PYRUVATE KINASE ISOENZYMES

PROEFSCHRIFT

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Voor Astrid,
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INTRODUCTION

Pyruvate kinase (EC. 2.7.1.40) catalyzes the following reaction

$$\text{Phospho-enolpyruvate} + \text{ADP} \longrightarrow \text{Pyruvate} + \text{ATP}$$

In liver this reaction is not only important as the last step of glycolysis, but it also needs to be inhibited under conditions in which P-enolpyruvate is synthesized during gluconeogenesis. The start of the investigations reported here was related to the mechanism of this inhibition.

STATEMENT OF THE PROBLEM

From the early investigations of the group of Tanaka[1-3] it was clear that several isoenzymes of pyruvate kinase exist. At the end of 1970 two types of pyruvate kinase were distinguished, the Liver- (L) and Muscle (M) type. The so-called L-type, the predominant type in liver, was known to show an allosteric response to one of its substrates, phospho-enolpyruvate, and its activity was further found to be influenced by glycolytic and gluconeogenic intermediates. Fru-1,6-P_2[2] and other phosphorylated hexoses[4] increase the apparent affinity for the substrate P-enolpyruvate. On the other hand ATP[3] and alanine[5] act as allosteric inhibitors. Furthermore the amount of enzymes is influenced by the diet and/or hormones[6].

The M-type of pyruvate kinase was considered to be a non-allosteric enzyme, and hormones and/or diet have no influence on the amount of enzyme present. The main differences between both isoenzymes known at the start of this investigation are summarized in TABLE I.

TABLE I

PROPERTIES OF L- AND M-TYPE PYRUVATE KINASES (known at the end of 1970)[2]

	M-type	L-type
Mol. weight	250,000	208,000
K_m ADP	0.27 mM	0.10 mM
$K_{0.5}$ P-enolpyruvate	0.075 mM	0.84 mM
K_i ATP	3.5 mM	0.10 mM
activators	unknown	Fru-1,6-P_2 and other phosphorylated hexoses[4]
inhibitors	ATP	ATP, alanine[5]
kinetics	Michaelis-Menten	allosteric[7-9]

The L-type was known to be present in liver[2], kidney[5] and erythrocytes[2], while the occurrence of the M-type was reported for liver, muscle, leucocytes, brain, heart and hepatoma cells[2]. The pyruvate kinase from adipose tissue was considered to be an intermediate type[10,11] with properties of both L- and M-types. For a more detailed review reference is made to Seubert and Schoner[12].

As already mentioned, this study was started with the problem of the mechanism to prevent pyruvate kinase activity under gluconeogenic conditions. The liver contains both L- and M-type pyruvate kinases. If both isoenzymes were located in the same compartment, the regulation of the allosteric L-type would be useless, since the M-type has a high affinity for P-enolpyruvate and its activity is not diminished during gluconeogenesis. Keeping in mind that the liver is quite a heterogeneous organ, containing parenchymal and non-parenchymal cells, the study of the cellular localisation of both L- and M-types was the start of the present study.

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CHAPTER I

INTERCELLULAR DISTRIBUTION AND REGULATION OF THE PYRUVATE KINASE ISOENZYMES

A. Intercellular distribution of liver pyruvate kinases.

The presence of both L- and M-type pyruvate kinase in the same cell would make the regulation of the enzyme with the lower apparent affinity for P-enolpyruvate (that is the L-type) rather useless. This regulatory problem would be overcome if the isoenzymes were located in different cells (parenchymal and non-parenchymal cells) or by a different compartmentation in the same cell. To test the first possibility rat liver parenchymal and non-parenchymal cells were separated from each other and the isoenzyme distribution in these cells was investigated (Appendix paper I). It could be shown that the L-type pyruvate kinase is the only type present in the parenchymal cell while the Kupffer cell preparations are enriched with the M-type as compared to a total liver homogenate. These Kupffer cell preparations still contained considerable L-type activity. At about the same time Crisp and Pogson[1] obtained similar results for mouse liver and subsequently Bonney et al.[2] and Garnett et al.[3] confirmed our findings for rat liver.

Later, the liver cell isolation techniques were improved (Appendix paper II). With the aid of collagenase and hyaluronidase[4] it is possible to isolate intact liver cells. A further purification of the non-parenchymal cell preparation made it possible to exclude the occurrence of the L-type pyruvate kinase in these cell types.

From the localisation studies on pyruvate kinase reported here

the conclusion can be reached that gluconeogenesis in liver is confined to the parenchymal cells. This is supported by Crisp and Pogson[1], who reported that the gluconeogenic enzymes glucose-6-phosphatase and fructose-1,6-phosphatase are respectively solely and mainly located in the parenchymal cell.

