5, found: 1434.1, 1452.1 and 1470.3. The masses 1452.1 and 1470.3 are the octaaldehyde plus 1 and 2 water molecules, respectively).

Figure 5. (A) RP-HPLC analysis of the Tetraoxime tree (see Figure 2 for structure); (B) ESI mass spectrum of the Tetraoxime tree (calculated Mr, 12059, found, 12059).

Figure 6. (A) RP-HPLC analysis of the Tetraoxime comb (see Figure 2 for structure); (B) ESI mass spectrum of the Tetraoxime comb (calculated Mr, 12216, found, 12218).

Figure 7. (A) RP-HPLC analysis of the Octaoxime tree (constructed on radial core), see Figure 2 for structure; (B) ESI mass spectrum of the Octaoxime tree (calculated Mr, 24157, found, 24156).

Figure 8. (A) RP-HPLC analysis of the Octaoxime comb (constructed on linear core), see Figure 2 for structure; (B) ESI mass spectrum of the Octaoxime comb (calculated Mr, 24314, found, 24313).

Figure 9. (A) RP-HPLC analysis of the Octaoxime I (constructed on radial core), see Figure 2 for structure; (B) MALDI mass spectrum of the Octaoxime I (calculated Mr, 23138, found, 23174).

Figure 10. (A) RP-HPLC analysis of the Octaoxime II (constructed on radial core), see Figure 2 for structure; (B) MALDI mass spectrum of the Octaoxime II (calculated Mr, 24543, found, 24600).

Figure 11. (A) RP-HPLC analysis of the '4 + 4' octaoxime (see Figure 2 for structure); (B) ESI mass spectrum of the '4+4' oxime (calculated Mr, 23876, found, 23871).

Figure 12. (A) RP-HPLC analysis of the control oxime (see Figure 3 for structure); (B) ESI mass spectrum of the control oxime (calculated Mr, 4334, found, 4334).

Table 1: Synthetic peptide sequences from residues 419-439 of gp120

Peptide 1	RIKQIINMWQEVGKAMYAPPI*GGGK*(*$COCH_2ONH_2$*)-amide
Peptide 2	RIKQIINMWQEVGKAMYAPPI*GGGC*-amide
Peptide 3	IPPAYMAKGVEQWMNIIQKIRGGG-*$COCH_2ONH_2$*
Peptide 4	RIKQIINMWQEVGKAMYAPPI-*$COCH_2ONH_2$*

Table 2: Core structures used in the project.

Core 1	$[Br\text{-}CH_2CO]_4\text{-}K_2K\text{-}K(NH_2OCH_2CO)$-amide
Core 2	S-K-amide | $[Br\text{-}CH_2CO]_4\text{-}K_2K$-PEG-succ
Core 3	$S_8K_4K_2K$-βA-OH
Core 4	$(S\text{-}GGG)_8K_4K_2K$-βA-OH
Core 5	$SG_3\text{-}[K(S)]_7$-G-OH
Core 6	$SG_3\text{-}[K(S)]_3$-G-OH
Core 7	$CH_3CO\text{-}G_3\text{-}[K(COCH_3)]_3\text{-}K(S)\text{-}[K(COCH_3)]_3$-G-OH

Table 3. MCP with eight peptide chains elicits antibodies recognizing envelope glycoprotein

Immunogen	gp120 IIIB titer
$(419\text{-}439)_8$MCP	39,000
$(419\text{-}439)_1$MCP*	<20

* Control MCP, with a single peptide chain on a polylysine core.

Table 4. Tetraoxime tree and comb design or octaoxime comb design elicit antibodies recognizing gp120

Immunogen	Sera collection day	Antigen				
		MCP	Octaoxime comb	Tetraoxime comb	Tetraoxime tree	gp120 LAV
		Titers				
MCP	29	9,200				90
	52	32,000				2,100
	72	109,000				2,400
	107	220,000				5,120
Octaoxime comb	29		12,150			28
	52		90,000			200
	72		520,000			1,800
	107		560,000			2,500
Tetraoxime comb	29			20,000		20
	52			280,000		76
	72			680,000		640
	107			108,000		2,560
Tetraoxime tree	29				32,000	20
	52				320,000	270
	72				960,000	105
	107				250,000	420

Table 5: MCP but not "4+4" oxime nor octaoxime I nor octaoxime II elicit antibodies recognizing gp120

Immunogen	Antigen					
	MCP	Monomer	Gp120	"4+4" oxime	Octaoxime I	Octaoxime II
	Titers					
MCP	125,000	4,300	2,600	ND	ND	ND
"4+4" oxime	ND	<20	<20	150	ND	ND
Octaoxime I	ND	800	20	ND	20	ND
Octaoxime II	ND	20	33	ND	ND	40

Table 6. Tetraoxime tree and octaoxime comb elicit high titers of antibodies recognizing gp120

Immunogen	Bleed Day	Antigen				
		Octaoxime I	Octaoxime tree	Octaoxime comb	Tetraoxime tree	gp120 MN
		Titers				
Octaoxime I	31	180				55
	59	8,500				58
	116	4,000				33
Octaoxime tree	31		2,100			25
	59		220,000			60
	116		328,000			125
Octaoxime comb	31			42,000		1,400
	59			220,000		2,500
	116			328,000		1,750
Tetraoxime tree	31				250,000	125
	59				250,000	2,560
	116				370,000	21,000

Table 7. Polyoximes constructs elicit antibodies that recognize conformational determinant(s) in gp120.

Antisera to	Gp120 MN	Gp120 MN denatured	% Control remaining after denaturation
	OD 405 mμ		
Octaoxime comb	1.240	0.010	0.7
Tetraoxime tree	1.770	0.090	5.5
MCP	0.990	0.026	2.6
Monomer	1.510	2.080	137.7

Figure 1

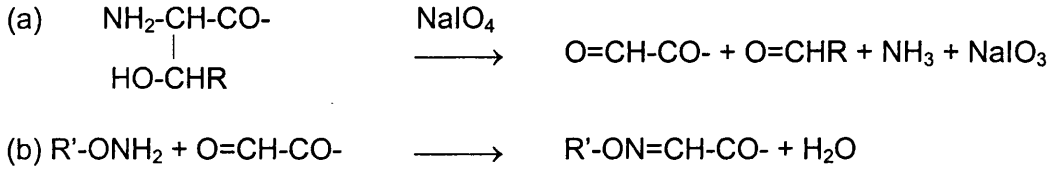

(a) NH2-CH-CO-
HO-CHR
NaIO4
O=CH-CO- + O=CHR + NH3 + NaIO3
(b) R'-ONH2 + O=CH-CO-
R'-ON=CH-CO- + H2O

Figure 2

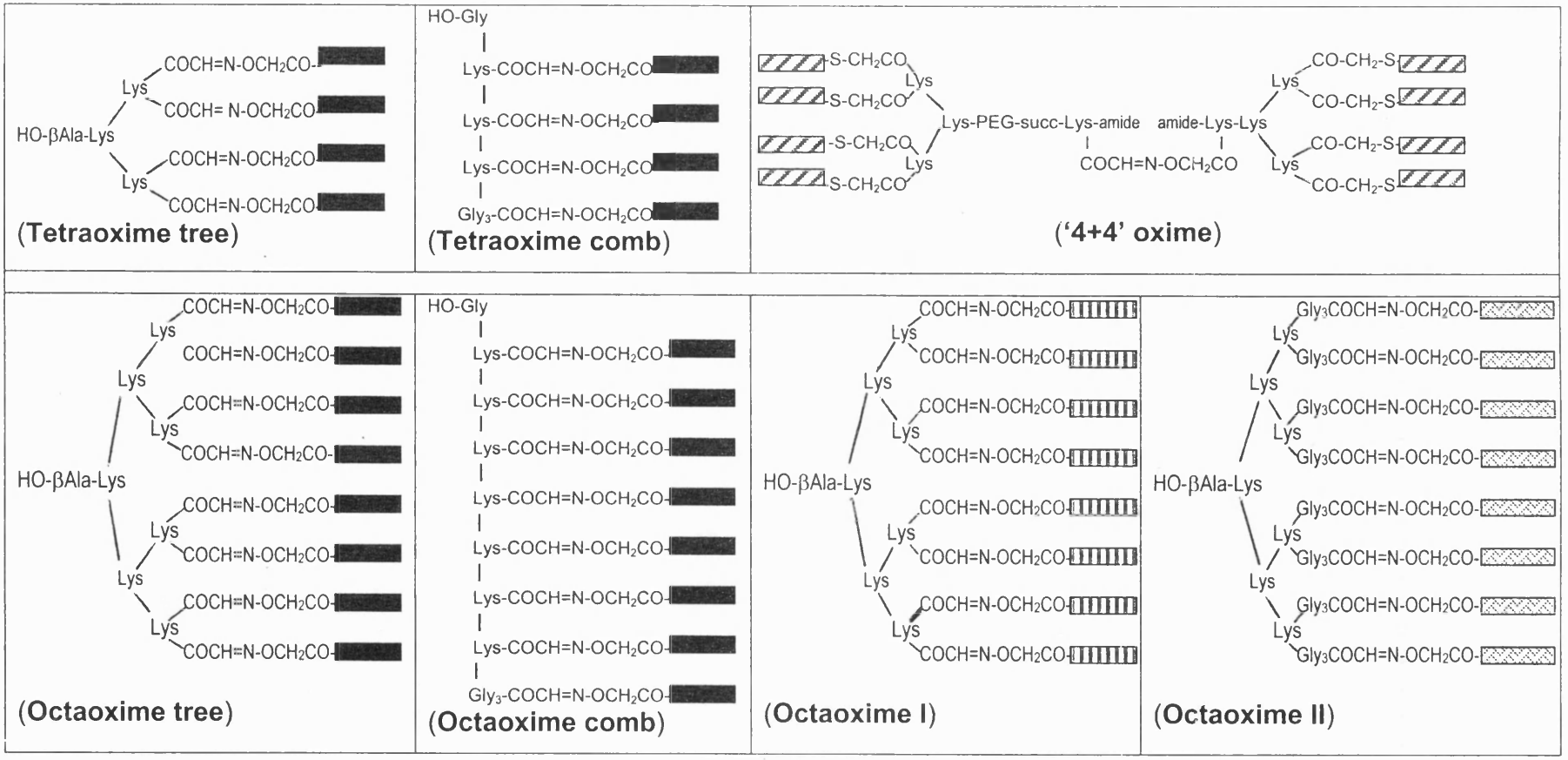
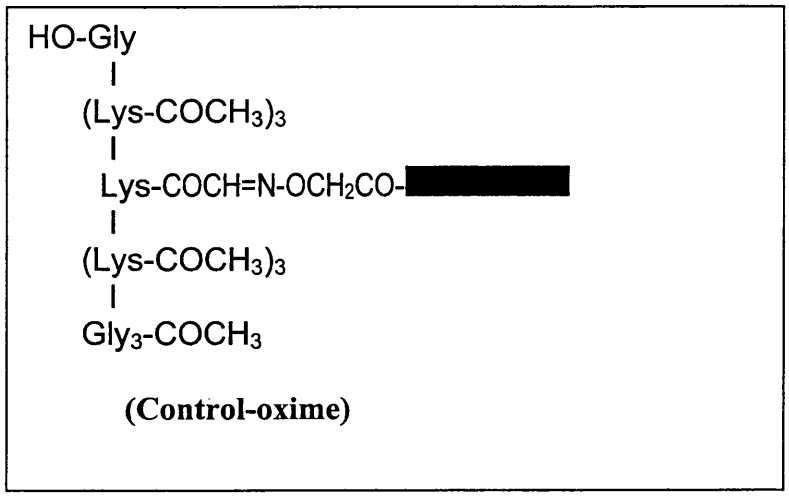

HO-βAla-Lys
Lys
COCH=N-OCH2CO-
COCH= N-OCH2CO
(Tetraoxime tree)
HO-Gly
Lys-COCH=N-OCH2CO
Gly3-COCH=N-OCH2CO
(Tetraoxime comb)
S-CH2CO
Lys-PEG-succ-Lys-amide
amide-Lys-Lys
COCH=N-OCH2CO
CO-CH2-S
('4+4' oxime)
(Octaoxime tree)
(Octaoxime comb)
(Octaoxime I)
Gly3COCH=N-OCH2CO-
(Octaoxime II)

Figure 3



(Control-oxime)

(A)

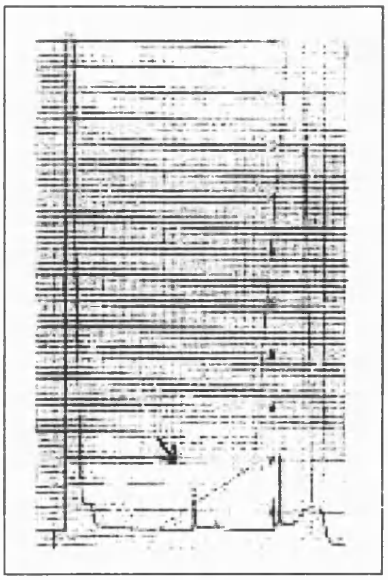


Figure 4

(B)

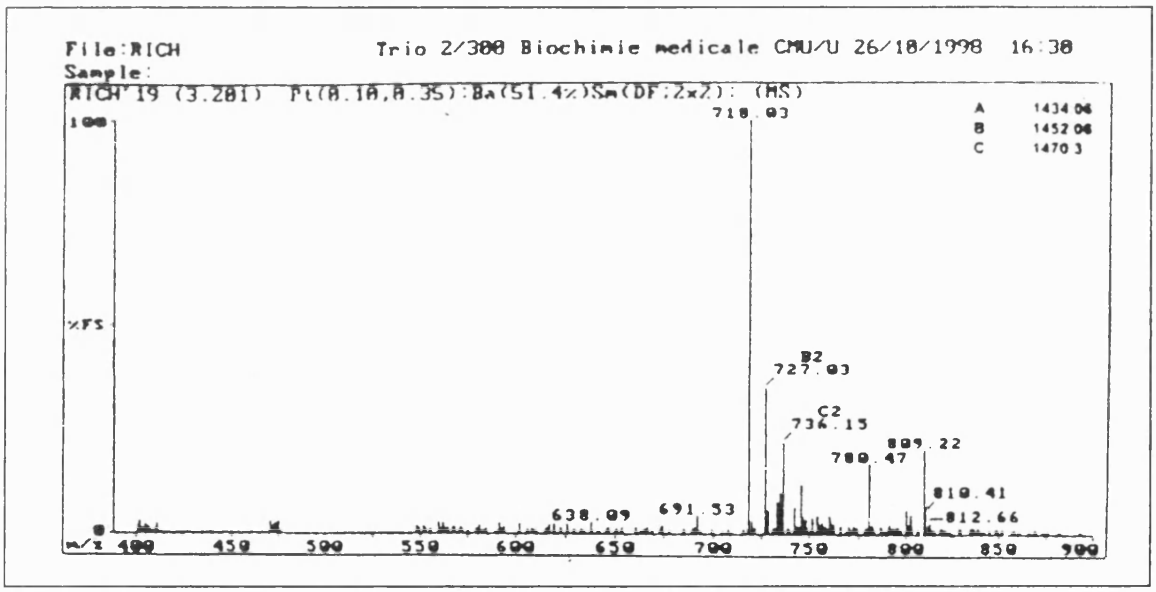

File:RICH
Trio 2/300 Biochimie medicale CMU/U 26/10/1998 16:30
Sample:
RICH'19 (3.281) Pt(0.10,0.35):Ba(51.4%)Sm(DF:2x2): (MS)
718.03
A 1434.06
B 1452.06
C 1470.3
%FS
727.03
734.15
789.47
809.22
810.41
812.66
638.09
691.53
m/z
400
450
500
550
600
650
700
750
800
850
900

Figure 5

(A) (B)

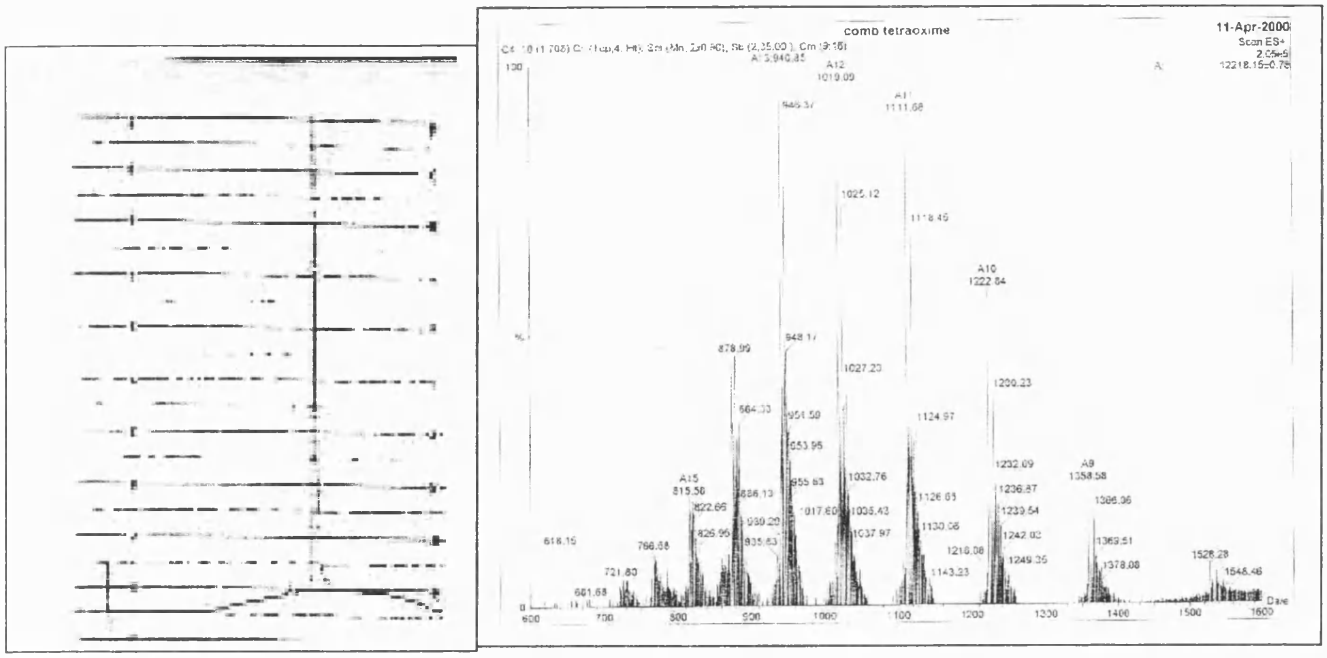


Figure 6

(A) (B)