The high peroxidase and relatively low catalase activities found in the non-parenchymal cells as compared to the parenchymal cells are consistent with the antimicrobial function of these cells in liver[5]. Localisation of peroxidase in the non-parenchymal cells and a low activity of catalase in these cells as compared to parenchymal cells, had been suggested earlier by histochemical[6] and ultrastructural[7] studies.

B. Regulation of L-type pyruvate kinase from rat liver

Tanaka and co-workers[8-10] have shown that the L-type pyruvate kinase from rat liver is under allosteric control. These findings were further explored by Carminatti et al.[11], Rozengurt et al.[12] and De Asúa et al.[13] who proposed that the allosteric model of Monod et al.[14] was valid for this isoenzyme. The enzymatic activity of the L-type can be modulated to a great extent (for a review see Seubert and Schoner[15]). In Appendix paper III is shown that Glc-1,6-P_2 is an allosteric activator of the L-type. Due to the presence of 15 μM Glc-1,6-P_2 in liver (a concentration which does not fluctuate between glycolytic and gluconeogenic conditions[16]) the regulation of the already activated enzyme by the fluctuating Fru-1,6-P_2 concentrations will be impossible. An active enzyme under gluconeogenic conditions, however, seems unlikely as the substrate P-enolpyruvate is needed for glucose formation. Appendix paper IV shows that the enzyme possesses additional properties to regulate its apparent affinity for P-enolpyruvate and its allosteric activators. An oxidation of thiol groups can result in an enzyme which will be inactive in the presence of effector concentrations found under gluconeogenic conditions[17]. A decreased sensitivity of the enzyme for its activators is also brought about by a lowering of the P-enolpyruvate concentration while in the presence of the physiological inhibitors alanine and ATP the decrease in

affinity is enlarged (Appendix paper V). It could be shown that in the presence of the physiological concentrations of ADP, P-enolpyruvate, GSH, ATP and alanine the enzyme is inactive. The only effective activator in a physiological concentration range is Fru-1,6-P_2. However, even the lowest reported Fru-1,6-P_2 concentration (5 μM)[18] will result in a nearly fully active enzyme. The same observation was made by Llorente et al.[19] and it has been suggested by Sols and Marco[18] that due to the binding of protein (mainly aldolase) the free Fru-1,6-P_2 concentration will be lowered. Two other additional possibilities exist: a) A change in the enzyme itself can influence its apparent affinity for Fru-1,6-P_2, as is shown in Appendix papers IV and V. b) The heterogeneity of the liver can influence the Fru-1,6-P_2 concentration available for the L-type (Appendix paper V). However, at the moment no definite answer can be given to the question of the extent to which the three possibilities mutually contribute to the overall regulation of pyruvate kinase by Fru-1,6-P_2.

As already mentioned the group of De Asúa et al.[11-13] applied the allosteric model of Monod et al.[14] to L-type pyruvate kinase from rat liver to explain the properties found. This model has also been applied to the L-type from other mammalian cells[20,21] and yeast[22-25]. The phosphorylated hexoses[26] can serve as an excellent tool to test the conformation of the activator site (which is a reflection of the conformation of the enzyme; see Appendix paper V). By comparison of the apparent affinities for various phosphorylated hexoses in the presence of ATP and alanine we were able to show that the latter two compounds introduce a different conformation probably because each binds to a different site. This conclusion is confirmed by stability experiments at 53°C. Alanine and ATP both stabilize the enzyme at this temperature. However, when under saturating alanine concentrations ATP is added, a further stabilization occurs. The same phenomenon is observed when alanine is added to the ATP-saturated enzyme. Rozengurt et al.[27] showed recently that the allosteric activators K^+ and Fru-1,6-P_2 each induce a different conformation. Together with our data this means that the model of Monod et al.[14] can no longer give an adequate description of

the properties of the L-type pyruvate kinase, and the evidence we obtained leads us to conclude that sequential conformation changes[28] are involved in the allosteric transitions of the enzyme.

C. Regulation and properties of the M-types of pyruvate kinase

Jimenez de Asúa et al.[29] showed in 1971 that the M-type pyruvate kinase from rat liver is inhibited by a series of amino acids while the M-type from muscle is only inhibited by phenylalanine[30], suggesting heterogeneity of M-type pyruvate kinases in various organs. For diagnostic purposes we are interested in the identification of certain leucocyte enzymes, since sometimes a loss of activity of a liver enzyme is reflected by a loss of the same type of enzyme in the leucocytes[31-33]. So we decided to investigate the properties of the leucocyte enzyme more closely (Appendix paper VI). From the amino acid inhibition pattern it could be concluded that the leucocyte enzyme resembles the M-type from liver and that it differs from the muscle M-type.