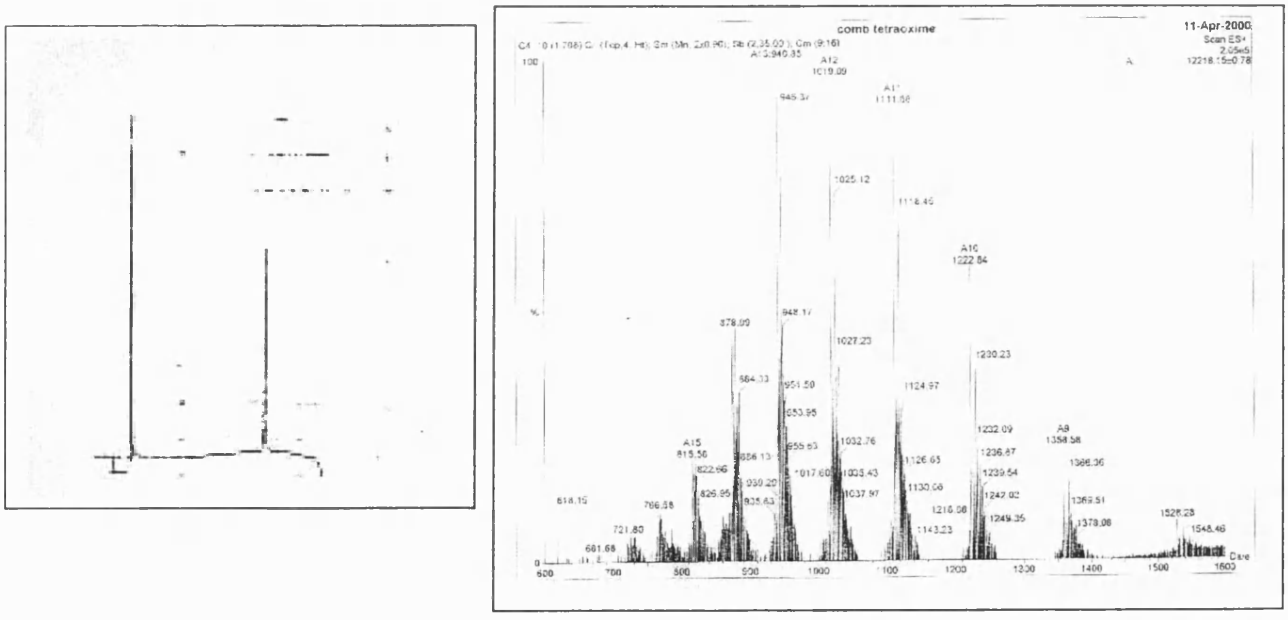
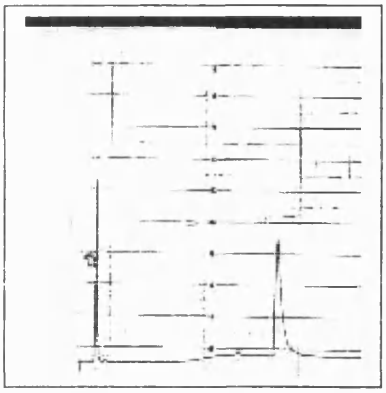


Figure 7

(A) (B)



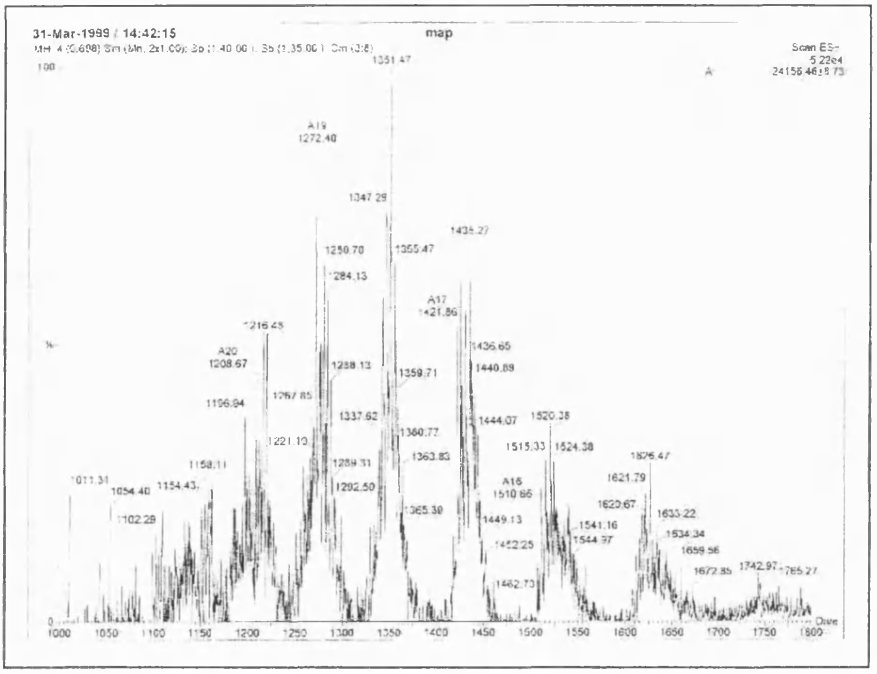
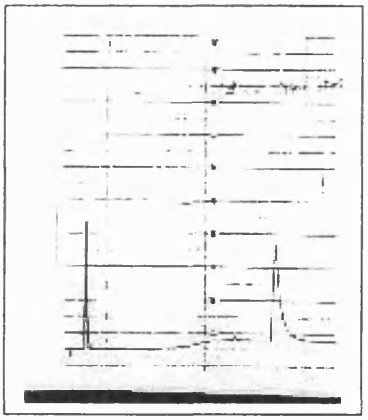


Figure 8

(A) (B)



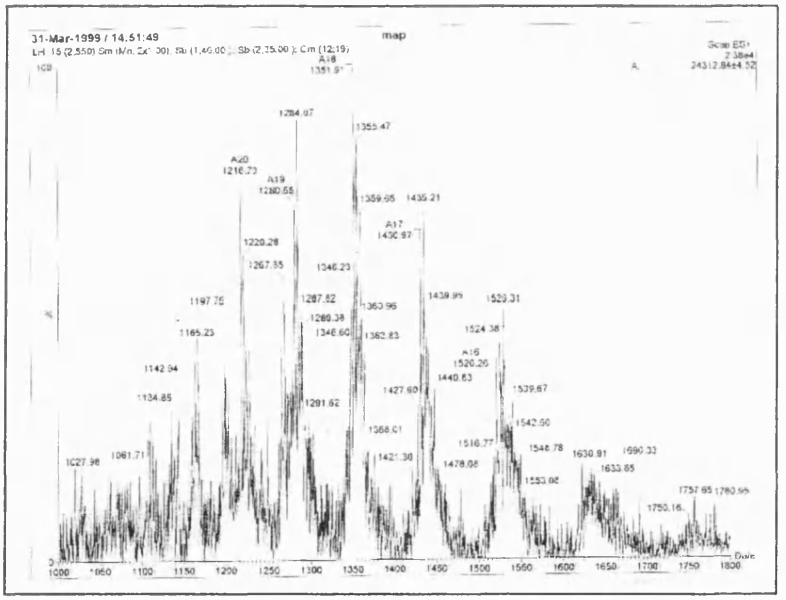
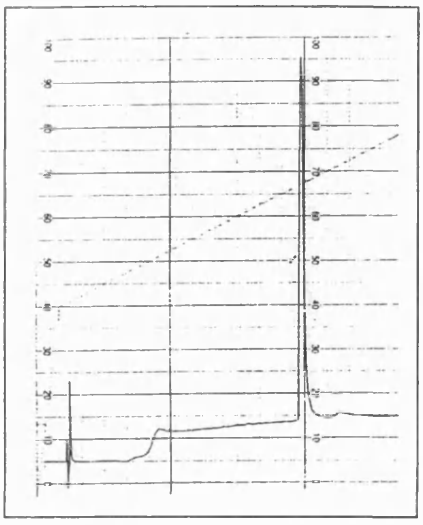


Figure 9

(A) (B)



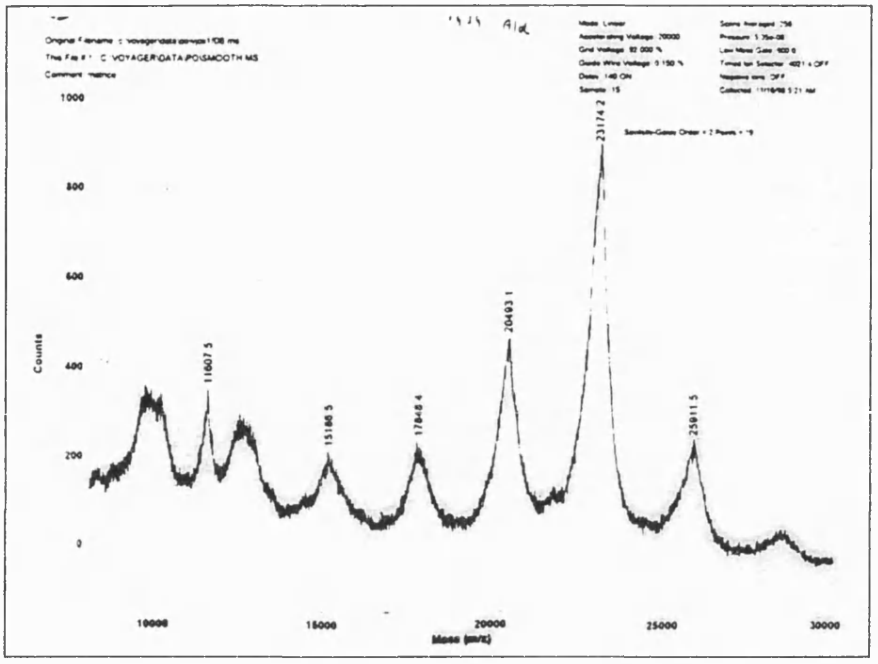
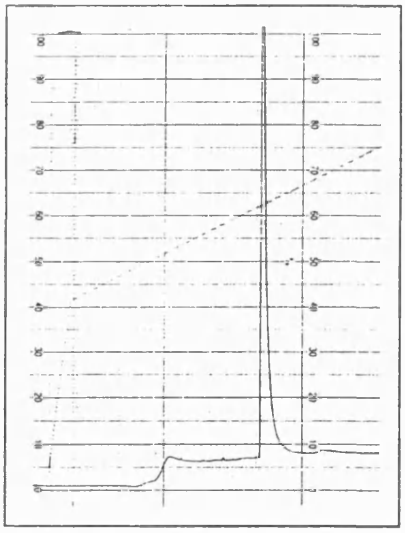


Figure 10

(A) (B)



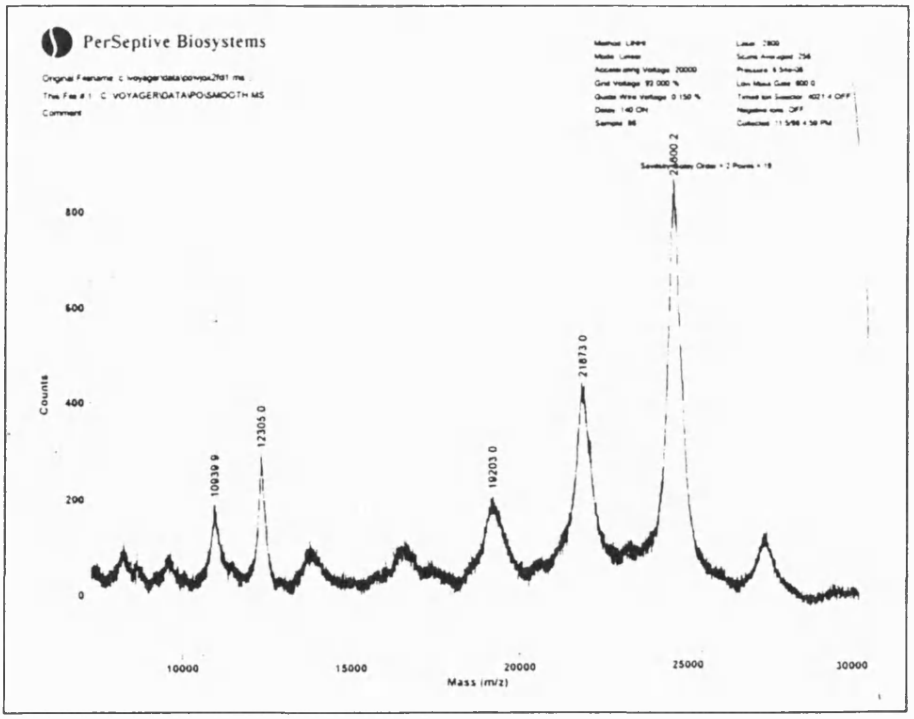


Figure 11

(A) (B)

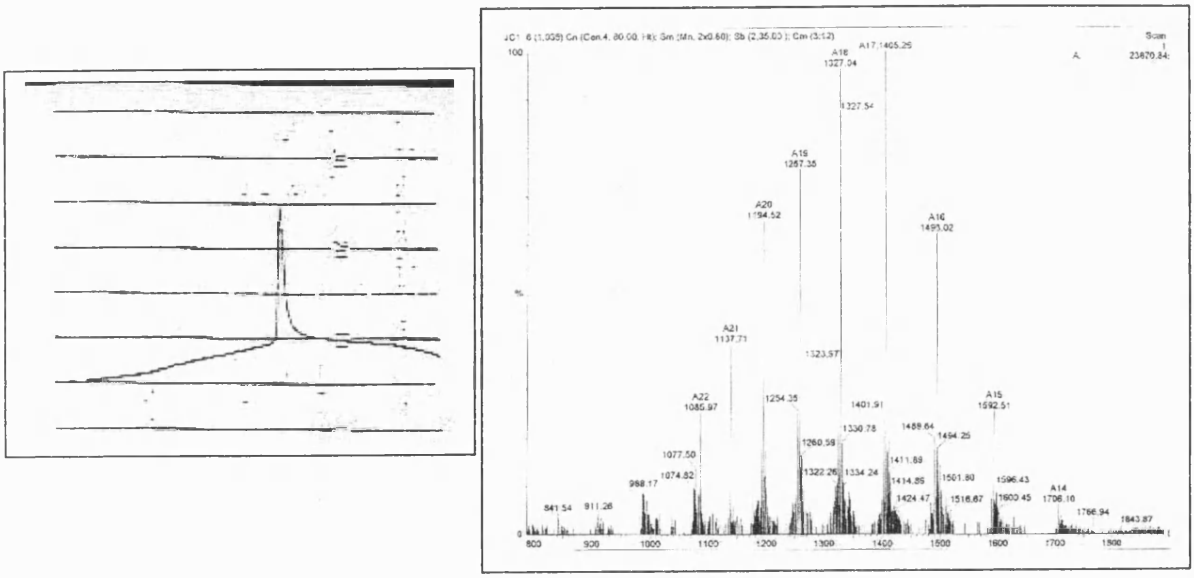
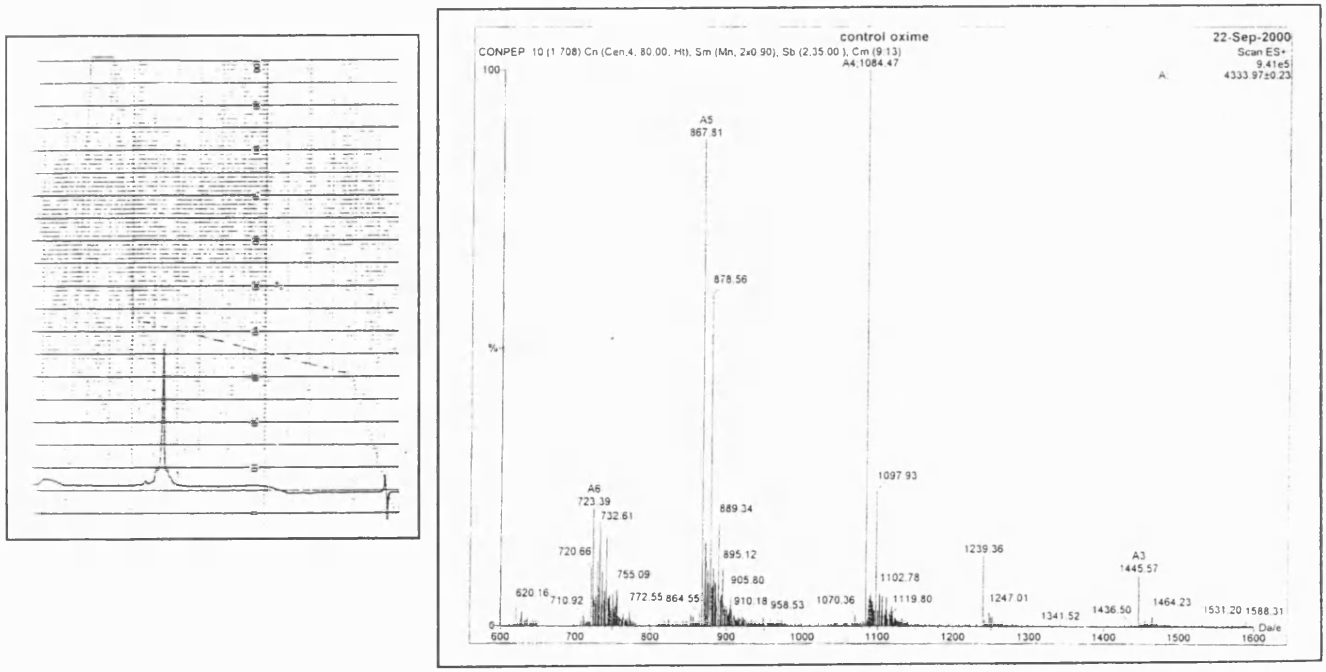


Figure 12

(A) (B)



Dissertation Equivalent C

K. Rose, J. Chen, M. Dragovic, W. Zeng, D. Jeannerat, P. Kamalaprija, U. Burger.

New cyclization reaction at the amino terminus of peptides and proteins.

Bioconjug Chem 1999 : 6(10), 1038-43

Jianhua Chen performed the experimental work involving cholera toxin B subunit and its conjugates. The NMR work was performed by the group from the Faculty of Science at the University of Geneva.

New cyclization reaction at the amino terminus of peptides and proteins

Rose K, Chen J, Dragovic M, Zeng W, Jeannerat D, Kamalaprija P, Burger U.

Department of Medical Biochemistry, University Medical Center and Department of Organic Chemistry, University of Geneva, CH-1211 Geneva 4, Switzerland. rose@cmu.unige.ch

Bioconjug Chem 1999 : 6(10), 1038-43

Correspondence and reprint requests: Prof. Keith Rose
Department of Medical Biochemistry
University Medical Center
1211 Geneva 4, Switzerland

Tel. +41 22 702 5523
Fax. +41 22 702 5502
Email. Keith.Rose@medicine.unige.ch

New Cyclization Reaction at the Amino Terminus of Peptides and Proteins

Keith Rose,*,† Jianhua Chen,† Marina Dragovic,† Weiguang Zeng,† Damien Jeannerat,‡ Philippe Kamalaprija,‡ and Ulrich Burger‡

Department of Medical Biochemistry, University Medical Center and Department of Organic Chemistry, University of Geneva, CH-1211 Geneva 4, Switzerland. Received May 20, 1999; Revised Manuscript Received September 8, 1999

Mild oxidation with periodate of the 1-amino-2-ol moiety of N-terminal seryl or threonyl peptides and proteins leads to a terminal aldehyde function O=CH−CO− which usually may be exploited for bioconjugate formation (e.g., via oxidation with an O-alkyl hydroxylamine). We report that, when followed by a prolyl residue, the O=CH−CO− group can undergo a rapid cyclization and dehydration reaction through nucleophilic attack by the amide nitrogen of the third amino acid residue of the chain. We have characterized the resulting heterocycle, which is stable in aqueous acid, by mass spectrometry and NMR. Quantitative oxidation can nevertheless be achieved in such cases by performing a one-pot oxidation-oxidation without isolation of the intermediate aldehyde, as is demonstrated with cholera toxin B subunit.

INTRODUCTION

The oxidation by periodate of N-terminal 1,2-amino alcohols Ser (R = CH_2OH) or Thr [R = $CH(OH)CH_3$] residues NH_2−CHR−CO− of a polypeptide to an aldehyde function O=CH−CO− has been known for many years (*1*). More recently, this mild oxidation reaction has been exploited for the synthesis of bioconjugates through formation of hydrazone, thiazolidine, or oxime (−ON=CH−CO-) bonds (e.g., refs *2*−*8*). Usually, the polypeptide aldehyde is stable in aqueous solution at pH values between 2 and 5 and may be lyophilized and stored as a powder at −20 °C (*4*). In some cases, however, the aldehyde can undergo side reactions. For example, formation of a 1,3,4-oxadiazolino peptide containing two peptide aldehyde units has been observed during attempted hydrazone formation (*9*).