It was further found that the amino acid inhibition could be fully relieved by the addition of Fru-1,6-P_2 (Appendix paper VI). These are properties, which usually are only found with allosteric enzymes. Therefore our interest was focussed on the kinetics of the enzyme. It appears that the M-type from leucocytes exhibits allosteric properties towards P-enolpyruvate in the presence of a physiological concentration of alanine (1 mM). Therefore, we extended the proposed $R \rightleftharpoons T$ model of the L-type also to the various M-types. Since the amino acid inhibition pattern of the leucocyte enzyme was similar to that of the liver M-type, we suggested that also the liver M-type possessed allosteric properties; this suggestion is in contrast to earlier reports[8,29,34] on the properties of this isoenzyme. Appendix paper VII shows that the amino acid inhibition is indeed of an allosteric nature, and that in the presence of alanine the response to P-enolpyruvate is sigmoidal. From the pH dependence of the $K_{0.5}$ for P-enolpyruvate it can be concluded that the enzyme has a pK_a value of 7.5. This finding is supported by the electrophoretic study of Whittell et al.[35], in which it was found that this isoenzyme at pH 7.0

slightly migrated to the anode and that at pH 8.0 the migration was greatly enhanced, whereas the mobilities of the other isoenzymes did not change.

In the presence of alanine, the activity as a function of the P-enolpyruvate concentration is characterized by two different n values, which suggests the possible presence of two interconvertible forms of M-type pyruvate kinase. These forms should then possess different affinities for alanine and/or P-enolpyruvate (Appendix paper VII).

From the electrophoratic study of Imamura et al.[36,37] it is clear that the pyruvate kinase from adipose tissue behaves similar to the M-type from liver. Pogson[38,39] showed for the adipose tissue enzyme that it can be isolated in two different forms depending on the isolation procedure. Isolation of the adipose tissue enzyme in the presence of EDTA yields an enzyme (called by Pogson Pyk-A) with a lower apparent affinity for P-enolpyruvate ($K_{0.5}$ of 0.6 mM). The enzyme isolated in the absence of EDTA (Pyk-B) has a relatively high apparent affinity for P-enolpyruvate ($K_{0.5}$ of 0.067 mM). Walker and Potter[40] further found that both these forms are present in cultured liver cells and that their relative concentrations are influenced by the culture conditions. Culture in the absence of added glucose results in an enzyme with properties identical to the Pyk-A form of Pogson[38,39], while the Pyk-B form is obtained when the cells are cultivated in the presence of added glucose. Although it is known that the enzyme pattern of the cell can be changed during cultivation (for example the L-type pyruvate kinase normally present in liver cells is replaced by the M-type), this finding suggests that the Pyk-A form of the M-type of pyruvate kinase is probably not only an isolation artifact. The presence of both forms in our M-type preparation of rat liver could explain the two different n values obtained in the presence of 1 mM alanine (Appendix paper VII). We, therefore, investigated the possibility whether both these forms can occur under physiological conditions (Appendix paper VIII). It appears that with Mg^{2+}_{free} concentrations reported to be present in vivo[41] two forms of the enzyme are present. In Appendix paper VIII a scheme is presented which explains the kinetic properties found. Recent reported studies[42-44]

have provided evidence that in the B⇌A equilibrium a tetramer-dimer transition may be involved.

The study of the B⇌A transition showed (Appendix paper VIII) that a prolonged storage of the enzyme in the A form makes the transition to the B form more difficult. This property allows the detection of the A form. When after isolation of the enzyme under conditions which favour the B form, the A form is still found, the conclusion is justified that the A form was present in vivo. With this approach we investigated the enzyme from liver, leucocytes, thrombocytes, intestine and fibroblasts (kindly made available by Dr. M.F. Niermeyer, Dept. of Cell Biology and Genetics). Only with rat small intestine a considerable amount of the enzyme was found in the A form (Appendix paper IX). This means that at least in rat small intestine the A form of M_2-type pyruvate kinase is present in vivo[45]. This finding is supported by Osterman and Fritz[46], who state that their electrophoretic finding might be related to our kinetic observations with the M-type of liver.

The physiological meaning of the B⇌A equilibrium is not completely clear at the moment. The main physiological regulator seems to be Fru-1,6-P_2 (Appendix paper VIII). At low Fru-1,6-P_2 levels the equilibrium can be shifted to the A form which is more sensitive to amino acid inhibition. This shift may induce a glucose-sparing effect and can be of importance during limited carbohydrate availability. Further investigations in intact cells (leucocytes) are however necessary to test this hypothesis.