We have observed that some polypeptides and proteins, including Cholera toxin B subunit (CTB), undergo rapid and quantitative oxidation to yield a compound which may be isolated and has the mass calculated for the expected O=CH−CO-polypeptide species, but oximation occurs very slowly indeed (half time estimated in weeks under conditions where normally the half time is of the order of very few minutes). The first protein which gave such difficulties was a β-lactamase (*5*), and in that case, the problem was solved by careful optimization of the conditions of oxidation of the N-terminus and storage of the aldehyde, but such an approach was not successful with CTB. We were able to reproduce the phenomenon of oxidation followed by very slow oximation using a small synthetic peptide consisting of the first six residues of the β-lactamase, Thr-Pro-Val-Ser-Glu-Lys-amide. In this article, we show that, when followed by a prolyl residue, the O=CH−CO− group formed by oxidation of N-terminal Ser or Thr can undergo a rapid cyclization and dehydration reaction through nucleophilic attack by the amide nitrogen of the third amino acid residue of the chain. The resulting heterocycle, which is stable in aqueous acid, was characterized by mass spectrometry and NMR. To achieve quantitative oximation in such cases, we developed a one-pot oxidation-oximation without isolation of the intermediate aldehyde and demonstrate here with CTB that it is possible to prepare in good yield a hybrid construct of which 60% of the mass is the B subunit and 40% is totally synthetic. CTB conjugates are potentially useful as vaccines: CTB can supply T helper epitopes and, if the construct assembles as its noncovalent pentamer, such constructs would be expected to elicit a mucosal immune response (e.g., refs *10* and *11*).

EXPERIMENTAL PROCEDURES

Materials. Synthetic peptides were prepared using Boc chemistry on a model 430A synthesizer (Applied Biosystems Inc.) modified according to Kent (*12*). ^{15}N (99 atom %) N^t-Boc-L-valine was purchased from Isotec. Methylbenzhydrylamine resin (NovaBiochem, Switzerland) was used to obtain peptide amides directly upon cleavage with HF. Synthetic peptides were purified by high-pressure liquid chromatography (HPLC), acetonitrile was removed by rotary evaporation at room temperature, and product was recovered by lyophilization. Purified peptides gave a single peak on analytical HPLC and the expected mass spectrum. The aminooxyacetyl peptide NH_2OCH_2CO-$Gly_3[Lys(Ser)]_5Gly$-OH was prepared as previously described (*13*). Cholera toxin B subunit (CTB, Inaba strain) was obtained from the Swiss Serum and Vaccine Institute, Berne, Switzerland. Sodium metaperiodate and guanidine hydrochloride (MicroSelect grade) were purchased from Fluka, Buchs, Switzerland. Boc-aminooxyacetic acid was from Calbiochem-NovaBiochem, Läufelfingen, Switzerland.

Chromatography. High-pressure liquid chromatography was performed using a column of Nucleosil 300A 5 μm C_8 (Macherey Nagel, Oensingen, Switzerland), 250

* To whom correspondence should be addressed. Phone: +41 22 702 5523. Fax: +41 22 346 8758. E-mail: rose@cmu.unige.ch.
† Department of Medical Biochemistry.
‡ Department of Organic Chemistry.

10.1021/bc9900587 CCC: $18.00
Published on Web 10/21/1999

× 4 mm id for analytical work (0.6 mL/min, 2 mL loop) and a radial compression system (210 × 25 mm id, Nova-Pak HR 60A 6 μm C_{18}, 20 mL/min, 50 mL loop) for preparative work, monitoring at 214 or 229 nm for analytical or preparative work, respectively. Linear gradients were established between solvent A [1 g of trifluoroacetic acid (TFA) added to 1 L of HPLC grade water and then vacuum filtered and degassed] and solvent B (1 g of TFA added to 100 mL of water, brought to 1 L with gradient grade acetonitrile then vacuum filtered and degassed). Gel filtration was performed on a Superdex 75 HR 10/30 column (Pharmacia, Uppsala, Sweden) at a flow rate of 0.4 mL/min using 0.25 M phosphate buffer, pH 7.0 (counterion sodium), which was 1 mM in EDTA, monitoring at 280 nm.

Mass Spectrometry. Spectra were obtained using electrospray ionization on either a Trio 2000 (infused at 2 μL/min) or Platform II instrument (infused at 7 μL/min); both machines were from Micromass, Altrincham, U.K. Samples of synthetic peptides were dissolved in water/acetonitrile 1:1 by volume, whereas protein samples were dissolved in water/acetonitrile/acetic acid (49.5:49.5:1 by volume).

NMR spectroscopy (*14*) was performed on a Bruker AMX-2−600 spectrometer operating at 14.1 T. Peptide samples were dissolved in D_2O at pD 4.7 (DCl). After acquisition of all data, samples were reanalyzed by HPLC to check for possible decomposition. Two-dimensional experiments were performed at 298 K. $^1H/^1H$-DQF−COSY spectra were acquired using 512 × 16K data points and a spectral width of 12 ppm in both dimensions. A shifted sine bell function was applied to the indirectly detected dimension during data processing. The heteronuclear HSQC (*15−17*) and HMBC experiments (*18−19*) were performed with 128 × 4K data points and spectral widths of 90 × 10.69 ppm in the carbon and proton dimension, respectively. Folded signals could easily be identified as such. For the processing, sine bells were applied in both dimensions.

Oxidation of TPVSEK-amide. To a solution of purified hexapeptide amide (17.6 mg, 26.8 μmol of peptide, 28 mL of water, 2.8 mL of 10% ammonium bicarbonate solution) was added 5.6 mL of sodium metaperiodate solution (0.184 g/L in water, 4.8 μmol) with vortex mixing. As soon as possible, the reaction was quenched by mixing in 5.6 mL of ethyleneglycol (10 M in water) and injecting the reaction mixture onto preparative HPLC. After a 5 min isocratic period, a linear gradient of 1% B/min was established. Once the three components had eluted within 30 min), the column was washed briefly at 50% B then reequilibrated at 100% A. Organic solvent was removed by rotary evaporation at room temperature and product recovered by lyophilization. From 85 mg of peptide, we isolated 39 mg of peak 1, 4.6 mg of peak 2 and 4.6 mg of peak 3.

Oximation of Oxidized TPVSEK-amide. For the study at pH 4.6, the buffer was 0.1 M acetate, counterion sodium. In separate experiments, both forms of the oxidized hexapeptide amide (peaks 2 and 3, 0.3 mg in 60 μL buffer) were treated with aminooxyacetic acid (60 μL, mM in buffer). For the study at pH 7.0, a phosphate buffer was used (0.1 M, counterion sodium) in place of acetate. The oximation reactions were monitored by HPLC, injecting 10 μL volumes with a gradient from 0 to 35% B over 30 min.

Oxidation and Oximation of CTB. CTB was supplied as a lyophilized powder in the presence of buffer salts, including Tris which might be expected to react with periodate. Vial contents (1 mg of protein) were

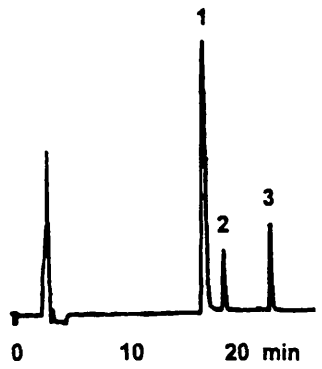


Figure 1. Preparative HPLC chromatogram of the oxidation of TPVSEK-amide with a limited quantity of periodate. After a 5 min isocratic period at 100% A, a gradient of 1%/min solvent B was applied, monitoring at 229 nm. Mass spectrometry and NMR identified components as peak 1, unmodified peptide; peak 2, hydrated glyoxylyl peptide $(HO)_2CHCO-$PVSEK-amide; peak 3, cyclic isomer of the expected O=CHCO−PVSEK-amide.

therefore dissolved in 1 mL of water, and the protein was isolated by gel filtration as the noncovalent pentamer. The protein was concentrated by ultrafiltration (Centricon, Amicon, Beverly, MA, 10000 molecular weight cutoff, precentrifuged twice with 1 mL of phosphate chromatography buffer to remove glycerol which interferes with the oxidation step) to 400 μL. Methionine (50 μL, 0.2 M in the phosphate buffer) was added to protect against thioether oxidation, then oxidation of the N-terminal Thr residue was initiated by addition of sodium periodate (25 μL, 40 mM in the phosphate buffer). After 5 min in the dark, excess periodate was quenched with ethyleneglycol (60 μL, 0.5 M in water), then 4 min later, 27.8 mg of aminooxyacetyl peptide NH_2OCH_2CO-$Gly_3[Lys(Ser)]_5$Gly-OH was added, followed by 50 μL of acetic acid. An aliquot (15 μL) was removed for HPLC and mass spectrometric analysis after 30 min, whereupon the sample was placed at −20 °C overnight. The next day, 500 mg of guanidine hydrochloride was added and the protein oxime isolated by gel filtration and ultrafiltration to a volume of about 400 μL. Oxidation of the Ser residues on the Lys side chains of the peptide attached to CTB was achieved by adding methionine (150 μL, 0.2 M in phosphate buffer) followed by periodate (62.5 μL, 80 mM in phosphate buffer). After 5 min in the dark, the reaction was quenched with 1,3-diamino-2-propanol (Fluka, 300 μL, 0.5 M in water adjusted to pH 7.8 with acetic acid). Four minutes later, 500 mg of guanidine hydrochloride was added, and the oxidized protein isolated again by gel filtration and concentrated by ultrafiltration to about 400 μL. A second 400 μL sample (from a separate experiment) was pooled at this stage. To the pool (800 μL), aminooxyacetyl peptide CH_3CO-$DC(CH_2CONH_2)$-TLIDALLGDPHK$(COCH_2ONH_2)$-NH_2 was added (5.7 mg), and the solution made 4% in acetic acid. For every microliter of this solution, 1 mg of guanidine hydrochloride was added and the oximation left overnight. Final product was isolated by semipreparative HPLC (1 mg after lyophilization) and characterized by mass spectrometry.

RESULTS AND DISCUSSION

Oxidation of TPVSEK-amide. To study the rapid oxidation of the terminal 1-amino-2-ol moiety of **1**, oxidation with periodate was performed with limiting amounts of periodate and a reaction time of a few seconds (to allow mixing). This allowed isolation of three components by HPLC (Figure 1). Peak 1 was identified by retention time and mass spectrum as unreacted starting

peptide (abundant signal at *m/z* 660.0 corresponding to the protonated molecular ion, most intense peak at *m/z* 330.9, the doubly protonated molecule, data not shown). Peak 2 gave a mass spectrum with signals at *m/z* 614.5, 632.5, and 654.4. The most intense peak *m/z* 632.5 corresponds to the hydrated form of the aldehyde, $(HO)_2CHCO-$PVSEK-amide (**2**) with one proton, and *m/z* 654.4 corresponds to sodium cationization of the same species. The small signal at *m/z* 614.5 in the spectrum of peak 2 became the most intense peak in the spectrum of peak 3, where it was accompanied by *m/z* 636.6. These two signals correspond to O=CHCO–PVSEK-amide (i.e., the aldehyde) with one proton and with one sodium ion, respectively, although we show below that peak 3 is actually a cyclic isomer (**3**) of the aldehyde. Peak 2 material, upon storage in aqueous solution at pH 4.6 (sodium acetate buffer), was found by HPLC and mass spectrometry to transform to peak 3 material over a number of days, but was more stable in 0.1% TFA at pH 2 (not shown). Thus, the signal at *m/z* 614.5 in the spectrum of Peak 2 material could be due to dehydration of the hydrate within the mass spectrometer to give either the aldehyde itself or its isomer (**3**). Peaks 1, 2, and 3 were found to be stable for days in aqueous solution at pH 2 at room temperature, and for months as lyophilized powders at −20 °C (not shown).

Oximation of oxidized TPVSEK-amide (peaks 2 and 3) was studied at pH 4.6 and pH 7.0. It has previously been shown that oximation proceeds faster at lower pH, and very slowly indeed at neutral pH (*7*). Oximation of peak 2 (the hydrated aldehyde) with aminooxyacetic acid at pH 4.6 was found to proceed extremely rapidly. Figure 2a shows almost quantitative reaction after 130 min: reaction halftime was approximately 10 min judging by earlier time points (not shown). The expected oxime product was identified by mass spectrometry through its protonated molecular ion at *m/z* 687.7. A trace of peak 3 material is also formed, which elutes 2 min after the oxime (ox). Oximation of peak 3 (which has the mass of the aldehyde) proceeded extremely slowly at pH 4.6, with very little product formed after 130 min (Figure 2b). The oxime was nevertheless being formed (it elutes 2 min earlier than peak 3 and is just visible in Figure 2b), and it represented about 12% after 35 h (product confirmed by mass spectrometry, not shown).

At pH 7.0, almost exactly the same chromatograms were obtained during oximation of peak 2 (Figure 2c) and peak 3 (Figure 2d). After 10 min, both showed presence of peak 2 and peak 3, with virtually no oxime product. At this pH, peaks 2 and 3 clearly interconvert rapidly: equilibrium is almost attained after 10 min, but not quite since we notice that Figure 2c is slightly richer in the starting peak 2 whereas Figure 2d is still slightly richer in the starting peak 3. In both cases, oxime formation proceeded very slowly, reaching about 30% after 10 h (not shown). Thus, although oximation proceeded very slowly at pH 7.0 for both peaks 2 and 3, it nonetheless proceeded more rapidly than for peak 3 at pH 4.6 (but much less rapidly than for peak 2 at pH 4.6).

These results were difficult to understand. For years it has been known that small aliphatic aldehydes (such as heptanal), many peptides and proteins carrying a O=CH–CO–NH–CHR–CO– group (where R is the side chain of a genetically coded amino acid), many polypeptides carrying a $-NHCH_2CH{=}O$ group, and many peptides carrying a O=CH–CO– group attached to the side chain of Lys undergo rapid and almost quantitative oximation at mildly acid pH. In the case of the oxidized small synthetic peptide TPVSEK-amide, the hydrated

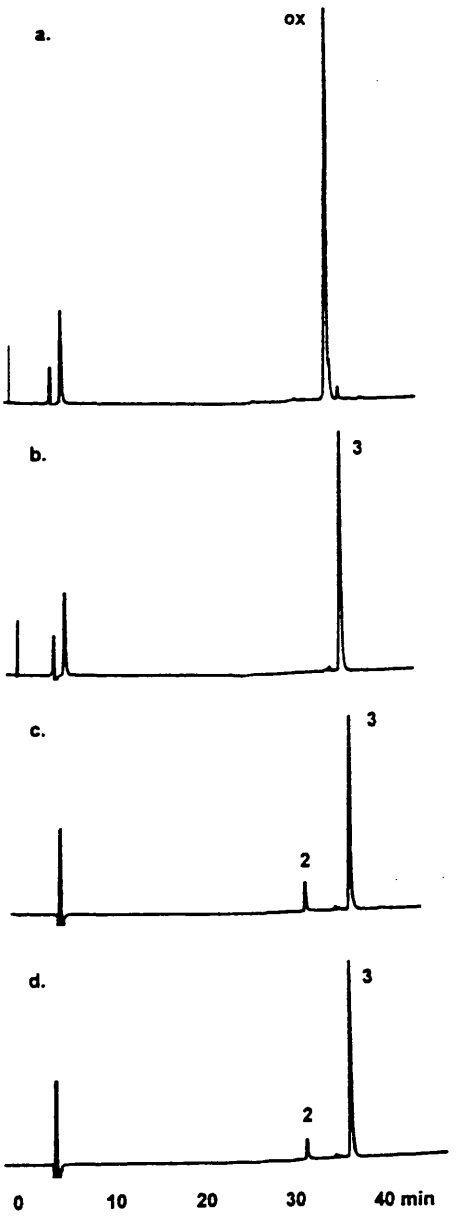


Figure 2. Analytical HPLC chromatograms of oximation reactions of peaks 2 and 3 of Figure 1. Remaining material is labeled 2 or 3, accordingly. Oxime product (ox) was identified by mass spectrometry. After a 5 min isocratic period at 100% A, a gradient of 1.17%/min solvent B was applied, monitoring at 214 nm. (a) Peak 2, pH 4.6, 130 min. (b) Peak 3, pH 4.6, 130 min. (c) Peak 2, pH 7, 10 min. (d) Peak 3, pH 7, 10 min.

aldehyde (peak 2) was able to undergo rapid oximation at pH 4.6, but the apparent aldehyde (peak 3) was not. This led us to investigate by NMR spectroscopy the structure of peaks 1, 2, and 3.

NMR Analysis. The $^1H/^1H$-DQF–COSY spectra of compounds **1**, **2**, and **3** allowed assignment of all resonances of hydrogen atoms bound to carbon (*14*). Since the amino acid residues differ all in their spin systems, the assignment was straightforward and unambiguous. Data for ^1H chemical shifts are compiled in Table 1.

Comparison of the ^1H-spectra of **1** and **2** immediately confirmed the Thr residue as the site of oxidative cleavage. The three signals of Thr of **1** are missing in compound **2** and are replaced by a singlet at δ (ppm) 5.58. This is the resonance of the H–C(2) proton of the newly formed dihydroxyacetyl residue [the former H–C(α) of Thr]. That we were dealing with a hydrated aldehyde was corroborated by an HSQC-experiment (*15–17*) which correlates this proton to a ^{13}C signal at δ (ppm) 86.0 via

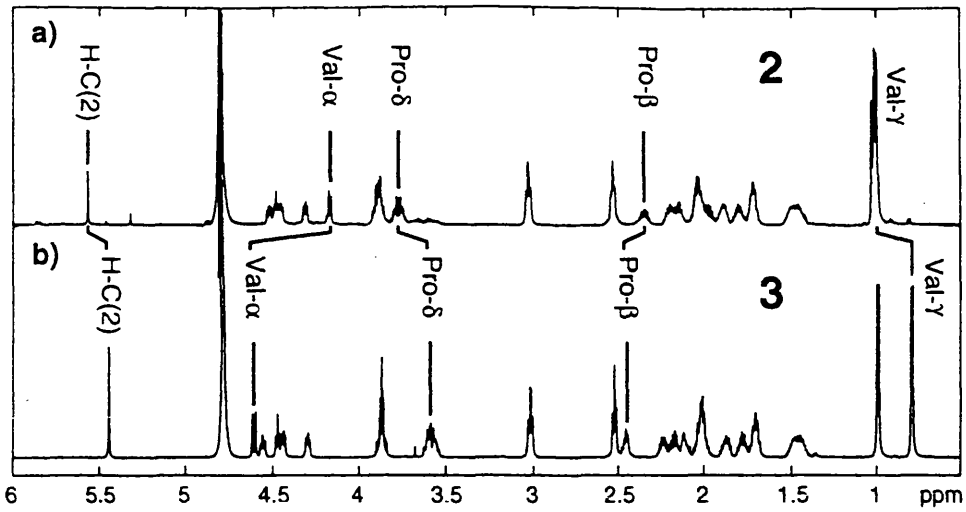


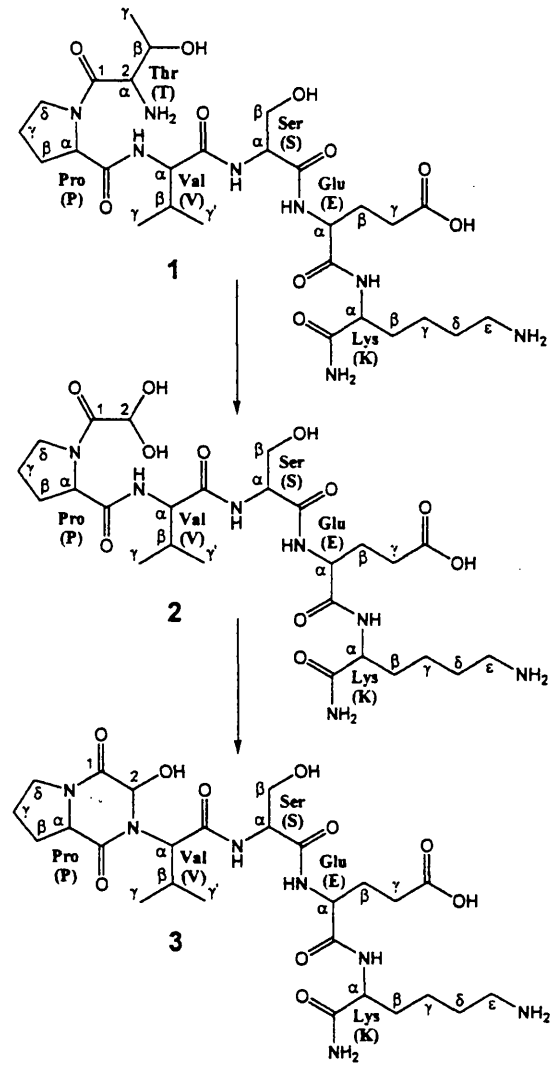
Figure 3. ^1H NMR spectra recorded at 600 MHz in D_2O of (a) compound **2** and (b) compound **3**, with assignment of those resonances which change significantly upon cyclization.