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CHAPTER II

ON THE MOLECULAR BASIS OF ERYTHROCYTE PYRUVATE KINASE DEFICIENCY

It was shown by Ibsen et al.[1], Jacobson and Black[2] and Staal et al.[3] that purified erythrocyte pyruvate kinase is very similar to the L-type pyruvate kinase from rat liver. During our investigations on the regulatory properties of L-type pryuvate kinase from rat liver we found that the kinetic properties of this type are markedly influenced by the reduction state of the thiol groups (Appendix paper IV). The lack of relationship between the pyruvate kinase activity and the degree of hemolysis in erythrocyte pyruvate kinase deficient patients prompted us to investigate the possibility that the alteration in pyruvate kinase might be a secondary defect. Because the reduction state of the thiol groups in the red blood cell is important for the lifespan of these cells[4,5], the degree of reduction could form a link between altered pyruvate kinase and the increased hemolytic activity found in the pyruvate kinase deficient patients.

Appendix paper X shows that the kinetic properties of the erythrocyte enzyme incubated with oxidized glutathione are identical with the kinetic data described in the literature for pyruvate kinase from most of the pyruvate kinase deficient patients. Also the thermostability of the oxidized enzyme and the enzyme from pyruvate kinase deficient patients seems to be identical[6]. These properties suggest that pyruvate kinase of the hemolytic patients might be altered by oxidation of the thiol groups of the enzyme. Further support for this possibility is the description of patients with a reduced glutathione reductase activity[7-9]. Deficiency in glutathione reductase increases the GSSG concentration which might affect pyruvate kinase.

Another way to approach the molecular basis of pyruvate kinase deficiency is presented in Appendix paper XI. Incubation of abnormal pyruvate kinase, from the class of patients characterized by a loss of allosteric interactions towards the substrate P-enolpyruvate, with mercaptoethanol changes the kinetics from abnormal to normal. From a comparison of the ATP inhibition curves of pyruvate kinase from the patients with the normal enzyme, it could be concluded that the loss of allosteric behaviour towards P-enolpyruvate was not the consequence of a loss of cooperative interaction between the subunits, but could be explained by a shift in the $R \rightleftharpoons T$ equilibrium to the R state (Appendix paper XI). The possibility of restoring abnormal kinetics to normal by in vitro incubation indicates that the abnormality observed in freshly isolated cells might be a secondary effect. Since thiol groups are involved in this alteration one patient (M.V.), who possesses also a lowered glutathione reductase activity[10], was treated with riboflavine. This treatment restored the glutathione reductase activity to its normal value. Furthermore when after six months treatment pyruvate kinase was isolated from the patient's red blood cells, the kinetic behaviour towards P-enolpyruvate appeared to be normal[11]. The clinical state of this patient had improved too as could be concluded from hematological data. During the six months treatment with riboflavine no blood transfusions were necessary, while before treatment these were required every month.

Of course the afore-mentioned hypothesis does not exclude the occurrence of a real pyruvate kinase deficiency as a primary cause for hematological diseases[12-15]. However, due to the high sensitivity of the enzyme to its cellular environment, an altered pyruvate kinase does not mean that this abnormality is the primary lesion for an increased hemolysis. By measurements of other enzymes a second abnormality may be detected and a therapy based upon this defect may help the patients.

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CHAPTER III

SUMMARY OF THE PROPERTIES OF THE VARIOUS ISOENZYMES OF PYRUVATE KINASE

In this chapter the properties of the various isoenzymes are summarized in TABLES and are meant to facilitate the comparison of the properties of the isoenzymes. TABLE I shows the nomenclature used by the most important groups working in this field. The different nomenclature is rather confusing and has not contributed to a clear understanding of the relation between the various isoenzymes. After the first report of Tanaka et al.[9] on the existence of two isoenzymes of pyruvate kinase, called L-type and M-type, the uniformity was given up by the groups of Susor[17] and Bigley[19] using their own nomenclature. Because there was no clear understanding of the relationship between the various isolated forms of pyruvate kinase, it was common use to give simply the source of the isolated enzyme. From the work of Jiménez de Asúa et al.[21] it became apparent that the M-type from liver was distinct from the M-type of muscle. Subsequently, Imamura et al.[1,2] were able to show by electrophoretic studies that the isoenzymes could be divided in three major forms; called L-type, M_2-type and M_1-type. This made it possible to identify the adipose tissue enzyme, which was studied earlier in detail[3,4], as M_2-type.

A comparison of the properties of the kidney enzymes, which were studied by Costa et al.[7,8], indicate that their type-II is similar to the L-type from liver. Since the so-called type-I from kidney is isolated in the presence of EDTA and the kinetic properties are identical to the Pyk-A form of the M_2-type, it seems likely that the A form of M_2-type pyruvate kinase is responsible for the "unique properties" ascribed to type-I. Carbonell et al.[11,12] suggested the

TABLE I

THE VARIOUS NOMENCLATURE AS THEY ARE USED IN THE LITERATURE

Author	Notation				
Imamura et al.[1,2]	L-Type		M_2-type	M_1-type	Erythrocyte Type
Pogson[3,4]		Pyk A	Pyk B		
Walker et al.[5,6]	Type I	Type III_A	Type III_B	Type II	
Costa et al.[7,8]	Type II	Type I			
Tanaka et al.[9,10]	L-Type		M-Type	M-Type	L-Type
Carbonell et al.[11,12]	Class L		Class A	Class M	
Ibsen et al.[13,14]	Type L		Type K	Type M	
Osterman et al.[15,16]	PK-1	PK-2	PK-4	PK-3	
Susor et al.[17,18]	Pyk-B		Pyk-C	Pyk-A	Pyk-D
Bigley et al.[19,20]	PK-I	PK-II	PK-III	PK-III	PK-I

use of the term Class A for the M_2-type because this enzyme was first investigated in adipose tissue[3,4] while Ibsen et al.[13,14] use the term type-K because this group studied this type first in kidney. Osterman et al.[15,16] follow the IUPAC-IUB Commission recommendations which are based upon the electrophoretic mobilities of the enzymes. However, this electrophoretic behaviour is dependent upon the pH and the buffer system used. (For example the mobility of the L-type is greatly influenced by the concentration of Fru-1,6-P_2 present in the buffer system[15,16,18].) Osterman et al.[15,16] prepare their tissue homogenates in the presence of EDTA and for this reason they detect two activity bands after electrophoresis of enzyme preparations from tissues which only contain M_2-type. Bigley et al.[19,20], too isolate the pyruvate kinase enzymes in the presence of EDTA (10 mM), a condition which again results in the presence of the Pyk-A form of the M_2-type (called by Bigley et al.[19,20] PK II (See TABLE I).

The kinetic properties of the purified erythrocyte pyruvate kinase are nearly identical to the L-type from liver[22]. However, Imamura et al.[1,2] showed that in fresh hemolysates the electrophoretic behaviour is slightly different from the L-type from liver. It is stated by Imamura et al.[23] that the enzyme in the hemolysate contains subunits of both L- and M_2-type. Purification of this enzyme can easily lead to a dissociation into subunits, while during reassociation preferentially L- and M_2-type isoenzymes are formed.

TABLE II shows the tissue localisation of the various isoenzymes. This TABLE is mainly based upon the electrophoretic studies of Imamura et al.[1]. The relative amounts of the L- and M_2-type in liver and kidney are valid for feeding conditions[12]. The amount of L-type can be influenced to a great extent (in liver from 97 µmol/min/g tissue under glycerol feeding to 17 µmoles/min/g tissue under 48 h of starvation [12]). Sandoval et al.[12] stated that in contrast to the adaptive L-type, the M_2-type is constitutive. However, for rat small intestine it was shown that in the tissue the M_2-type could be adaptive (Osterman and Fritz and unpublished results).

TABLE II

THE OCCURRENCE OF THE ISOENZYMES IN THE VARIOUS MAMMALIAN TISSUES

Tissue	Type of isoenzyme (nomenclature of Imamura et al.[1,2])			
	L-Type	M_2-Type	M_1-Type	Erythrocyte Type
Liver	90% (parenchymal cells)	10% (non-parenchymal cells)		
Kidney	30%	70%		
Adipose tissue		+		
Leucocytes		+		
Thrombocytes		+		
Fibroplasts		+		
Intestine		+		
Testis		+		
Ehrlich ascites turnover cells		+		
Hepatomas		+		
Placenta		+		
Spleen		+		
Stomach		+		
Lung		+		
Ovary		+		
Muscle			+	
Heart			+	
Brain			+	
Erythrocytes				+

TABLE III summarizes the kinetic constants of the main effectors for the various isoenzymes. The reported constants are valid at pH 7.5 and were measured under comparable circumstances. With all three major isoenzymes the kinetic behaviour towards ADP obeys Michaelis-Menten kinetics with about the same K_m values. In contrast to the equal apparent affinity for the substrate ADP the $K_{0.5}$ for P-enolpyruvate of the L-type is markedly higher as compared to both M-types. This difference disappears in the presence of high Fru-1,6-P_2 concentrations. The main difference between the M_2-type and L-type is the relative insensitivity of the M_2-type towards ATP inhibition. Recently Dunaway and Weber[24,25] pointed out that also for the two forms of phosphofructokinase in rat liver the main difference is related to the difference in ATP sensitivity. Whether these differences in ATP inhibition are related to the regulation of gluconeogenesis deserves further attention. This relation between phosphofructokinase and pyruvate kinase is of special interest because, as pointed out in Appendix paper V and VIII, the activity of both L- and M_2-type are dependent upon the product of the phosphofructokinase reaction Fru-1,6-P_2.