Table 1. ^1H Chemical Shifts for the Amino Acid Residues of Compounds 1, 2, and 3 at 600 MHz in D_2O (pD 4.7)

residue	position	1	2	3
Thr (T) or modified residue[a]	H−C(2)	4.26	5.58	5.46
	H−C(β)	4.20		
	$H_3C(\gamma)$	1.39		
Pro (P)	H−C(α)	4.58	4.52	4.58
	$H_2C(\beta)$	1.93/2.36	1.97/2.34	2.03/2.47
	$H_2C(\gamma)$	~2.10	~2.03	2.04/2.13
	$H_2C(\delta)$	3.72/3.83	3.75/3.77	3.57/3.61
Val (V)	H−C(α)	4.12	4.17	4.63
	H−C(β)	2.10	2.14	2.25
	$H_3C(\gamma)$	0.97/1.00	0.99/1.01	0.81/1.01
Ser (S)	H−C(α)	4.46	4.47	4.49
	$H_2C(\beta)$	3.86/3.88	3.86/3.88	3.89
Glu (E)	H−C(α)	4.45	4.44	4.45
	$H_2C(\beta)$	2.00/2.19	1.96/2.18	2.04/2.18
	$H_2C(\gamma)$	2.50	2.51	2.55
Lys (K)−(amide)	H−C(α)	4.30	4.30	4.31
	$H_2C(\beta)$	1.78/1.86	1.79/1.88	1.80/1.89
	$H_2C(\gamma)$	1.42/1.46	1.45/1.49	1.47/1.50
	$H_2C(\delta)$	1.70	1.70	1.72
	$H_2C(\epsilon)$	3.03	3.01	3.04

[a] Dihydroxyacetate for **2**, and H−C(2) of the new six-membered heterocycle for **3**.

Scheme 1. Structures of Unoxidized Peptide (1), Hydrated Glyoxylyl Peptide (2), and Cyclic Isomer of Glyoxylyl Peptide (3)



^1J scalar coupling. The ^1H chemical shifts of the remaining five residues (Pro, Val, Ser, Glu, and Lys-amide) show a remarkable similarity between compounds **1** and **2**. Clearly, these amino acid residues were not altered.

However, when comparing compounds **2** and **3** (Table 1 and Figure 3), it was evident that the dehydration reaction resulted in significant changes of the Pro and Val resonances. One of the methyl groups of Val had undergone an upfield shift of 0.2 ppm, whereas H−C(α) of Val changed from 4.17 to 4.63. A significant change (5.58 → 5.46) was observed also for the H−C(2) resonance of the oxidized residue. The signals of the three remaining amino acid residues Ser, Glu, and Lys-amide are almost identical for **2** and **3**. These findings clearly show that only Val and Pro sense the new molecular environment in **3**. Bond formation between the nitrogen atom of Val and C(2) of the oxidized residue, i.e. the disguised aldehyde, best accounts for our observations (Scheme 1).

The conclusions found strong support by a HMBC experiment (*18*, *19*), which unveiled scalar $^1H/^{13}C$ coupling between H−C(α) of Val and C(2) of the oxidized residue. The absolute value of this $^3J_{C/H}$ coupling is 4.6 Hz. The existence of the crucial $C(2)-N_{(Val)}$ bond was eventually ascertained by specific ^{15}N-labeling of compound **3** in the $N_{(Val)}$ position. The ^{13}C NMR spectrum of this labeled material [$^{15}N_{(Val)}$-**3**] shows $^1J_{C/N}$ coupling for three carbon atoms, which are C(2) [at δ = 79.9, $^1J_{C/N}$ = 7.6 Hz], $C(\alpha)_{(Val)}$ [at δ = 64.4, $^1J_{C/N}$ = 8.4 Hz], and $CO_{(Pro)}$ [at δ = 175.9, $^1J_{C/N}$ = 12.8 Hz]. In addition, $^{15}N_{(Val)}$ couples

Table 2. Ease of Oximation of Various Oxidized Polypeptides

N-terminal sequence	source	oximation after isolation of oxidized polypeptide
SAKELR.....	IL-8	easy
SLAADT.....	MIP-1α	easy
SQLHSG.....	GCSF fragment	easy
TPVSEK.....	β-lactamase	possible[a]
TPVSEK-amide	synthetic	difficult
SPYSSD.....	Rantes	difficult
TPQNIT.....	Cholera toxin B subunit	difficult

[a] After much optimization (5).

over two bonds with $C(\alpha)_{(Pro)}$ [at δ = 61.58, $^2J_{C/N}$ = 5.4 Hz]. These values are close to what is expected for **3** (*20*), and they leave no doubt that $N_{(Val)}$ is bound to three carbon atoms. The ring closure creates, with high stereoselectivity, a new center of chirality. We see a single epimer of **3**. The absolute configuration at H–C(2), however, is unknown.

The cyclization process **2** → **3** is entropically favored by the rigidity of the proline ring, which keeps the two reacting centers in proximity. This propensity to cyclize is probably responsible for the difficulty sometimes encountered to oximate a polypeptide which, by mass spectrometry, has the mass of the aldehyde. All of our difficult oximations have involved X-Pro sequences, where X is Thr or Ser (Table 2, where only a small selection of easily oximated polypeptides is shown).

Studying "glyoxylyl-Pro-Ile-Val-amide" made by treatment of diisopropylthioacetyl peptide with N-bromosuccinimide and isolation under acidic conditions, Qasmi et al. (*21*) found the mass of the aldehyde form (which is the same as that of the cyclic form) by isobutane CI mass spectrometry, but found evidence by NMR (in DMSO) and FAB mass spectrometry (thioglycerol matrix) that the polypeptide can also exist in hydrated form, as we found for our synthetic peptide. By proton NMR, they found no aldehydic proton at all for "glyoxylyl-Pro-Ile-Val-amide", but found a singlet at δ(ppm) 9.2–9.3 integrating 0.5 H for the aldehyde proton of "glyoxylyl-Phe-Pro-Ile-Val-amide" and "glyoxylyl-Asn-Phe-Pro-Ile-Val-amide".

Oxidation of CTB and oximation with the carrier molecule NH_2OCH_2CO-Gly_3[Lys(Ser)]$_5$Gly-OH under the "one-pot" conditions described led to quantitative conversion (Figure 4a) to the expected oxime CTB=$NOCH_2CO$-Gly_3[Lys(Ser)]$_5$Gly-OH: relative molecular mass found by electrospray ionization mass spectrometry, 12 938 Da (theoretical mass, 12 937 Da). CTB normally exists as a noncovalent pentamer, but treatment with acid and organic solvents leads to the monomeric form exclusively. Oxidation of the side-chain Ser residues of the ligated carrier molecule followed by isolation of the oxidized protein possessing five aldehyde groups and oximation with the peptide CH_3CO-DC(CH_2CONH_2)TLIDALLGDPH–K($COCH_2ONH_2$)-NH_2 (NH_2OCH_2CO-peptide) yielded 1 mg of product from 2 mg of starting CTB. Characterization by mass spectrometry showed this product (relative molecular mass, 21 102 Da, theoretical mass, 21 101 Da) to be the expected branched hybrid CTB=$NOCH_2CO$-Gly_3[Lys($COCH$=$NOCH_2CO$-peptide)]$_5$Gly-OH.

A similar product was made earlier (*22*) using the "one-pot" conditions and different form of the peptide, CH_3CO-DC($SCH_2CH_2NHCOCH_2ONH_2$)TLID-ALLGDPH–NH_2. At that time, we did not know why it was necessary to use "one-pot" conditions for the first oximation step, while it was possible to isolate the oxidized protein prior to the second oximation step. By performing the "one-pot" oxidation-oximation at pH 6.5 instead of 7.0, and quenching after 2 min, it was possible to isolate four components from the reaction mixture (Figure 4b). Mass spectrometry identified peaks 1–4: peak 1 was the expected oxime (found, 12 938; calcd, 12 937); peak 2 was unoxidized CTB (found, 11 602; calcd, 11 604); peak 3 was hydrated glyoxylyl CTB $(HO)_2CHCO$–CTB (found, 11 580; calcd, 11 577); and peak 4 had the mass expected for "glyoxylyl-CTB" but was most probably the cyclic isomer; found, 11 558; calcd, 11 559. Incubation of peak 3 material with NH_2OCH_2CO-Gly_3[Lys(Ser)]$_5$Gly-OH led to formation of peak 1 material (oxime), while oximation of peak 4 material failed. Use of methanol, which leads to hemiacetal formation, in place of acetonitrile as cosolvent for electrospray ionization shifted the mass of peak 3 to, found, 11 589 and, calcd, 11591, but did not shift the other masses, in particular that of peak 4, providing further evidence of lack of a free aldehyde moiety for peak 4. Taken together with the mass spectrometric and NMR results obtained with the model peptide, it would seem that cyclization of glyoxylyl-Pro polypeptides to a cyclic isomer which oximate with difficulty may also occur with proteins.

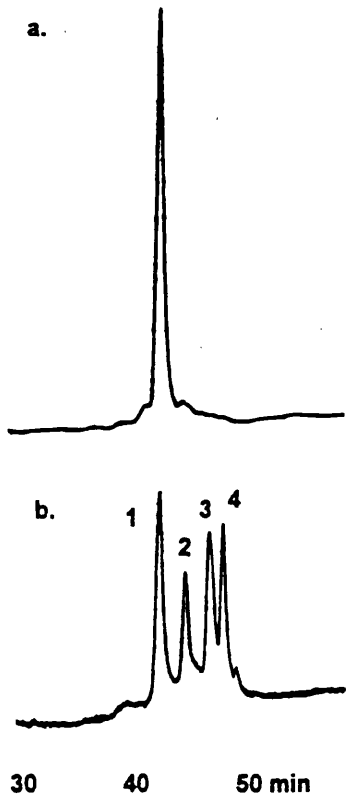


Figure 4. Analytical HPLC chromatograms of oximation reactions of oxidized CTB with NH_2OCH_2CO-Gly_3[Lys(Ser)]$_5$-Gly-OH. Compounds were identified by mass spectrometry. After a 5 min isocratic period at 35% B, a gradient of 0.2%/min solvent B was applied, monitoring at 214 nm. (a) "One-pot" oxidation-oximation under optimized conditions, showing quantitative conversion to the oxime. (b) "One-pot" reaction at suboptimal pH and time.

CONCLUSIONS

It is generally an advantage to isolate an oxidized (glyoxylyl-) polypeptide prior to oximation to avoid the necessity to use an excess of aminooxy component over the aldehydes coproduced during the oxidation reaction (formaldehyde from Ser, acetaldehyde from Thr, and formaldehyde from oxidation of the quenching agent ethyleneglycol). However, particularly when the N-ter-

minal Ser/Thr residue to be oxidized is followed by Pro, we recommend "one-pot" oxidation-oximation to avoid possible formation of the cyclic isomer of the aldehyde described here.

We have not studied the biological properties of the heterocyclic isomer of the aldehyde. It might be interesting as a scaffold for combinatorial chemistry of small (or even protein-sized) molecules, or for other purposes.

ACKNOWLEDGMENT

We are indebted to the Swiss National Science Foundation (Grants 31-50826.97 and 20-52905.97) and the Schmidheiny Foundation. We thank Marie-Laure Curchod, Irène Rossitto-Borlat, and Pierre-Olivier Regamey for expert technical assistance. The allocation of spectrometer time in the regional high-field NMR facilities in Lausanne-Dorigny is also gratefully acknowledged.

LITERATURE CITED

(1) Fields, R., and Dixon, H. F. (1968) A spectrophotometric method for the microdetermination of periodate. *Biochem. J. 108,* 883−887.

(2) Geoghegan, K. F., and Stroh, J. G. (1992) Site-directed conjugation of non-peptide groups to peptides and proteins via periodate oxidation of a 2-amino alcohol. (1992) *Bioconjugate Chem. 3,* 138−146.

(3) Gaertner, H. F., Rose, K., Cotton, R., Timms, D., Camble, R., and Offord, R. E. (1992) Construction of protein analogues by site-specific condensation of unprotected fragments. *Bioconjugate Chem. 3,* 262−268.

(4) Rose, K. (1994) Facile synthesis of homogeneous artificial proteins. *J. Am. Chem. Soc. 116,* 30−33.

(5) Mikolajczyk, S. D., Meyer, D. L., Starling, J. J., Law, K. L., Rose, K., Dufour, B., and Offord, R. E. (1994) High yield, site-specific coupling of N-terminally modified β-lactamase to a proteolytically derived single-sulfhydryl murine Fab'. *Bioconjugate Chem. 5,* 636−646.

(6) Zhang, L., and Tam, J. P. (1996) Thiazolidine formation as a general and site-specific conjugation method for synthetic peptides and proteins. *Anal. Biochem. 233,* 87−93.

(7) Rose, K., Zeng, W., Regamey, P.-O., Chernushevich, I. V., Standing, K. G., and Gaertner, H. F. (1996) Natural peptides as building blocks for the synthesis of large protein-like molecules with hydrazone and oxime linkages. *Bioconjugate Chem. 7,* 552−556.

(8) Nardin, E. H., Calvo-Calle, J. M., Oliveira, G. A., Clavijo, P., Nussenzweig, R., Simon, R., Zeng, W., and Rose, K. (1998) *Plasmodium falciparum* polyoximes: highly immunogenic synthetic vaccines constructed by chemoselective ligation of repeat B cell epitopes and a universal T-cell epitope of CS protein. *Vaccine 16,* 590−600.

(9) Melnyk, O., Bossus, M., David, D., Rommens, C., and Gras-Masse, H. (1998) Synthesis of lipopeptides using hydrazone chemical ligation. *J. Pept. Res. 52,* 180−184.

(10) Walker, R. I. (1994) New strategies for using mucosal vaccination to achieve more effective immunization. *Vaccine 12,* 387−400.

(11) Hathaway, L. J., Obeid, O. E., and Steward, M. W. (1998) Protection against measles virus-induced encephalitis by antibodies from mice immunized intranasally with a synthetic peptide immunogen. *Vaccine 16,* 135−141.

(12) Schnölzer, M., Alewood, P., Jones, A., Alewood, D., and Kent, S. B. H. (1992) *In situ* neutralization in Boc-chemistry solid phase peptide synthesis. Rapid, high yield assembly of difficult sequences. *Int. J. Pept. Protein Res. 40,* 180−193.

(13) Vilaseca, L. A., Rose, K., Werlen, R., Meunier, A., Offord, R. E., Nichols, C. L., and Scott, W. L. (1993) Protein conjugates of defined structure: synthesis and use of a new carrier molecule. *Bioconjugate Chem. 4,* 515−520.

(14) Cavanagh, J., Fairbrother, W. J., Palmer, A. G., III, Skelton, N. J. (1996) *Protein NMR Spectroscopy − Principles and Practice,* Academic Press, Inc., San Diego, CA.

(15) Palmer, A. G., III, Cavanagh, J., Wright, P. E., and Rance, M. (1991) Sensitivity improvement in proton-detected two-dimensional heteronuclear correlation NMR spectroscopy. *J. Magn. Reson. 93,* 151−170.

(16) Schleucher, J., Schwendinger M., Sattler, M., Schmidt, P., Schedletzky, O., Glaser, S. J., Sorensen, O. W., and Griesinger, C. (1994) A general enhancement scheme in heteronuclear multidimensional NMR employing pulsed field gradients. *J. Biomol. NMR 4,* 301−306.

(17) Kay, L. E., Keifer, P., and Saarinen, T. (1992) Pure absorption gradient enhanced heteronuclear single quantum correlation spectroscopy with improved sensitivity. *J. Am. Chem. Soc. 114,* 10663−5.

(18) Bax, A., Griffey, R. H., and Hawkins, B. L., (1983) Correlation of proton and nitrogen-15 chemical shifts by multiple quantum NMR. *J. Magn. Reson. 55,* 301.

(19) Willker, W., Leibfritz, D., Kerssebaum, R., and Bermel W., (1993) Gradient selection in inverse heteronuclear correlation spectroscopy. *Magn. Reson. Chem. 31,* 287−292.

(20) Berger, S., Braun, S., and Kalinowski, (1992) *^{15}N NMR-Spektroskopie,* Vol. 2 of *NMR−Spektroskopie von Nichtmetallen,* Georg Thieme Verlag, Stuttgart, Germany.

(21) Qasmi, D., de Rosny, E., René, L., Badet, B., Vergely, I., Boggetto, N., and Reboud-Ravaux, M. (1997) Synthesis of *N*-glyoxylyl peptides and their in vitro evaluation as HIV-1 protease inhibitors. *Bioorg. Med. Chem. 5,* 707−714.

(22) Rose, K., Zeng, W., Dragovic, M., Brown, L. E., and Jackson, D. C. (1996) Polyoxime artificial proteins as vaccines, in *Peptides: Chemistry, Structure, Biology* (P. T. P. Kaumaya and R. S. Hodges, Eds., Mayflower Scientific, Kingswinford, England, pp 767−769.

BC9900587

Dissertation Equivalent D

J. Chen, W. Zeng, K. Rose

Enhanced binding properties of cholera toxin B subunit when conjugated with a polyoxime

Jianhua Chen performed all of the work described in this paper.

Enhanced binding properties of cholera toxin B subunit when conjugated with a polyoxime

Jianhua Chen[1], Weiguang Zeng[2], Keith Rose[1]

[1] Department of Medical Biochemistry, University Medical Center, Geneva, Switzerland;
[2] Cooperative Research Centre for Vaccine Technology, Department of Microbiology and Immunology, University of Melbourne, Parkville, Australia.

Correspondence and reprint requests: Prof. Keith Rose
Department of Medical Biochemistry
University Medical Center
1211 Geneva 4, Switzerland

Tel. +41 22 702 5523
Fax. +41 22 702 5502
Email. Keith.Rose@medicine.unige.ch

Summary

Cholera Toxin B subunit (CTB), capable of binding to all mucous membranes in its pentameric form, is a potential carrier of mucosal vaccines. In our previous work we reported that the N-terminus of CTB, a threonine, could in principle undergo oxidation and oximation to form conjugates with a cascade of immunogenic peptides. In this study, we set up a model by chemically coupling CTB to a polyoxime that possessed five copies of influenzavirus-derived peptides displayed in comb-like form. The construct was reconstituted into pentameric form when eluted from a Superdex column after conjugation, and the pentameric nature of this CTB-like complex was confirmed by SDS-PAGE. GM_1-ELISA assay showed that the binding properties of CTB conjugate were increased 4 - 5 fold over native CTB.

Introduction

Cholera toxin, an AB_5 hexameric protein composed of 5 identical B subunits and a single A subunit, is the primary enterotoxin produced by *Vibrio cholerae* bacteria, and is the causative agent of cholera infection. The B subunits of Cholera toxin (CTB) are the nontoxic binding components of the CT holotoxin [1] and function in pentameric form to specifically recognize the GM_1 ganglioside receptor present on the surface of mucous cells. It has been demonstrated in a large field trial that CTB afforded, when orally administered, protection against both cholera and enterotoxigenic *E. coli*-caused diarrhea [2]; and when conjugated to a poor immunogen and given orally, CTB showed the ability to dramatically increase the mucosal immunogenicity [3, 4]. This raised the possibility of using CTB as a potential carrier of mucosal vaccines. Holmgren, J. et al and other workers also reported that the physical coupling of foreign antigens to CTB is a very effective way of inducing such a mucosal immune response after oral vaccination [5, 6]. In our previous work we reported that the N-terminus of CTB, a threonine, could in principle undergo oxidation and oximation to form conjugates with a cascade of immunogenic peptides [7]. Here we explore a possible way to generate novel mucosal vaccines that are chemically coupled to peptide and reconstituted into a CTB-like complex *in vitro*. Since the mucosal immune response from oral vaccination is not restricted to the intestine but also partly disseminates to other mucosal tissues, this approach raises the prospect to use CTB as a vaccine carrier against infections in the intestinal as well as in any other mucosal tissues.

Synthetic peptides can serve as immunogens to induce antibodies, but a small linear peptide may not be effective as an immunogen because of the influence of protein conformation [8] and because of its lack of high molecular mass that is necessary for enhanced immunogenicity. One way to solve these problems is to create an artificial protein by coupling the antigenic peptide to protein carriers

such as mucosal adhering proteins [e.g. CTB or LTB (*E. coli* heat-labile toxin B-subunit)]. In this way, a synthetic immunogenic peptide is attached to part of a protein carrier capable of localizing in a specific tissue (mucosa of gastrointestinal tract, for example). In order to enhance peptide immunogenicity further, it is possible to cross-link antigenic peptides to a synthetic core in a branched (radial or comb) manner. For instance, Tam [9] in 1988 designed an amide-linked branched artificial protein, a multiple antigenic peptide (MAP) system, to increase molecular weight. This is a macromolecule containing several copies of synthetic epitopes or protein antigens that are coupled to a peptide core matrix (based on lysine) by peptide bond formation. MAP directs the immune system to a specific response. As MAPs are entirely synthetic, they are in principle safer vaccines as they are free of replicative agents (viruses, DNA, prions) and endotoxin. Unfortunately, with this approach it is impossible to produce homogeneous artificial proteins due to inevitable side-reactions and coupling failures, and the wanted material, when the peptide epitope is longer, cannot be purified from the many impurities present with similar structure. One solution is to couple the pre-purified synthetic peptides to the MAP-core matrix by site-specific conjugation. Oximation chemistry is one of the mild and effective methods for such site-specific tagging that permits the modification of proteins without damaging their intricate structure [10].

In this study we set up a model by grafting a MAP system to the N-terminus of CTB. An antigenic peptide derived from hemagglutinin protein of inluenza virus was selected [11, 12]. We aim to use this model composed of polyoxime and mucosal carrier protein to study the possibility of attaching a cascade of immunogenic peptide to CTB and retaining or reconstituting its pentameric form.

Materials and Methods

1. Reagents and chemicals

Unless otherwise specified, all solvents and reagents were obtained from Fluka, Buchs, Switzerland, were of analytical or higher grade and were used without further purification. All amino acids were purchased from Peptide Institute Inc., Japan. The resins were from Applied Biosystems, USA; Novabiochem, Switzerland, or Bachem, Switzerland. CTB was from Swiss Serum & Vaccine Institute, Bern, Switzerland. Water was repurified using a Milli-Q system (Millipore, Inc.).

2. HPLC and MS

Analytical RP-HPLC (reverse-phase high performance liquid chromatography) was performed using a column 250 x 4 mm i.d. packed with Nucleosil 300-A 5 μm C_8 particles at a flow rate of 0.6 ml/min and effluent was monitored at 214nm. Peptides were purified on a C_8 semi-preparative column (250 x 10 mm i.d. Nucleosil 300-A 5 μm particle size) at a flow rate of 4 ml/min monitoring at 214 nm, or purified on a C_{18} preparative column (210 x 25 mm i.d. Novapak 6 μm particle size) at 20 ml/min monitoring at 229 nm. Solvents used in RP-HPLC were as follows: A, 0.1% TFA (1 g TFA in 1 liter H_2O); B, 0.1% TFA in 90% acetonitrile (1 g TFA mixed with 100 ml H_2O and then brought to 1 liter with acetonitrile). Generally, the conditions used in analytical work were 5 min isocratic at 100% A followed by a linear gradient 2% B/min to 100% B, and in preparative work a shallower linear gradient (usually 0.5% B/min) was used. Components were collected manually at the detector exit, evaporated at room temperature, frozen and then recovered by lyophilization.

Electrospray ionization mass spectrometry (ESI-MS) was performed in positive ion mode on a Trio 2000 machine or a Platform-II instrument (both from Micromass, Manchester, England). Samples were introduced either at 2 μl/min (Trio) in an acidified solvent acetonitrile/water/AcOH (49.5 : 49.5 : 1) or at 10 μl/min (Platform) in solvent acetonitrile/water/formic acid (49.9 : 49.9 : 0.2). MALDI-TOF mass spectrometry was performed in linear mode using sinapinic acid as matrix on a Voyager Elite machine (Perkin Elmer) equipped with delayed extraction. External calibration was performed on the electrospray machines using a solution of horse heart apomyoglobin, and on the MALDI-TOF machine using the mixture of peptides supplied by the manufacturer.

3. Synthesis of Peptides

The antigenic peptide sequences *NH_2OCH_2CO*-KTYQRTRALV derived from Influenza virus [11, 12] were synthesized by Boc chemistry on 0.5 mmol Boc-Val-PAM resin (phenylacetamidomethyl resin, Perkin Elmer ABI division) using an automated solid-phase synthesizer (model ABI 430A, Applied Biosystems Inc.). Italic type denotes linker residues not present in the corresponding protein sequences. Side-chain protection of Boc-protected amino acid was Lys(2ClZ), Thr(Bzl), Arg(Tos). After chain elongation with Boc chemistry, the N-terminal Boc- protection of Lys was deprotected with TFA. The Boc-aminooxyacetyl (AOA) was attached to the Lys residue with an activated mixture solution (1.0 mmol of Boc-AOA-OSu, 2-fold excess over the substitution of the resin in 5ml DMSO, 1 ml N-methylmorpholine) to form aminooxyacetyl precusor peptide.

The linker NH_2OCH_2CO-Gly_3-[Lys(Ser)]$_5$-Gly-OH was synthesized manually on 0.5 mmol Boc-Gly-PAM resin. After Boc deprotection, 5 cycles of coupling with Fmoc-Lys(Boc) and deprotection were performed by Fmoc-chemistry to form the linear polylysine core, followed by three cycles of Fmoc-Gly. The side-chain protection of Lys core was then removed, and Boc-Ser(Bzl) groups were attached to the core with an activated mixture solution (8 mmol Boc-Ser(Bzl)-

OSu in 8 ml DMSO and 2 ml N-methylmorpholine), followed by Fmoc deprotection of the N-terminal amino group and attachment of a Boc-AOA group as above.

The resin and all other side-protection groups were cleaved with HF (0°C for 1 h in the presence of 5% p-cresol). Peptides were precipitated with cold ether, extracted with 50% acetonitrile, filtered and lyophilized. The crude peptides were purified by preparative HPLC and characterized by ESI-MS.

4. Preparation of Polyoxime conjugated to CTB

1 mg CTB, supplied as a lyophilized powder together with buffer salts, was dissolved in 1 ml water and isolated by gel filtration as the non-covalent pentamer on a Superdex 75 HR column (Amersham Pharmacia Biotech) at a flow rate of 0.4 ml/min. A phosphate buffer prepared by mixing NaH_2PO_4 (0.25 M in H_2O) with Na_2HPO_4 (0.25 M in H_2O) to obtain pH 7.0 and containing EDTA (to a final concentration of 1 mM) was used as eluent solution. The protein was concentrated by ultrafiltration (Centricon, Amicon, Beverly, MA, 10,000 Mwt cut off, precentrifuged twice with 1 ml of the phosphate buffer) to 400 µl. Methionine (50 µl, 0.2 M in H_2O) was added. N-terminal threonine was then oxidized for 5 min by adding 25 µl sodium periodate (40 mM in H_2O). The reaction was quenched with 60 µl ethylene glycol (0.5 M in H_2O). After 4 min, 27.8 mg of aminooxyacetyl linker was added, followed by 50 µl acetic acid. 30 min later, 15 µl of the reaction solution was removed for HPLC and ESI-MS, while 500 mg guanidine hydrochloride was added to the rest. After a reaction time of 16-18 h, the protein oxime was isolated by gel filtration and concentrated to 400 µl as before.

The five Ser residues on the linker $NH_2OCH_2CO\text{-}Gly_3\text{-}[Lys(Ser)]_5\text{-}Gly\text{-}OH$ ligated to CTB were oxdized for 5 min by addition of 150 µl methionine (0.2 M in H_2O)

and 62.5 µl sodium metaperiodate (80 mM in H_2O). The reaction was quenched with 300 µl of 1,3-diamino-2-propanol (0.5 M in H_2O, adjusted to pH 7.8 with acetic acid). 4 min later, 500 mg guanidine hydrochloride was added and the oxidized protein isolated again by gel filtration and concentrated by ultrafiltration to 400 µl. A second 400 µl of sample from a separate experiment was pooled at this stage. Then the powder of aminooxyacetyl influenza peptide (5.7 mg) was added to the pool (800 µl), followed by acetic acid to a final concentration of 4%. The reaction was left overnight, and the final product was isolated by gel filtration and the CTB complex was isolated in pentameric form. An aliquot of the final product was analysed by analytical HPLC and characterized by ESI-MS.

5. Concentration determination

CTB complex was analyzed by UV spectrophotometry (Ultrospec III, Pharmacia Biotech) scanning the region 240-320 nm, and the concentration was calculated according to the formula $Absorbance_{280} = \sum l \bullet C$ [C, concentration of the product; $\sum l$, total of molar absorptivity (ε) of Tryptophan and Tyrosine in CTB complex: Absorptivity ($\varepsilon \times 10^{-3}$) of Tryptophan at 279 nm is 5.6, and absorptivity ($\varepsilon \times 10^{-3}$) of Tyrosine at 275 nm is 1.4.]

6. SDS-PAGE

A sample of CTB complex was heated in a boiling water bath prior to the electrophoresis. 3 µl of heated and un-heated samples (1 µg/µl) of CTB complex were then loaded and run on a Homogeneous-20 Phast gel using a PhastGel System (Amersham Pharmacia Biotech) for approximately 30 minutes using a standard method. Two samples of commercial CTB (one native, one heated) were used as positive controls for the pentamer and monomer of CTB, and SDS-buffer served as negative control. A LMW Calibration kit of Protein Standards

was used as a marker. The gel was then developed with silver staining automatically on PhastGel system for 90 minutes.

7. GM_1-ELISA

A polystyrene 96-well ELISA plate was coated by incubation at 4°C overnight with 200 µl/well of 1.5µM Ganglioside GM_1 coating buffer (PBS). The plate was washed 3 times with PBS, and the additional binding sites were blocked by incubating plates with 200µl/well of a 2% (vol/vol) skim milk-PBS solution for 30-60 min at 37°C followed by washing 3 times with PBS. 100µl of the test samples was pipetted into a well immediately and the plates incubated at 37°C for 1h. The plate was washed again with PBS buffer, and 100 µl of 1:2000 goat anti-CTB was added to each well followed by incubation for 1h at 37°C. After washing with PBS buffer, each well received 100 µl of a 1:24000 dilution of anti-Goat IgG (peroxidase) and the plate was incubated for 1h at 37°C. The plate was washed, then 100 µl of the chromogenic reagent (POD substrate) was added and the plate incubated 3-5 min to obtain a blue coloration. The reaction was finally stopped by addition of 100 µl of 1N H_2SO_4 (color change to yellow) and results were read at 450 nm using an ELISA reader.

Results and Discussion

1. "One pot" oxidation and oximation of CTB.

Oxidation and oximation of CTB with the linker molecule NH_2OCH_2CO-Gly_3-[Lys(Ser)]$_5$-Gly-OH was performed under "one-pot" conditions as previously described [7]. The expected oxime CTB=$NOCH_2CO$-Gly_3-[Lys(Ser)]$_5$-Gly-OH was purified by gel filtration, and one aliquot was analyzed by HPLC and characterized. Relative molecular mass found on ESI-MS was 12,938, close to the expected 12,937. Ser residues on the CTB linker complex were oxidized and the product purified by gel filtration. The oxidized protein possessed five aldehyde groups which were then oximated with aminooxyacetyl influenza virus peptide NH_2OCH_2CO-KTYQRTRALV (NH_2OCH_2CO-peptide) and isolated by gel filtration. One aliquot was analyzed by HPLC and characterized by ESI-MS which showed this product (relative molecular mass found, 19,236; theoretical, 19,234) to be the expected branched hybrid CTB=$NOCH_2CO$-Gly_3-[Lys(COCH=$NOCH_2CO$-peptide)]$_5$-Gly-OH. Generally, there is an advantage to isolate an oxidized N-terminal Threonyl or Seryl polypeptide prior to oximation, to avoid the necessity to use an excess of aminooxy component over the aldehydes coproduced from the oxidation reaction (formaldehyde from Ser, acetaldehyde from Thr, and formaldehyde from oxidation of the quenching agent ethyleneglycol). In our case, however, the N-terminal sequence of CTB is Thr-Pro- [13]. We demonstrated in our previous study that when N-terminal Thr-Pro- is oxidized the resulting aldehyde group O=CH-CO- can undergo a rapid cyclization and dehydration reaction through nucleophilic attack by the amide nitrogen of the third amino acid residue because of the rigidity of the proline ring which keeps the two reaction centers in proximity [7]. "One pot" oxidation-oximation was adopted here so as to avoid possible formation of the cyclic isomer of the aldehyde.

2. CTB complex in pentameric form

CTB normally exists as a noncovalent pentamer which is necessary for CTB to specifically recognize the GM_1 ganglioside receptor present on the surface of mucous cells, but treatment with acid and organic solvents leads to quantitative dissociation to the monomeric form. A stepwise denaturation-renaturation process has been reported which allows CTB to regain its active form [14]. However, using this method in our previous study most of the CTB complex could not be obtained in pentameric form. The protocol was thus modified to obtain CTB=$NOCH_2CO$-Gly_3-[Lys(COCH=$NOCH_2CO$-peptide)]$_5$-Gly-OH from gel filtration in pentameric form. An aliquot of the eluate was taken for HPLC analysis and characterization, and the rest was kept for concentration determination by UV spectrophotometry and then used directly for the immunization assay. 1.5 ml of such pentameric solution was obtained in this way, with an absorbance after pooling of OD_{280} 0.068. Thus the concentration of CTB complex was determined as 0.81 μM by use of the formula $OD_{280} = \sum l \bullet C$ ($\sum l = 84 \times 10^3$ l/mol cm, total of molar absorptivity (ε) of Tryptophan and Tyrosine in CTB complex; C, concentration of the product).

The pentameric form of CTB complex was further confirmed by SDS-PAGE. This was done using the standard method on PhastGel System (Amersham Pharmacia Biotech). Two samples of CTB complex pentamer (one was heated in boiling water for denaturation) obtained from gel filtration were detected on the electrophoresis gel, alongside two samples of commercial CTB (one was denatured) as positive control. The result (Figure 1) showed that the denatured CTB complex (Mr, 12,938) and its control, denatured commercial CTB (Mr, 11,604), were close to the molecular marker 14,400, while the CTB complex (Mr, 64,690) and native commercial CTB (Mr, 58,020) were close to molecular marker 67,000. This indicated that the CTB complex like native commercial CTB was its noncovalent pentameric form and when denatured it can be dissociated into its monomeric form. Moreover, there were several parallel bands for CTB complex

on the electrophoresis gel, which might be due to CTB complex lacking a copy of aminooxyacetyl peptide on one or more subunits. This loss can occur during gel electrophoresis. Our previous work [7] showed that the purified pentaoxime is not contaminated with species lacking a copy of aminooxyacetyl peptide.

3. CTB complex shows enhanced binding to GM_1

To determine the ability of the CTB complex to bind to the cholera toxin receptor, GM_1 ganglioside was bound to the surface of plastic microtitre plates and reacted with the CTB complex. ELISA assay was performed using a modified method from Svennerholm AM and Holmgren J [15]. Results were read at 450 nm using an ELISA reader, and a standard ELISA curve was obtained by testing the binding property of native CTB pentamer (Figure 2). The same GM_1-ELISA experiment was also done on CTB complex, from which a ELISA curve was obtained (Figure 2). The curve of CTB complex was shifted to the left of the standard one and its value at 1.5 (OD 450 nm) showed that the affinity of CTB complex was 4 – 5 fold higher than that of commercial CTB, which may result from favorable comfomational change or stabilization of the CTB complex. This indicates that binding properties of the the CTB complex are at least as strong as those of the native CTB, in spite of the conjugation of antigenic peptides to its N-terminus. Since CTB complex pentamer possesses 5 x 5 antigenic peptides (see Scheme) it represents a useful candidate immunogen. This approach is general, and opens the way to create vaccine candidates using other immunogenic peptides.

Conclusion

A polyoxime employing influenza virus-derived peptides as building block was successfully conjugated to the N-terminus of CTB, a potential carrier of mucosal vaccines, via a "one-pot" oxidation-oximation process. The construct was reconstituted into pentameric form when eluted from a Superdex column after conjugation, and this CTB-like complex was confirmed by SDS-PAGE. GM_1-ELISA assay showed that the binding properties of CTB conjugate were improved 4 – 5 fold over native CTB. Although we have not studied the immunogenicity of the CTB-conjugate, this general approach opens an interesting approach to create vaccine candidates using other immunogenic peptides.

References

[1] Sun J.B., Holmgren J. and Czerkinsky C. (1994) Cholera toxin B subunit: an efficient transmucosal carrier-delivery system for induction of peripheral immunological tolerance. *Proc Natl Acad Sci U S A* 91, 10795-9

[2] Holmgren J, Lycke N, Czerkinsky C. (1993) Cholera toxin and cholera B subunit as oral-mucosal adjuvant and antigen vector systems. *Vaccine* 11(12), 1179-84

[3] Rask C., Fredriksson M., Lindblad M., et al. (2000) Mucosal and systemic antibody responses after peroral or intranasal immunization: effects of conjugation to enterotoxin B subunits and/or of co-administration with free toxin as adjuvant. *APMIS* 108(3), 178-86

[4] McKenzie S.J. and Halsey J.F. (1984) Cholera toxin B subunit as a carrier protein to stimulate a mucosal immune response *J Immunol* 133(4), 1818-24

[5] Yuki Y., Byun Y., Fujita M., et al. (2001) Production of a recombinant hybrid molecule of cholera toxin-B-subunit and proteolipid-protein-peptide for the treatment of experimental encephalomyelitis. *Biotechnol Bioeng* 74(1), 62-9

[6] Holmgren J., Czerkinsky C. and Lycke N., et al. (1994) Strategies for the induction of immune responses at mucosal surfaces making use of cholera toxin B subunit as immunogen, carrier, and adjuvant. *Am J Trop Med Hyg* 50(5 Suppl), 42-54

[7] Rose K., Chen J., Dragovic M., et al. (1999) A new cyclization reaction at the amino terminus of peptides and proteins. *Bioconjug Chem* 10,1038-43

[8] Janeway, C.A. Jr. (1999) Manipulation of the immune response: Synthetic peptides of protective antigens can elicit protective immunity. *Immuno Biology 4th edition* (Austin P. and Lawrence E. eds) pp568-9. Elsevier Science Ltd, London, UK

[9] Tam J.P. (1988) Synthetic peptide vaccine design: synthesis and properties of a high-density multiple antigenic peptide system. *Proc Natl Acad Sci USA* 85, 5409-13

[10] Rose K. (1994) Facile synthesis of artificial proteins *J Am Chem Soc* 116, 30-3

[11] Bodmer, H. C., R. M. Pemberton, J. B. Rothbard, et al. (1988) Enhanced recognition of a modified peptide antigen by cytotoxic T cells specific for influenza nucleoprotein. *Cell.* 52, 253-8

[12] Sherman, L. A., T. A. Burke, and J. A. Biggs (1992) Extracellular processing of peptide antigens that bind class I major histocompatibility molecules *J Exp Med.* 175, 1221-6

[13] Takao T, Watanabe H, and Shimonishi Y (1985) Facile identification of protein sequences by mass spectrometry: B subunit of Vibrio cholerae classical biotype Inaba 569B toxin *Eur J Biochem* 146(3), 503-8

[14] Hatic SO 2nd, McCann JA, and Picking WD (2001) In vitro assembly of novel cholera toxin-like complexes *Anal Biochem* 292(2), 171-7

[15] Svennerholm AM and Holmgren J (1978) *Curr Microbiol* 1, 19-23

Figure Legends

Figure 1. SDS-PAGE of commercial CTB and CTB complex

CTB complex (Mr, 64,690) is in noncovalent pentameric form like its control CTB native (Mr, 58,020); while CTB complex (Mr, 12,938) when denatured by heat runs close to monomeric CTB standard (Mr, 11,604) produced by denaturation of the native pentamer. Negative control (loading buffer) and molecular weight markers are shown in the figure.