TABLE III
COMPARISON OF THE VARIOUS PROPERTIES OF THE ISOENZYMES

Property		L-Type	M_2-Type		M_1-Type
Mol. W.		208.000	Pyk A 100,000	Pyk B 200,000	250,000
K_m ADP	(mM)	0.27	0.30	0.30	0.28
$K_{0.5}$ P-enolpyruvate	(mM)	0.8	0.5	0.05	0.075
$K_i^{(app)}$ ATP	(mM)	0.1	-	-	3.5
$K_i^{(app)}$ ala	(mM)	0.10	0.01	0.10	-
$K_i^{(app)}$ phe	(mM)	1.0	0.01	0.07	5
Relief of amino acid inhibition by Fru-1,6-P_2		complete	none	complete	none
Kinetics		allosteric, sequential model	allosteric	allosteric	allosteric

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SUMMARY

The intercellular distribution of the isoenzymes of pyruvate kinase of rat liver has been investigated. The L-type pyruvate kinase is located in the parenchymal cells. The M-type is located in the non-parenchymal cells. From this distribution we can conclude that the non-parenchymal cells cannot significantly contribute to gluconeogenesis. The intercellular distribution of catalase and peroxidase also showed main functional differences between non-parenchymal and parenchymal cells, the former only possessing a major phagocytic and antimicrobial action.

The regulatory properties of the various types of pyruvate kinase were investigated. The kinetic behaviour of the L-type pyruvate kinase from rat liver can be influenced to a great extent by the reduction state of the thiol groups of the enzyme. It can be concluded that under physiological effector concentrations the enzymatic activity is mainly dependent upon the Fru-1,6-P_2 concentration in the parenchymal cells. A comparison of the effect of various phosphorylated hexoses on the L-type leads to the conclusion that sequential conformation changes are involved in the allosteric transitions of the enzyme.

The M-type pyruvate kinase from leucocytes and liver shows in the presence of a physiological concentration of alanine an allosteric response towards the substrate P-enolpyruvate. The kinetic data obtained at physiological effector concentrations allow one to conclude that the activity of this type of pyruvate kinase is also dependent upon the Fru-1,6-P_2 concentration in the cell. An additional phenomenon is the observation of two kinetic forms of the enzyme under physiological conditions. At least in rat small intestine these forms with different kinetic properties are present in vivo.

The molecular basis of erythrocyte pyruvate kinase deficiency was investigated. Oxidation of enzyme thiol groups reveals an altered pyruvate kinase. The kinetic and thermostability properties of this oxidized enzyme are identical with the enzyme from most of the pyruvate kinase deficient patients. Pyruvate kinase, isolated from erythrocytes of patients and characterized by a loss of allosteric behaviour towards P-enolpyruvate, can be converted (by incubation with mercaptoethanol) into normal enzyme with respect to kinetic behaviour. It is concluded that an altered pyruvate kinase in erythrocytes from patients suffering erythrocyte pyruvate kinase deficiency might be caused by the high sensitivity of the enzyme to its cellular environment. It is suggested that the primary cause(s) for altered pyruvate kinase might be related to activities of enzymes that regulate the redox state of the thiol groups within the red blood cell.

SAMENVATTING

De intercellulaire verdeling van de isoenzymen van pyruvaat kinase in rattelever is onderzocht. Het L-type pyruvaat kinase is gelocaliseerd in de lever parenchymcellen. Het M-type daarentegen in de niet-parenchymale cellen. Uit deze verdeling kan geconcludeerd worden dat de niet-parenchymale cellen niet significant bij kunnen dragen tot de gluconeogenese activiteit in de lever. De intercellulaire verdeling van de enzymen catalase en peroxidase wijst ook op belangrijke functionele verschillen tussen parenchymale en niet-parenchymale cellen en is in overeenstemming met een fagocyterende en bacteriedodende functie van de niet-parenchymale cellen.

De kinetische eigenschappen van het L- en M-type pyruvaat kinase werden bestudeerd. De kinetische eigenschappen van het L-type pyruvaat kinase uit rattelever kunnen sterk beïnvloed worden door de reductietoestand van de -SH groepen van het enzym. Uit een onderzoek naar de regulatie van het enzym onder fysiologische effector concentraties kan geconcludeerd worden dat de enzymatische activiteit voornamelijk afhankelijk is van de fructose-1,6-difosfaat concentratie in de parenchymcel. Uit een vergelijking van het effect van diverse gefosforyleerde hexosen op het L-type bleek dat opeenvolgende structurele veranderingen optreden bij de allosterische overgangen in het enzym.

Het M-type pyruvaat kinase uit leucocyten en lever vertoont in aanwezigheid van de fysiologische concentratie alanine een allosterisch gedrag ten opzichte van het substraat fosfoenolpyruvaat. Uit de kinetische onderzoekingen gemeten bij fysiologische effector concentraties, kan geconcludeerd worden dat ook de activiteit van dit meest voorkomende type pyruvaat kinase afhangt van de fructose-1,6-difosfaat concentratie in de verschillende cellen. Verder werd waargenomen dat dit type in twee verschillende vormen voor kan

komen met verschillende kinetische eigenschappen. Voor rattedarm kon worden aangetoond dat beide vormen in vivo aanwezig zijn.

Een onderzoek werd verricht naar de moleculaire basis van erythrocytaire pyruvaat kinase deficiëntie. Aangetoond werd dat de eigenschappen van het erythrocytaire enzym sterk beïnvloed worden door oxidatie van -SH groepen in het enzym. De kinetische eigenschappen en thermostabiliteit van het geoxideerde enzym zijn identiek met het in de literatuur meest beschreven "mutant" enzym dat geïsoleerd kan worden uit pyruvaat kinase deficiënte personen. Een andere groep patiënten bezit een "gemuteerd" enzym dat zijn allosterische respons ten opzichte van fosfoenolpyruvaat verloren heeft. Als dit enzym geïncubeerd wordt in aanwezigheid van mercaptoëthanol dan wordt een enzym verkregen dat kinetisch niet te onderscheiden is van het normale enzym. De conclusie kan getrokken worden dat een veranderd erythrocytair pyruvaat kinase in patiënten met erythrocytaire pyruvaat kinase deficiëntie veroorzaakt kan worden doordat het enzym zeer gevoelig is voor de reductietoestand in de cel. De primaire oorzaak voor een veranderd pyruvaat kinase zal dan zijn een mutatie in één van de enzymen die deze reductietoestand van de thiol groepen in de rode bloedcel reguleren.

NAWOORD

Na het behalen van het eindexamen HBS-B in 1964 aan het Mendel-College te Haarlem begon in in hetzelfde jaar met de studie scheikunden aan de Universiteit van Amsterdam. Het doctoraalexamen met hoofdvak biochemie werd afgelegd op 20 januari 1971. Tijdens mijn studie werd onderwijservaring opgedaan als leraar scheikunde aan het Stedelijk Gymnasium te Hilversum en als student-assistent aan het B.C.P. Jansen Instituut te Amsterdam. Op 21 januari 1971 trad ik in dienst als wetenschappelijk medewerker op de afdeling Biochemie I aan de Medische Faculteit te Rotterdam. Vanaf 1 juni 1972 verschafte FUNGO de middelen om dit onderzoek lichamelijk vol te houden.

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PAPERS

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DISTRIBUTION OF L- AND M-TYPE PYRUVATE KINASE BETWEEN PARENCHYMAL AND KUPFFER CELLS OF RAT LIVER

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SUMMARY

1. Parenchymal cells were isolated from rat liver by using EDTA + lysozyme or citrate. These cells contained only the L-type pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40).

2. Kupffer cells were isolated from rat liver by using pronase. The Kupffer cell preparation showed a ratio of M-type to L-type pyruvate kinase 10–20 times higher than the ratio in a total liver homogenate, suggesting that Kupffer cells only (probably) contain the M-type pyruvate kinase.

3. The results obtained suggest that gluconeogenesis is confined to the parenchymal cells of rat liver.

INTRODUCTION

Pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40) catalyses the last step of glycolysis. Its activity is important in the regulation of the dynamic balance between gluconeogenesis and glycolysis in the liver. During glycolysis the enzyme must be active; during gluconeogenesis, on the other hand, activity leads to wastage of energy. In liver there are two types of pyruvate kinase[1]. The L-type pyruvate kinase shows an allosteric response to one of its substrates, phosphoenolpyruvate (PEP), and its activity is further influenced by glycolytic and gluconeogenic intermediates[2]. Fru-1,6-P_2 (ref. 3), Glc-1,6-P_2 (ref. 4) and other phosphorylated hexoses[5] increase the apparent affinity for the substrate PEP. On the other hand ATP acts as an allosteric inhibitor[3]. L-Alanine, a precursor of gluconeogenesis inhibits the L-type pyruvate kinase[6]. The abundance of this type of pyruvate kinase is regulated by the diet and by hormones[7].

The M-type pyruvate kinase is a non-allosteric enzyme as can be concluded from the Michaelis–Menten kinetics. From the properties of the two types of pyruvate kinase one could conclude that only the L-type is involved in the regulation of the

Abbreviation: PEP, phosphoenolpyruvate.

dynamic balance between glycolysis and gluconeogenesis. Since the liver contains different types of cells, it is possible that the two types of pyruvate kinase have a different localization. In order to investigate the localization differences of pyruvate kinases, we isolated Kupffer and parenchymal cells from rat liver.