Figure 2. GM_1-ELISA of CTB

ELISA plates were coated with 1.5μM Ganglioside GM_1 in PBS. Commercial CTB or CTB complex were tested for their binding properties using a modified method from Svennerholm AM and Holmgren J [14]. Result shows the avidity of CTB-complex pentamer are 40-50 fold higher than commercial CTB pentamer.

Scheme. Structure of CTB complex in pentameric form.

Five copies of CTB complex were pertamerized unconvalently and each subunit of CTB complex possesses a linear pentalysine linker and an antigenic peptide on the side chain of each lysine. Thus, the pentamer of CTB complex possesses 5 x 5 copies of antigenic peptides.

Figure 1

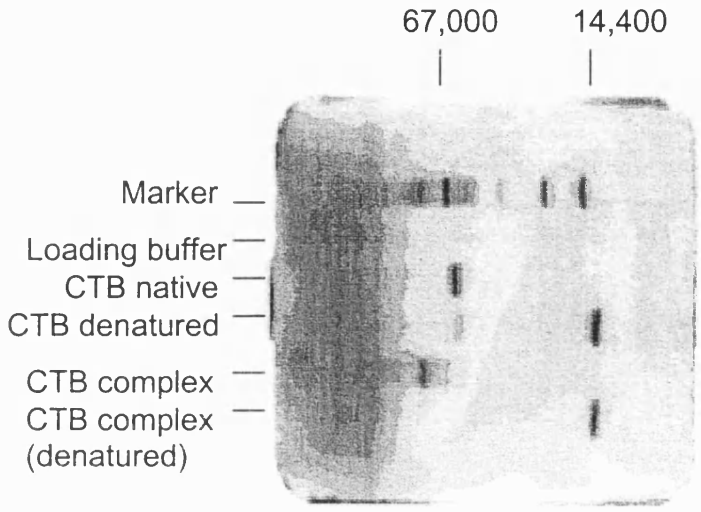
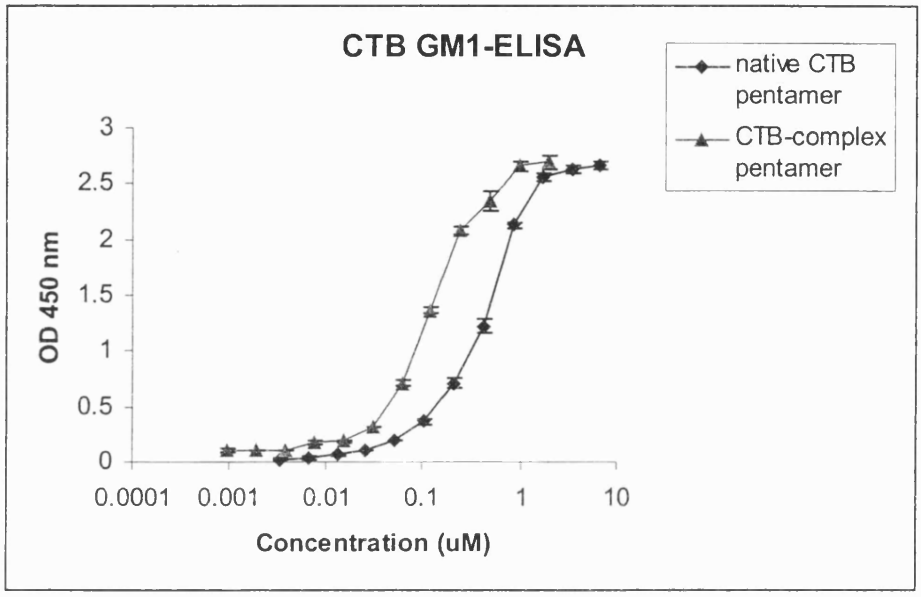
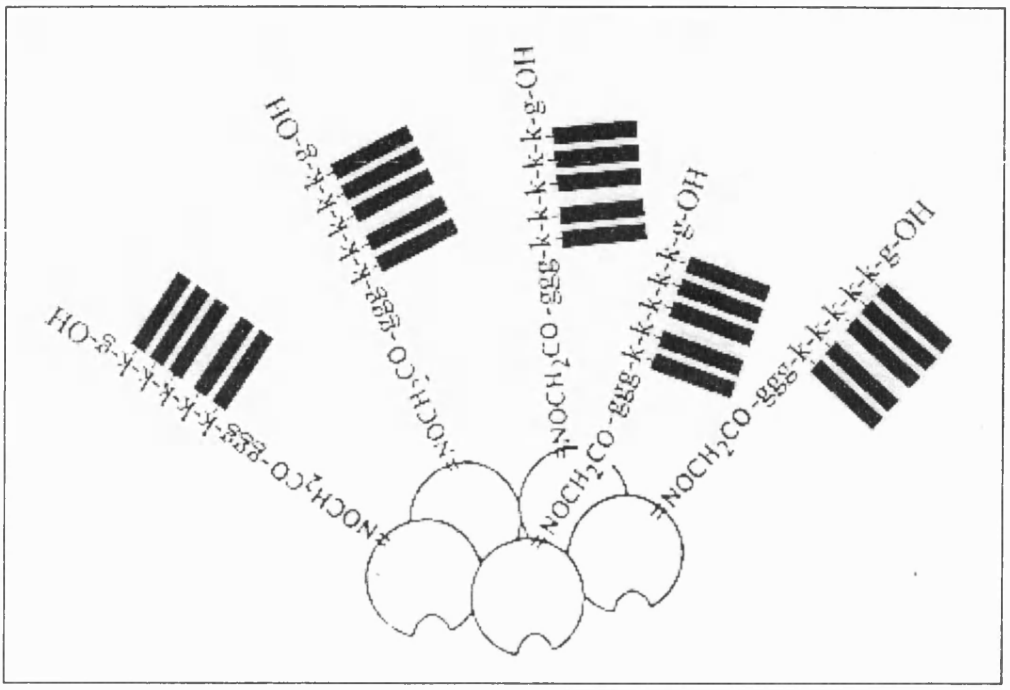

67,000
14,400
Marker
Loading buffer
CTB native
CTB denatured
CTB complex
CTB complex
(denatured)

Figure 2


CTB GM1-ELISA
native CTB pentamer
CTB-complex pentamer
OD 450 nm
3
2.5
2
1.5
1
0.5
0
0.0001
0.001
0.01
0.1
1
10
Concentration (uM)

Scheme


HO-g-k-k-k-k-k-k-g-OH
HO-g-k-k-k-k-k-g-OH
HO-g-k-k-k-k-g-OH
HO-g-k-k-k-g-OH
+NOCH2CO-ggg-k-k-k-k-k-g-OH
+NOCH2CO-ggg-k-k-k-k-g-OH
+NOCH2CO-ggg-k-k-k-g-OH
+NOCH2CO-ggg-k-k-g-OH

Dissertation Equivalent E

J. Chen, C. Sauser, K. Rose, C. Bonny

Chemobodies as Targeting Moieties of Insulin-Secreting Cells

Dr Bonny performed phage work and biochemical experiments. Jianhua Chen designed and synthesized chemobodies and was involved in the biochemical work.

Chemobodies as Targeting Moieties of Insulin-Secreting Cells

Jianhua Chen[1], Christelle Sauser[2], Keith Rose[1], Christophe Bonny[2]

[1] Department of Medical Biochemistry, University Medical Center, Geneva, Switzerland;

[2] Division of Medical Genetics, CHUV, Lausanne, Switzerland

Correspondence and reprint requests: Dr Christophe Bonny
Division of Medical Genetics, CHUV
Chief Research Unit
1011 Lausanne, Switzerland
Tel. +41 21 314 3379
Fax. +41 21 314 3385
Email. Christophe.Bonny@chuv.hospvd.ch

Summary

Peptide ligands that mimic natural ligands are often identified using the powerful phage display technique. However, the obtained ligands are usually of low-affinity (micromolar range) due to the high degree of conformational freedom and small number of contact residues within a short peptide molecule. One way to circumvent this drawback is to present several copies of the low-affinity peptides in a single multimeric molecule. Avidities (and bioactivities) can thus be greatly enhanced over the affinity or activity of the monomeric peptide. In this study, we prepared a series of multimeric constructs employing cell-targeting peptides as building blocks through oxime chemistry. These peptides were derived from phage display experiments selecting specific binding to pancreatic β-cells, and capable of eliciting uptake by the cells via receptor-mediated endocytosis. The multimeric molecules were taken up efficiently by the cells. Our results indicate that the avidity of the multimeric molecules for pancreatic receptors is high enough to elicit uptake. This study opens a promising way to develop novel chemical entities to specifically deliver therapeutic agents to control diabetes.

Introduction

Progress in the transportation of peptides or proteins into cells *in vivo* may benefit from the transduction of large size molecules, which tends to be limited due to size and biochemical properties. For example, Schwarze SR and his colleagues described a 10-mer peptide derived from HIV, TAT_{48-57}, that is capable of transporting conjugated peptides or proteins intracellularly into tissues and across the blood-brain barrier [1]. These peptides or proteins are internalized by cells via a so-called protein transduction process that does not involve endocytosis. This discovery opens a new methodology for biomedical research and for direct delivery of drugs into patients. However, an important limitation of this approach is secondary to the lack of cellular specificity of these types of transporters. Thus, adverse side effects on normal tissues limit their potential usefulness, a point that is particularly problematic for the treatment of chronic diseases like diabetes, for example.

Cell-type specific delivery might be achieved by successfully targeting cell-specific endocytotic receptors. Receptor-mediated endocytosis is indeed widely exploited in experimental systems for the targeted delivery of therapeutic agents into cells [2]. Endocytotic activity is a common property that has been described for many receptors including IgG Fc, somatostatin, insulin, IGF-I and II, transferrin, EGF, GLP-1, VLDL or integrin receptors [3-10]. Recently, the isolation of peptide sequences that direct efficient receptor-mediated endocytosis has been profoundly boosted by the use of phage display technologies [11]. Phage display libraries are extremely powerful tools that provide for a practically unlimited source of molecule variants including modifications of natural ligands to cell receptors [12] and short peptides [13]. Using this technology, evidence that cell-type specific receptors mediate endocytosis has been reported [14]. Similar libraries have been injected directly into mice and peptide sequences that show a 13-fold selectivity for brain and kidney have been successfully isolated [15-16].

The advantages of small peptide carriers such as those obtained using phage display libraries are numerous and include chemical synthesis and associated high quality and purity, low immunogenicity and potential highly efficient delivery to all cells in an organism [1]. Accordingly, peptide carriers have the potential to outscore more conventional transporters including liposomes or viruses for the efficient delivery of many macromolecules (see for example [17-18]). Unfortunately, the obtained ligands are usually of low-affinity (micromolar range) [19-20]. This may be due to the high degree of conformational freedom and small number of contact residues within a short peptide molecule.

If several copies of the peptide molecule with low-affinity binding sites are present in a single multimeric molecule, avidities (and bioactivities) can be greatly enhanced over the affinity or activity of monomeric peptide. IgM is an example of low-affinity protein, but, when presents in a pentameric form, the avidity is highly enhanced toward repetitive antigenic determinants present on the surface of bacteria or viruses [21]. Such enhancement (10^5-fold) occurred with the peptabody [22], a protein produced through recombinant DNA techniques. In this protein, the low affinity of polypeptides derived from phage libraries is compensated by its pentameric structure resulting in a high avidity towards their targets. Recently, Rose K [23] also designed such a molecule named "chemobody". This is a synthetic molecule displaying multiple copies of a peptide subunit capable of binding non-covalently to a complementary structure, thus mimicking a major feature of the antibody molecule. The amino acid sequence of the binding peptide was identified using phage library techniques and the subunits themselves were assembled using oxime chemistry. To be able to bind through two or more subunits simultaneously, an appropriate spacer polyethyleneglycol (PEG) chain and a linker were also introduced, which are flexible, amphiphilic, non-immunogenic, and unsusceptible to proteases. Some reporter group such as fluorescein or biotin can also be introduced into the linker moiety of the Chemobody much more easily than into the Peptabody. Thus the

Chemobody shows greater flexibility that renders it more convenient for biological use.

We previously created a model chemobody [23] that displayed 4 copies of a phage-derived peptide from influenza virus together with flexible polyethyleneglycol (PEG) linkers. In this study, we isolated by phage display panning a series of peptides that target pancreatic insulin secreting cells. We then prepared multimeric constructs employing these cell-targeting peptides as building blocks through oxime chemistry with the aim of exploiting enhanced avidity through multivalent binding. These molecules potentiallly represent novel chemical entities to specifically deliver therapeutic agents to control diabetes.

Methods

Materials and cell lines

Unless otherwise specified, all solvents and reagents were obtained from Fluka, Buchs, Switzerland, were of analytical or higher grade and were used without further purification. All amino acids were purchased from Peptide Institute Inc. Japan. Resins were from Applied Biosystems, USA; Novabiochem, Switzerland, or Bachem, Switzerland. Water was repurified using a Milli-Q system (Millipore, Inc.). The insulin-secreting cell line βTC-3 [24] was used in the bioassay.

Phage preparation and enrichment procedures

A library of $3x10^8$ independent phages displaying random 15-mer epitopes at the surface of the capsids was generated using standard procedure in the T7413b phage (Novagen). Phages were amplified then purified by polyethylene glycol (PEG) precipitation and finally resuspended at a concentration of 10^{10} infective particles per μl in Tris-EDTA buffer (10:1 mM, TE) as described [25]. Phages (10^{12}) were added to cells in culture medium for 1 to 24 hours. Longer incubation times were preferred to favor isolation of phages that escaped proteolytic degradation in endocytotic vesicles. Following binding and internalization, cells were washed and non-internalized phages were destroyed by digestion with subtilisin (3mg/ml) [11]. Following extensive washing, internalized phages were then recovered by lysing cells in a buffer containing 2% deoxycholate, 10 mM Tris-HCl and 2mM EDTA, pH 8.0. Recovered phages were finally amplified in *E.coli* cells (XL-1-Blue) and purified as described above. This preparation of selected phages was then used for a second round of panning. Three to five sequential rounds were performed to obtain enrichment of specific phage-bearing peptide sequences.

Peptides synthesis

The peptide sequence RRTK was obtained from the phage display experiments that showed the peptide consensus motif R/K-X-X-R/K for binding to pancreatic β-cells. Peptides modified as seen in Table 1 were synthesized manually on 0.5 mmol Boc-Lys(ClZ)-PAM resin by Boc chemistry. Italic type denotes linker residues and space not present in the corresponding protein sequences. Several core structures used in this project were shown in Table 2. Synthesis was performed on 0.5 mmol methylbenzhydrylamine resin (MBHA resin) using Boc-chemistry.

All peptides were cleaved from the resin with HF (0°C for 1 h in the presence of 5% p-cresol), precipitated with cold ether, extracted with 50% acetonitrile, filtered and lyophilized. The crude peptides were purified by preparative HPLC and characterized by ESI-MS.

Preparation of a Fluorescein linker

A fluorescein linker NH_2OCH_2CO-Lys(FITC)-OH was prepared according to Offord's method [26]. In brief, 2 mmol of Boc-aminooxyacetyl (Boc-AOA-OSu) and 1.2 mmol of N^{ε}-(TFA)-Lys were added into 3 ml of DMSO solution, and N-Ethylmorpholine was added until pH value 8-9. After incubation at room temperature for 15 hours and then at 37°C for 1 hour, the pH value was brought to 3.0 by carefully addition of glacial acetic acid under constant agitation. The product [Boc-AOA-Lys(TFA)-OH] was isolated on a preparative-scale HPLC and then dried. 4 ml water was added and then 0.44 ml piperidine (final concentration 1 M) into the dried compound, which was incubated 4 hour at room temperature, followed by pH adjustment to 3.0 on ice. The mixture was diluted with 10 ml of 0.1% TFA. The deprotected material (Boc-AOA-Lys-OH) was isolated by preparative HPLC and dried again. The latter was dissolved in 300 µl N,N-dimethylformamide (DMF), 40 mg fluorescein isothiocyanate (FITC) was added,

and the mixture was adjusted to pH 8.0 with N-ethylmorpholine. After incubation for 15 hours in the dark, the product was purified by chromatography on a silica column [Kieselgel 60 (Fluka Chemie, Buchs, Switzerland), 1.5 x 20 cm] equilibrated in methanol/CH_3Cl (1:1, v/v). The excess FITC elutes in the flow-through fraction, and a second yellow fraction containing the expected product was eluted with methanol/CH_3Cl (4:1, v/v). The solvent was removed by rotary evaporation, and the dried compound Boc-AOA-Lys(FITC)-OH was deprotected in TFA at a concentration not exceeding 20 mg/ml and left for 45 min at room temperature. The majority of TFA was then evaporated and drying was completed by lyophilization. The final product [AOA-Lys(FITC)-OH] was isolated by preparative HPLC and characterized by ESI-MS.

Alkylation of peptide ligands to the core

0.9 μmol of Cys core peptide (1.19 mg) was dissolved in 50 μl of acetonitrile and 100 μl of water (peptide not fully soluble). A fresh solution of bromoacetyl peptide was prepared (3.6 μmol in 0.1M sodium phosphate buffer, pH 7.0). Then the alkylation was started by mixing two solutions at room temperature in dark. After 40 min, the product was purified on semi-preparative RP-HPLC, lyophilized, and characterized.

Periodate oxidation

0.375 μmol of peptide 3 or 4 or alkylated peptides, listed as follows,

H-**S**-(peg-succ)$_2$-**RRTK**-OH
H-**S**-(peg-succ)$_2$-**RRTK**-OH (D-form)
[HO-**KTRR**-$COCH_2$-S-**C**]$_4$-K_2K(succ-peg-**S**)-amide
[HO-**KTRR**-(succ-peg)$_2$-$COCH_2$-S-**C**]$_4$-K_2K(succ-peg-**S**)-amide
HO-**KTRR**-$COCH_2$-S-**C**-**G**$_3$-[**K**(**C**-S-CH_2CO-**RRTK**-OH)]$_3$-K(succ-peg-**S**)-amide

Note, a bold letter denote an amino acid residue

was dissolved, respectively, in 433 µl acetonitrile and 1.26 ml imidazole buffer (50 mM, pH 6.95, chloride counter ion). 113 µl of methionine (200 mM) was added. Then 17 µl of $NaIO_4$ (0.1 M in water) was added to start the oxidation. After 5 min the reaction was quenched by adding 40 µl of ethylene glycol (500 mM in water). The product was purified on preparative HPLC and characterized by mass spectrometry.

Oximation

The aldehyde obtained by oxidation was then reacted with core 3 or 4 (containing an AOA group in each core), or AOA-Lys(FITC)-OH, to form an oxime. Oximes 1 to 6 were obtained by this way (see Figure 1 and 2). For oximations with AOA-Lys(FITC)-OH, 0.129 µmol of oxidized construct was dissolved in 5 µl of acetonitrile and 200 µl of NaOAc buffer (acetic acid 0.57 ml in 100 ml of water, sodium acetate 0.82 g dissolved in 100 ml of water, mixed part of both until pH 4.0). 0.516 µmol AOA-Lys(FITC)-OH (8.8 mg/ml in 100 µl acetonitrile and 100 µl of NaOAc buffer) was added into the mixture, followed by addition of acetic acid to a final concentration of 4%. For oximation with core 3 or 4, 3.8 µmol of oxidized peptide 3 or 4 was dissolved in 100 µl of acetonitrile and 200 µl of NaOAc buffer. 0.475 µmol core 3 or 0.237 µmol core 4 was added into the solution, followed by addition of acetic acid to a final concentration of 4%. After 24 h reaction at room temperature, the product was purified on semi-preparative RP-HPLC and characterized by mass spectrometry.

Immunocytochemistry and fluorescence studies

Single phages isolated according to the enrichment scheme above were amplified. The phage peptides and synthesized oxime 1 – 6, respectively, were added and incubated for 24 hours with cells in culture medium. Medium was then washed off and cells fixed in cold methanol-acetone (1:1) for 5 minutes.

Antibodies directed against the phage capsid or against biotin (peptides) were used with a fluorescein-conjugated (phage) or Texas Red labeled (peptides) secondary antibody. Classical fluorescence microscopic studies and confocal microscopic assays were performed.

Results and Discussion

1. Construction of a novel phage system

Phage peptide libraries are traditionally constructed in derivatives of the filamentous phage M13. Peptide libraries are fused to the minor coat protein pIII of the capsid that displays 1-5 copies of the peptide motif [13]. Alternatively, high-valent display is attained by using the major coat protein pVIII. These libraries however have not been optimized for the isolation of receptor-mediated endocytotic peptide sequences, because mono- or low-valent display of peptides is essentially insufficient for efficient uptake of such giant structures as filamentous phages and only a multivalent display allows for efficient uptake [11]. In addition, the internalization of receptor-bound ligands involves concentration of cell surface receptors in specialized areas of the plasma membrane and subsequent formation of clathrin-coated vesicles [27], and receptor-mediated internalization by these specialized and highly efficient structures is not expected to occur with the conventional M13 phages.

We therefore constructed a phage display library in the T7 415 phage system. This phage exhibits 415 copies of the displayed peptides within a reduced volume (capsid is ~50nM diameter).

2. Identification of binding peptide RRTK

We first panned the pancreatic β-cell line βTC-3 with our phage display library. Selective enrichment of the number of recovered phages is observed at each cycle of selection (Table 3).

Sequence analysis of 20 recovered phages at the final cycle of enrichment is presented in Table 4. Importantly, all sequences strictly obeyed a conserved

consensus sequence of 5 amino acids (see below). This suggests the specific selection/enrichment of a conserved motif that directs efficient uptake of the phages.

Phage P1 was used for further analyses. Titration experiments indicated that as much as 10% of the initial P1 phage input could be recovered (Table 5).

Determination of the specificity of uptake was performed by titrating the number of recovered phages in 5 different cell lines. This indicates that P1 is uptaken by βTC-3 cells 10'000 to 1'000'000 fold more efficiently than by any other cell line tested (Table 6).

3. Generation of multiple transporter peptides

It is known that ligand peptides obtained from phage library are usually of low-affinity (micromolar range) [19, 20], and that their binding affinity or avidity can be enhanced by multimerize copies of the peptide molecule such as peptabody [22]. We here designed and synthesized a series of multimers (Figure 1 and 2) which displayed multiple copies (four or eight) of a β-cell specific peptide, RRTK, obtained from the phage library. A reporter group either FITC (fluorescein isothiocyanate) or Biotin was introduced into these multimer molecules.

The first construct (oxime 1, Figure 1) was built on a radial trilysine core (core 1) possessing a copy of RRTK peptide (peptide 1) at the tip of each of 4 branches. They were N-terminally conjugated through a thioether bond arising from a specific reaction between bromoacetyl and thiol groups. The trilylsine core also contained a PEG spacer and Ser capable of undergoing oxidation to generate an aldehyde, and thus a reporter group aminooxyacetyl-Lys(FITC) was introduced into the tetramer via oxime formation. HPLC and ESI-MS analysis revealed the desired product: Mr found 4289.31, Mr theoretical 4289.04. It was stable at acidic and neutral pH over 24 h.

Oximes 2 and 3 (Figure 1) were formed in a similar way to oxime 1, but on oxime 3 a two-unit PEG spacer was placed between the RRTK peptide and the core, and on oxime 2 the RRTK peptide was N-terminally conjugated to core 2 instead of core 1. Oxime 2: Mr found 4459.48, Mr theoretical 4460.19; Oxime 3: Mr found 6708.29, Mr theoretical 6708.00.

Oxime 4 (Figure 2) was synthesized similarly to Oxime 3, but on its trilysine core, a Biotin group was attached directly via amide bond, instead of a FITC group as reporter. Mr found 6160.0, Mr theoretical 6160.3. Oxime 5 (Figure 3) was similar to Oxime 4, but its RRTK residues were all D-form. Mr found 6158.6, Mr theoretical 6160.3.

Oxime 6 (Figure 2) was synthesized as Oxime 4, but it possessed eight copies of RRTK peptide instead of four copies. Mr found 11646.1, Mr theoretical 11643.6.

4. Multimer transporter as novel chemical entities

Phage experiments indicated that the phage peptide RRTK was taken up specifically by βTC-3 cells (Table 6) via receptor-mediated endocytosis, though the efficiency of internalization was low, which might be due to its low-affinity binding (~x00 micromolar range). A serie of multimers displaying multiple copies (four or eight) of this peptide with a reporter tag, either FITC or Biotin, was built (Figure 1 and 2) in order to determine whether the uptake of the peptide could be enhanced.

Oximes 1 – 3 were first tested for their entry into βTC-3 cells. Fluorescence studies showed only oxime 3, a tetraoxime (tree design) employing four copies of RRTK peptide with 2 units of PEG space as building blocks, clearly bound to the receptors on the surface of βTC-3 cells and seemed to enter into the cells (Figure 3). Whereas, oximes 1 or 2, tetraoxime tree-design or comb-design with four

copies of RRTK peptide without any spacer, did not bind to the receptor and thus did not enter the cells. This may be due to the flexibility of PEG spacer in the oxime 3, which increases its binding properties to the receptors on the cells. However, the localization of the fluorescence peptide within the cells was not clear in this case.

We then designed oximes 4 – 6 (Figure 2) according to oxime 3 for an enhanced binding avidity and entry efficiency. The copies of the RRTK peptide was increased up to eight in oxime 6, whereas, the peptide was changed into its D enantiomeric form in oxime 5, because D-peptides had been demonstrated previously to possess an increased stability (and also lower immunogenicity, a point to consider when injecting peptides into animals [28]). Microscopic examination showed that the oxime 6 with eight copies of peptides entered cells, whereas the others did not (Figure 4) including one control. Texas Red labeled secondary antibody to the Biotin demonstrated that the octaoxime localized either inside cells as "vacuolar" structures, or at the cell membrane. Therefore, these results indicate that multimers, when the peptide copies are increased up to eight, greatly enhanced the avidity of the binding peptides and thus their transport efficiency.

Work in future

The structure of the multimeric molecules (chemobodies) needs to be further optimized to get higher avidity, and other reporters will be introduced as well for a more sensitive detection. The effect of conjugated inhibitor peptide will be investigated for specific delivery of therapeutic agents in order to control diabetes.

Conclusion

To characterize multivalent binding peptides for enhanced avidity, we prepared a serie of multimeric constructs employing phage-derived cell-targeting peptides as building blocks through oxime chemistry, in which a reporter tag, either fluorescein isothiocyanate (FITC) or Biotin, was introduced. The selected peptide is specific for binding to the receptor on the surface of pancreatic β-cells and is then taken up by cells via receptor-mediated endocytosis. Microscopy showed that FITC-labelled molecules or Texas Red labeled secondary antibody to Biotin were taken up by the cell efficiently. Our results indicate that the multimeric molecules are of high avidity to the receptors and capable of entering cells efficiently. The study opens a promising way to develop novel chemical entities to specifically deliver therapeutic agents to control diabetes.

References

[1] Schwarze, S.R., A. Ho, A. Vocero-Akbani, et al. (1999) *In Vivo* Protein Transduction: Delivery of a Biologically Active Protein into the Mouse. *Science* 285, 1573

[2] Kato, Y. and Y. Sugiyama (1997) Targeted delivery of peptides, proteins, and genes by receptor-mediated endocytosis *Crit.Rev.Ther.Drug Carrier.Syst.* 14, 287

[3] Anderson, C.L. (1989) Human IgG Fc receptors *Clin.Immunol.Immunopathol.* 53, S63

[4] Hofland, L.J., W.A. Breeman, E.P. Krenning, et al. (1999) Internalization of [DOTA degrees,125I-Tyr3]Octreotide by somatostatin receptor-positive cells in vitro and in vivo: implications for somatostatin receptor-targeted radio-guided surgery. *Proc.Assoc.Am.Physicians.* 111, 63

[5] Lund, K.A., L.K. Opresko, C. Starbuck, et al. (1990) Quantitative analysis of the endocytic system involved in hormone-induced receptor internalization. *J.Biol.Chem.* 265, 15713

[6] Smith, R.M. and L. Jarett (1988) Receptor-mediated endocytosis and intracellular processing of insulin: ultrastructural and biochemical evidence for cell-specific heterogeneity and distinction from nonhormonal ligands *Lab.Invest.* 58, 613

[7] Soler, A.P., J. Alemany, R.M. Smith, et al (1990) The state of differentiation of embryonic chicken lens cells determines insulin-like growth factor I internalization *Endocrinology* 127, 595

[8] Widmann, C., W. Dolci, and B. Thorens (1995) Agonist-induced internalization and recycling of the glucagon-like peptide-1 receptor in transfected fibroblasts and in insulinomas *Biochem.J.* 310, 203

[9] York, S.J., L.S. Arneson, W.T. Gregory, et al. (1999) The rate of internalization of the mannose 6-phosphate/insulin-like growth factor II receptor is enhanced by multivalent ligand binding *J.Biol.Chem.* 274, 1164

[10] Mukherjee, S., R.N. Ghosh, and F.R. Maxfield (1997) Endocytosis *Physiol.Rev.* 77,759

[11] Ivanenkov, V., F. Felici, and A.G. Menon (1999) Uptake and intracellular fate of phage display vectors in mammalian cells. *Biochim.Biophys.Acta* 1448, 450-62

[12] Cabibbo, A., E. Sporeno, C. Toniatti, et al. (1995) Monovalent phage display of human interleukin (hIL)-6: selection of superbinder variants from a complex molecular repertoire in the hIL-6 D-helix *Gene* 167, 41

[13] Zwick, M.B., J. Shen, and J.K. Scott (1998) Phage-displayed peptide libraries *Curr.Opin.Biotechnol.* 9, 427-36

[14] Ivanenkov, V.V., F. Felici, and A.G. Menon (1999) Targeted delivery of multivalent phage display vectors into mammalian cells *Biochim.Biophys.Acta* 1448, 463

[15] Pasqualini, R. and E. Ruoslahti (1996) Organ targeting in vivo using phage display peptide libraries *Nature* 380, 364

[16] Pasqualini, R. and E. Ruoslahti (1996) Tissue targeting with phage peptide libraries *Mol.Psychiatry* 1, 423

[17] Mahato, R.I., Y. Takakura, and M. Hashida. (1997) Development of targeted delivery systems for nucleic acid drugs *J.Drug Target.* 4, 337

[18] Mahato, R.I., Y. Takakura, and M. Hashida. (1997) Nonviral vectors for in vivo gene delivery: physicochemical and pharmacokinetic considerations. *Crit.Rev.Ther.Drug Carrier.Syst.* 14, 133

[19] Scott J.K. and Smith G.P. (1990) Searching for peptide ligands with an epitope library *Science* 249(4967), 386-90

[20] Renschler M.F., Bhatt R.R., Dower W.J., et al. (1994) Synthetic peptide ligands of the antigen binding receptor induce programmed cell death in a human B-cell lymphoma. *Proc Natl Acad Sci U S A* 91(9), 3623-7

[21] Roitt, I. M. (1991) Essential Immunology (Oxford/Blackwell, London), pp. 65-84

[22] Alexey V., Terskikh, Jean-Marc Le Doussal, et al. (1997) "Peptabody": A new type of high avidity binding protein *Proc. Natl. Acad. Sci. USA* 94,1663-8

[23] Rose K. and Vizzavona J. (1999) Stepwise solid-phase synthesis of polyamides as linkers *J. Am. Chem. Soc.* 121,7034-8

[24] Efrat S, Linde S, Kofod H, et al (1988) Beta-cell lines derived from transgenic mice expressing a hybrid insulin gene-oncogene. *Proc Natl Acad Sci U S A* 85(23), 9037-41

[25] Smith, G.P. and J.K. Scott (1993) Libraries of peptides and proteins displayed on filamentous phage. *Methods Enzymol.* 217:228.

[26] Offord RE, Gaertner HF, Wells TN, et al. (1997) Synthesis and evaluation of fluorescent chemokines labeled at the amino terminal. *Methods Enzymol* 287, 348-69

[27] Damke H (1996) Dynamin and receptor-mediated endocytosis *FEBS Lett* 389(1), 48-51

[28] Sela M, Zisman E (1997) Different roles of D-amino acids in immune phenomena *FASEB J* 11(6), 449-56

Tables and Figures

Table 1. Synthetic peptide sequences modified from RRTK

Table 2. Core structures used in the project.

Table 3. Panning experiments using βTC-3 cells were performed as described in Methods, with 10^9 phages used at each step of the enrichment procedure. The number of phages recovered at 0°C (no endocytosis) is less 100, indicating that the background of extracellular (but not internalized) bound phages is extremely low in our conditions.

Table 4. Occurrence of phages recovered after three steps of panning in the βTC-3 cell line. 20 isolated phages were sequenced and grouped according to the sequence of the peptide displayed

Table 5. Titration experiments were performed with the phage P1 incubated for the indicated times with βTC-3 cells. Ratio of input/recovered phages is presented

Table 6. Phages (10^8) were incubated for 16 hours with the indicated cell lines, and the number of internalized and recovered phages was calculated. Control phages displaying an integrin internalization motif {Ivanenkov, Felici, et al. 1999 ID: 305} showed a similar number (1-3x10^6) of recovered phages for all cell lines

Figure 1. Structure schemes for Oxime 1 – 3. The branching Lys residues of the cores are acylated on both alpha and epsilon amino groups with the N-terminus of RRTK peptide with or without a spacer. A reporter tag, FITC, together with a spacer is introduced into the core of each construct.

Figure 2. Structure schemes for Oxime 4 – 6. The branching Lys residues of the cores are acylated on both alpha and epsilon amino groups with the N-terminus of RRTK peptide together with a spacer. A reporter tag, Biotin, together with a spacer is introduced into the core of each construct.

Figure 3. Fluoresence microscopy on a tetraoxime (oxime 3). Oxime 3 (tree design) employing four copies of RRTK peptide with 2 units of PEG spacer as building blocks was incubated with βTC-3 cells. After 1 hour, the construct clearly bound to the receptors on the surface of βTC-3 cells and seemed to enter into the cells.

Figure 4. Biotin assay on octaoxime (oxime 6). Oxime 6 possessing 8 copies of RRTK peptide was incubated with βTC-3 cells. Texas Red labeled secondary antibody to the Biotin demonstrated that the octaoxime localized either inside cells as "vacuolar" structures, or at the cell membrane, whereas, a control not.

Table 1: Synthetic peptide sequences modified from RRTK

Peptide 1	*$BrCH_2CO$*-RRTK-OH
Peptide 2	*$BrCH_2CO$-(peg-succ)$_2$*-RRTK-OH
Peptide 3	*H-S-(peg-succ)$_2$*-RRTK-OH (L-form)
Peptide 4	*H-S-(peg-succ)$_2$*-RRTK-OH (D-form)

Table 2: Core structures used in the project.

Core 1	C_4-K_2K-K(succ-peg-S)-amide
Core 2	C-GGG-[K(C)]$_3$-K(succ-peg-S)-amide
Core 3	$(NH_2OCH_2CO)_4$-K_2K-K(succ-peg-Biotin)-amide
Core 3	$(NH_2OCH_2CO)_8$-K_4K_2K-K(GGG-Biotin)-amide

Panning	Phages recovered
1st	$<1x10^3$
2nd	$2x10^5$
3rd	$3x10^8$

Table 3.

Phage	Occurrence
P1	61%
P6	17%
P8	5,5%
P10	5,5%
P65	5,5%
P66	5,5%

Table 4.

Phage	Incubation	% Recovered
P1	1h	0.01
	5h	1
	17h	10

Table 5.

Cells	Phages recovered
βTC-3	$1x10^7$
HeLa	$<1x10^2$
WiDr	$2x10^2$
HepG2	$<10^1$
A549	10^3

Table 6.

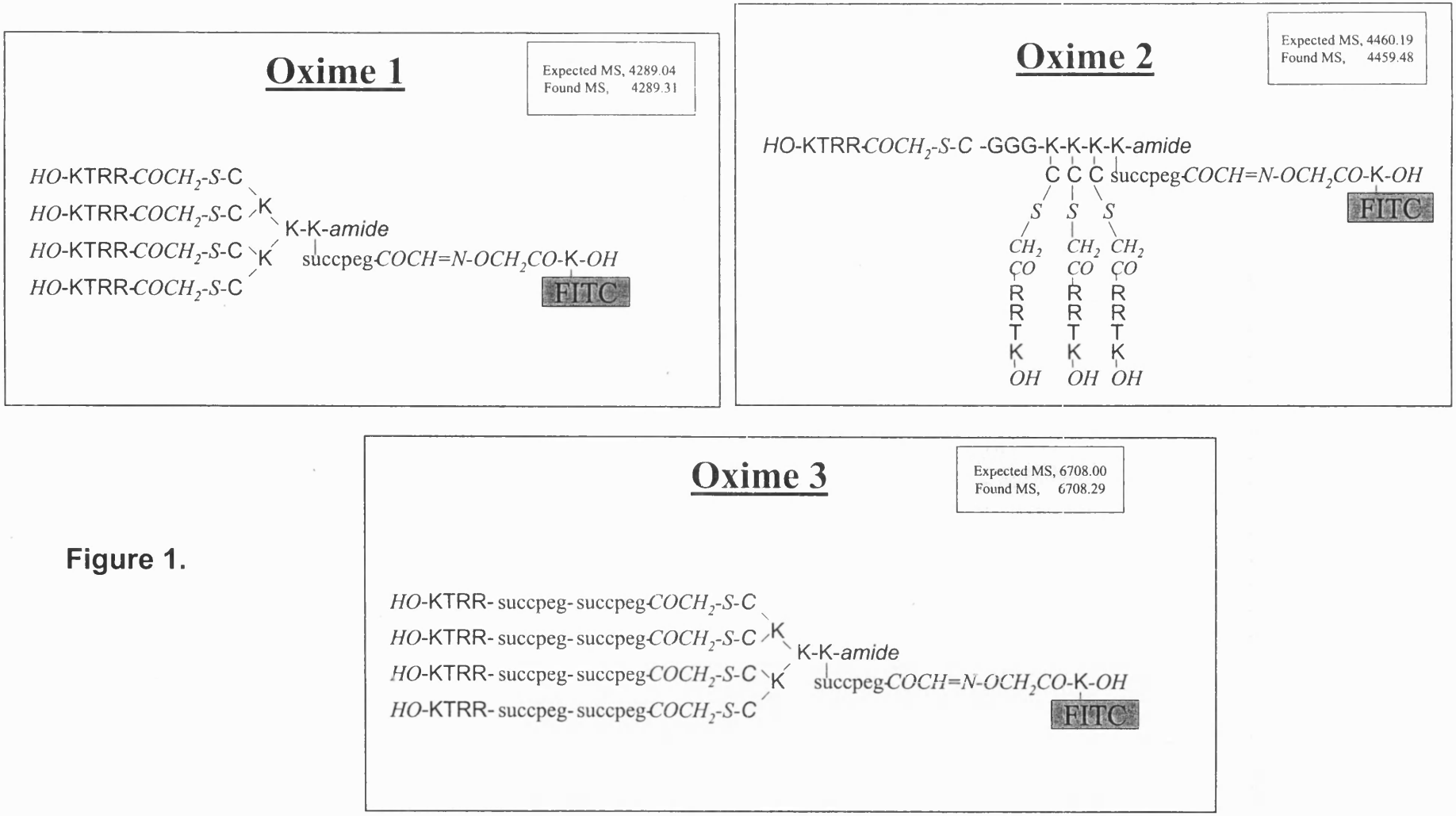

Oxime 1
Expected MS, 4289.04
Found MS, 4289.31
HO-KTRR-COCH2-S-C
HO-KTRR-COCH2-S-C
HO-KTRR-COCH2-S-C
HO-KTRR-COCH2-S-C
K
K
K-K-amide
succpeg-COCH=N-OCH2CO-K-OH
FITC
Oxime 2
Expected MS, 4460.19
Found MS, 4459.48
HO-KTRR-COCH2-S-C -GGG-K-K-K-K-amide
succpeg-COCH=N-OCH2CO-K-OH
FITC
Oxime 3
Expected MS, 6708.00
Found MS, 6708.29
HO-KTRR- succpeg- succpeg-COCH2-S-C
HO-KTRR- succpeg- succpeg-COCH2-S-C
HO-KTRR- succpeg- succpeg-COCH2-S-C
HO-KTRR- succpeg- succpeg-COCH2-S-C
K
K
K-K-amide
succpeg-COCH=N-OCH2CO-K-OH
FITC

Figure 1.

Figure 2.

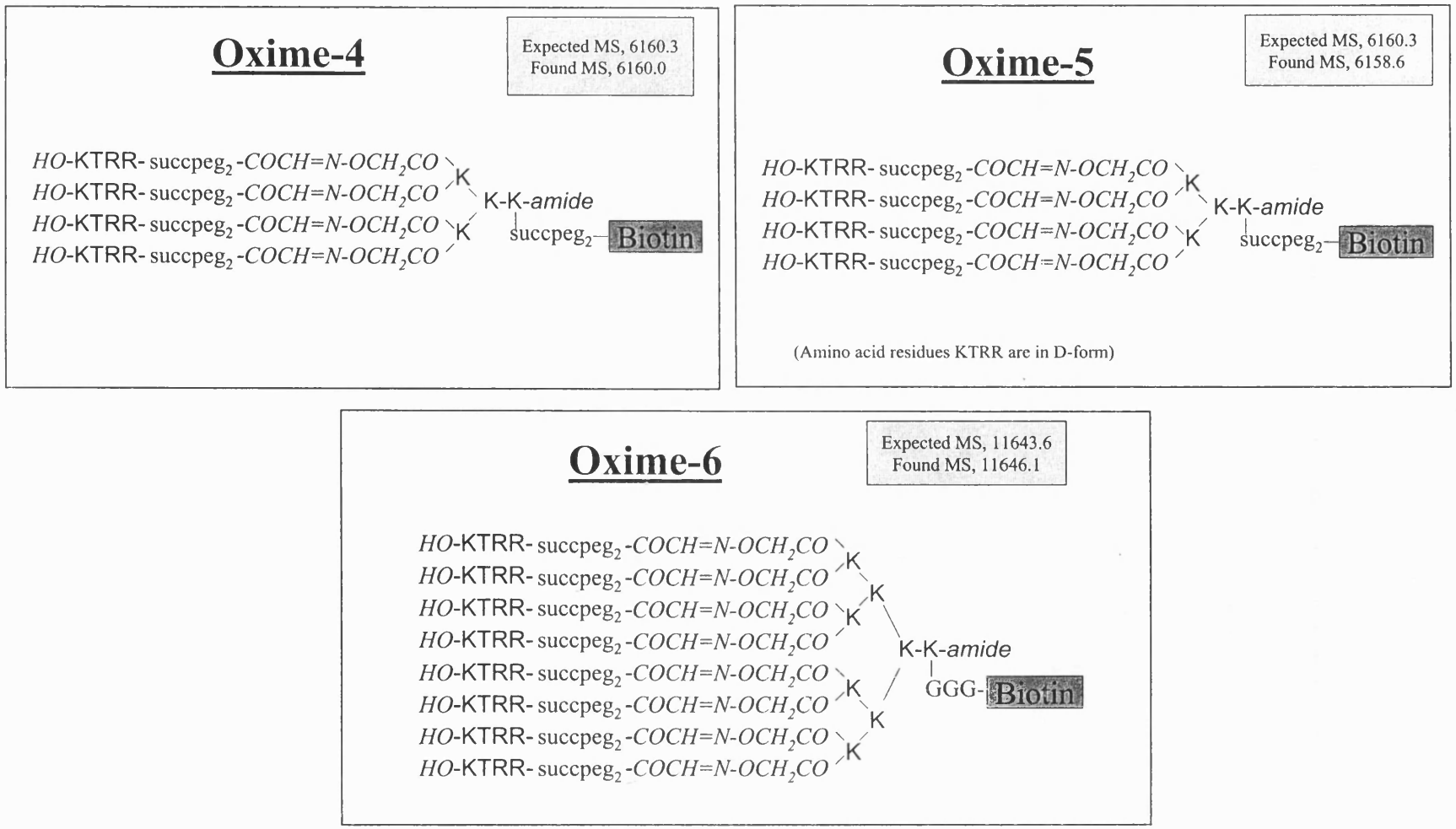

Oxime-4
Expected MS, 6160.3
Found MS, 6160.0
HO-KTRR- succpeg2 -COCH=N-OCH2CO
HO-KTRR- succpeg2 -COCH=N-OCH2CO
HO-KTRR- succpeg2 -COCH=N-OCH2CO
HO-KTRR- succpeg2 -COCH=N-OCH2CO
K
K
K-K-amide
succpeg2
Biotin
Oxime-5
Expected MS, 6160.3
Found MS, 6158.6
HO-KTRR- succpeg2 -COCH=N-OCH2CO
HO-KTRR- succpeg2 -COCH=N-OCH2CO
HO-KTRR- succpeg2 -COCH=N-OCH2CO
HO-KTRR- succpeg2 -COCH=N-OCH2CO
K
K
K-K-amide
succpeg2
Biotin
(Amino acid residues KTRR are in D-form)
Oxime-6
Expected MS, 11643.6
Found MS, 11646.1
HO-KTRR- succpeg2 -COCH=N-OCH2CO
HO-KTRR- succpeg2 -COCH=N-OCH2CO
HO-KTRR- succpeg2 -COCH=N-OCH2CO
HO-KTRR- succpeg2 -COCH=N-OCH2CO
HO-KTRR- succpeg2 -COCH=N-OCH2CO
HO-KTRR- succpeg2 -COCH=N-OCH2CO
HO-KTRR- succpeg2 -COCH=N-OCH2CO
HO-KTRR- succpeg2 -COCH=N-OCH2CO
K
K
K
K
K
K-K-amide
GGG-
Biotin

Figure 3.

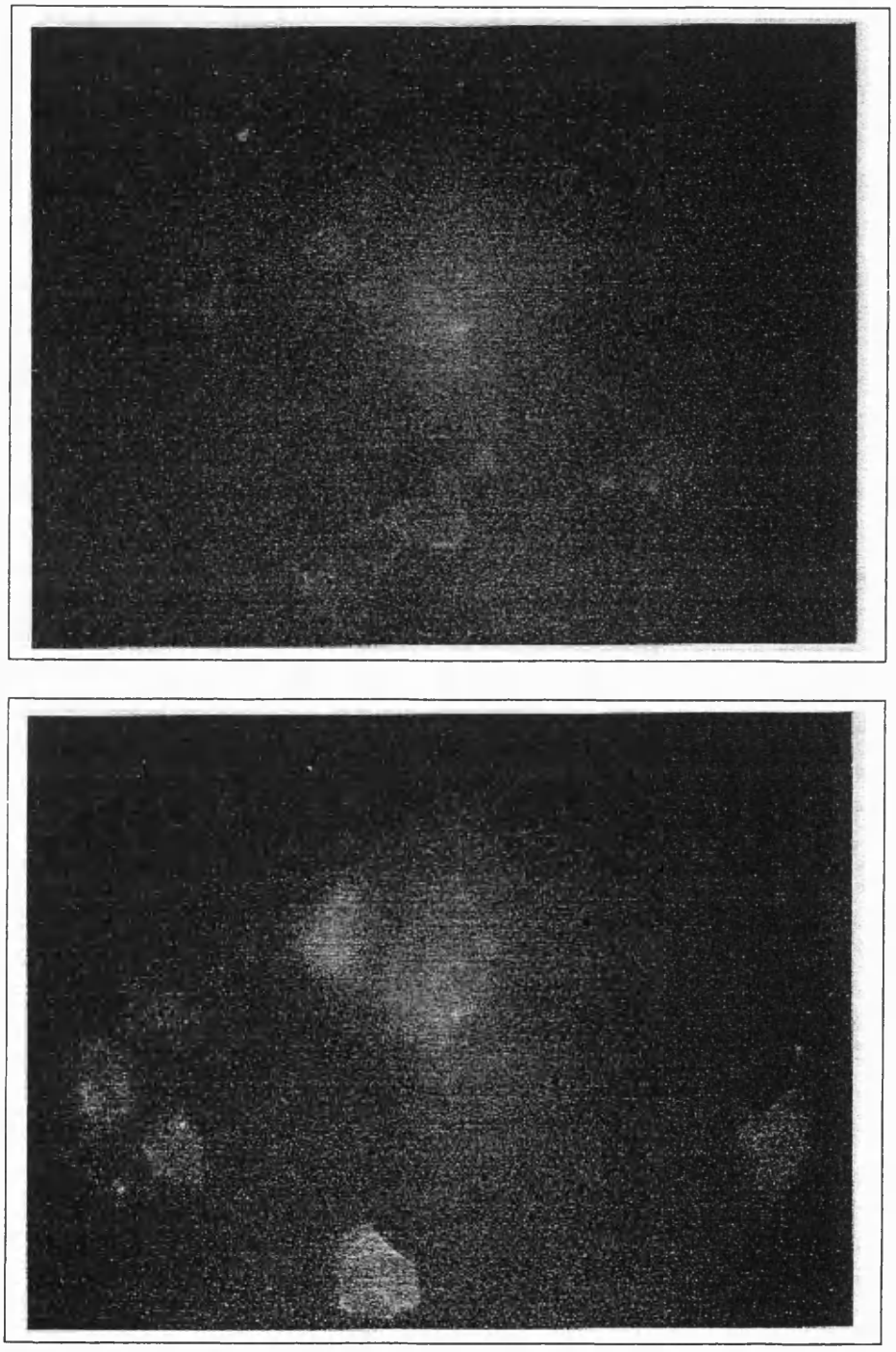
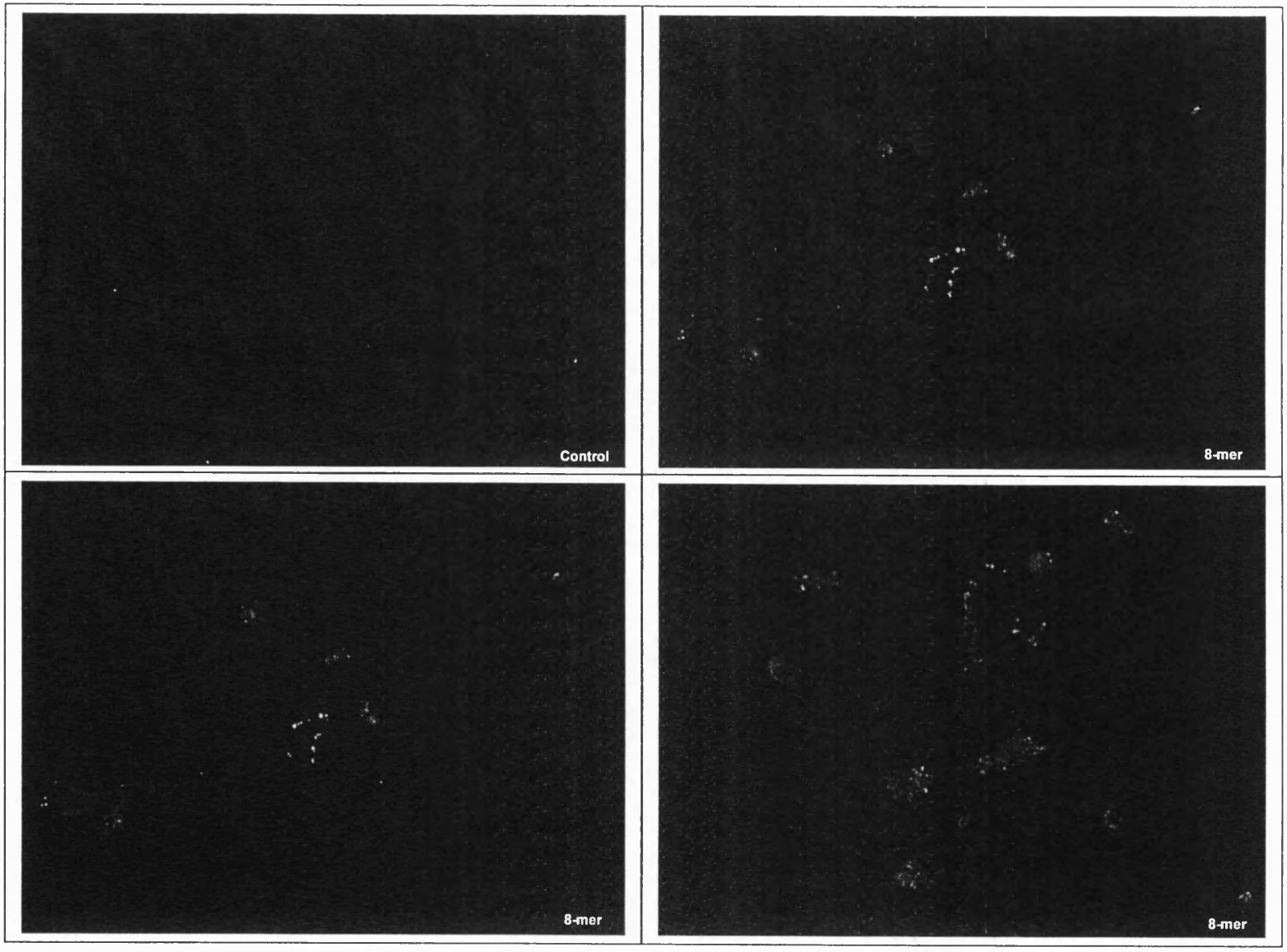


Figure 4



D. Acknowledgements

I would like to thank Prof. Keith Rose for giving me the opportunity to do my Ph.D. in his group and for his kind supervision and constant support during my study. I would also like to thank Prof. Anthony Rees for accepting me toward my Doctorate in the University of Bath. I extend my thanks to Prof. Robin Offord, the director of the Department of Medical Biochemistry, University of Geneva. I owe my gratitude to Dr. Alan Wheals, Dr. Hubert Gaertner, Dr. Oliver Hartley and Dr. Matteo Villain for their kind guidance during my study; and to Dr. Christophe Bonny, Dr. Weiguang Zeng and Dr. Hanna Kelker for good collaboration. Many thanks also to Irène Rossitto-Borlat and Pierre-Olivier Regamey. I also thank all other members at the Department of Medical Biochemistry, University Medical Center in Geneva, Switzerland as well as at the Department of Biology and Biochemistry, University of Bath in U.K.

Especially, I would like to thank my wife, Ms Zhang Yufen, and my son, Chen Mai, for supporting my studies.

E. BIBLIOGRAPHY

Dissertations

Molecular typing for the strain variability of *Helicobacter pylori*

Supervised by Prof. M. Fred and Dr. med. P. Bauerfeind
Conferred *Doctor degree of Medicine* by University of Zurich, Switzerland

Detection of the related sequence of *Human Papillomavirus*-16 in gastrointestinal tumors by Polymerase Chain Reaction

Supervised by Prof. Yu Jieping and Prof. Sheng Zhixiang
Conferred *Master of Medicine* by Hubei Medical University, PR China

List of Publications

1. Rose K., Chen J., Dragovic M, etc. (1999) New cyclization reaction at the amino terminus of peptides and proteins *Bioconjug Chem* 10(6):1038-43
2. Chen J. Yu J.P. and Shen Z.X. (1996) The relation between *Human Papillomavirus* and colorectal carcinoma in Human. *Chin J Gastroenterol & Hepat* 5(1): 45-8
3. Chen J. and Yu J.P. (1995) Progress of molecular biology in gastrointestinal tumors (review). *Foreign Med* 22(3):121-3
4. Yu J.P. and Che

F. CURRICULUM VITAE

Name:	Jianhua CHEN
Gender:	Male
Birth/place	5 May 1965, Hubei, PR China
Health:	Excellent
Marital status:	Married with one child
Home address:	3, rue Dancet, 1205 Geneva, Switzerland
Work address:	Department of Medical Biochemistry, Center Medical University (CMU), Geneva, Switzerland; Tel. +41/22-702 5523, Fax. +41/22-7025502 Email. jianhua.chen@medicine.unige.ch
Current post:	**Ph.D. candidate**, joint supervised by Department of Medical Biochemistry, Center Medical University, (CMU), Geneva, Switzerland; and Department of Biology and Biochemistry, University of Bath, U.K.
Education:	1998-present: **Ph.D. candidate in Biochemistry** Department of Medical Biochemistry, CMU, Geneva, Switzerland Department of Biology and Biochemistry, University of Bath, UK 1996-1997: **Doctor of Medicine** Faculty of Medicine, University of Zurich, Switzerland 1990-1993: Master of Medicine 1st Affiliated Hospital of Hubei Medical University, PR China 1983-1988: Bachelor of Medicine Faculty of Medicine, Hubei Medical University, PR China
Positions:	Aug.1993 – Dec.1995: Research Assistant Lab of Digestive Medicine, Division of Gastroenterology 1st Affiliated Hospital of Hubei Medical University, PR China Aug.1988 – Aug.1990: Clinic assistant Div. of Gastroenterology, Department of Internal Medicine 1st Affiliated Hospital of Hubei Medical University, PR China

Research experiences:

(1) Study on artificial proteins as vaccines and as binding moities
(supported by NIH and by Swiss National Science Foundation)
(2) Strain variability of *Helicobacter pylori* in corpus and antrum in humans
(supported by Swiss National Science Foundation)
(3) Identification and characterization of *Human Papillomavirus*-16 in the carcinomas of esophagus, stomach and colon
(supported by China National Science Foundation)

Research Skills:

Bio-organical chemistry

- 'Nuts and bolts' protein engineering at protein level
- Design and synthesis of peptide/derivatives for specific use
- Purification of proteins/peptides by HPLC, and gel filtration
- Peptide cleavage (TFMSA cleavage)
- Charaterization of proteins/peptides by ESI-MS, MALDI-TOF, ELISA, SDS-PAGE, 2D-electrophoresis

Biology

- Cell and Bacterial culture
- DNA isolation from tissue and cell culture
- Primers design and PCR assay
- Restriction enzyme analysis
- Cloning
- DNA Sequencing
- Preparation of DIG-labelled DNA probes
- Southern blot hybridization assay

Others

- Organization of laboratory
- Instrument maintenance and trouble-shooting.
- Student teaching

Language ability: English, fluent in speaking, listening, reading and writing.