MATERIALS AND METHODS

Type L pyruvate kinase was isolated from rat liver according to the isolation procedure described earlier[4]. Type M pyruvate kinase was isolated by the method of Passeron *et al.*[8]. Pyruvate kinase was assayed by following the decrease in absorbance at 340 nm in the coupled reaction with lactate dehydrogenase at room temperature according to Valentine and Tanaka[9]. The triethanol–HCl buffer (0.4 M, pH 7.5) was replaced by Tris–HCl buffer (0.25 M, pH 8.0). Parenchymal cells were obtained by a method described by Hommes *et al.*[10] and were centrifuged for 5 min at 50 × *g* and washed with the isolation medium except that EDTA and lysozyme were omitted. This procedure was repeated five times and the final precipitate was homogenized in 0.25 M Tris–HCl (pH 8.0) containing 1 mM mercaptoethanol. After centrifugation at 20 000 × *g* for 20 min the pyruvate kinase activity was measured. The final precipitate contained only parenchymal cells, as shown by light microscopy.

Kupffer cells were isolated by a method based on that described by Mills and Zucker-Franklin[11]. Wistar rats were anaesthesized with nembutal; the liver was perfused with 0.9% NaCl or Hank's balanced salt solution and after it became a light tan colour it was sliced into 3–5 mm fragments and placed in 10 ml Hank's balanced salt solution containing 0.25% pronase. After 30 min vigorous shaking at 37 °C the mixture was filtered and subsequently centrifuged for 5 min at 600 × *g*. The cells were resuspended by addition of Hank's balanced salt solution and recentrifuged. The procedure was repeated four times. The cells were then homogenized, centrifuged for 20 min at 20 000 × *g* and the pyruvate kinase activity was measured.

Pronase was obtained from Cal Biochem. Lysozyme, ADP, PEP, lactate dehydrogenase and NADH were obtained from Boehringer (Mannheim, Germany). All other reagents were of analytical grade purity.

RESULTS

In Fig. 1 are plotted the activities of the isolated L- and M-type pyruvate kinases *versus* the PEP concentration at fixed [ADP] and pH 8.0. The isolated M-type pyruvate kinase follows the Michaelis–Menten kinetics, and is not influenced by Fru-1,6-P_2. In contrast, the L-type pyruvate kinase shows a sigmoidal curve, which can be converted into a hyperbolic curve by adding 0.5 mM Fru-1,6-P_2. From the curves obtained with the isolated L-type it can be concluded that Fru-1,6-P_2, at least under our assay conditions, stimulates the enzyme activity at 1 mM PEP by a factor of 11.0 ± 0.3 ($n = 6$). From these results (Fig. 1) it is possible to calculate the activity ratio of the L- and M-types. It was found that in crude liver homogenates at 1 mM PEP an average activation of 7.1 was obtained by adding 0.5 mM Fru-1,6-P_2. From this it was calculated that the L-type contributes about 61% to the overall pyruvate kinase activity prior to Fru-1,6-P_2 addition.

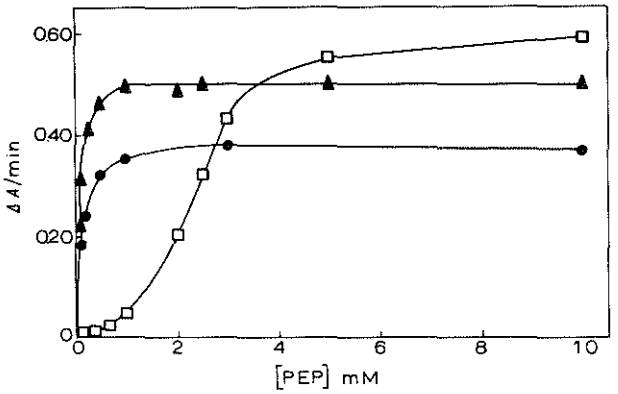

Fig. 1. The *v vs* [PEP] plot of the partly purified L- and M-type pyruvate kinase at [ADP] = 2 mM, in presence and absence of Fru-1,6-P_2 (0.5 mM). □—□, L-type pyruvate kinase; ▲—▲, L-type pyruvate kinase in the presence of Fru-1,6-P_2; ●—●, M-type pyruvate kinase in the absence or presence of Fru-1,6-P_2.

Fig. 2 shows the pyruvate kinase activity of the homogenate of isolated parenchymal cells, tested under the same conditions as in Fig. 1. From this plot we conclude that only the L-type pyruvate kinase is present in parenchymal cells. Also, when parenchymal cells were isolated with citrate (27 mM) in Hank's balanced salt solution instead of EDTA and the lysozyme-containing medium (described in Materials and Methods) the same result was obtained.

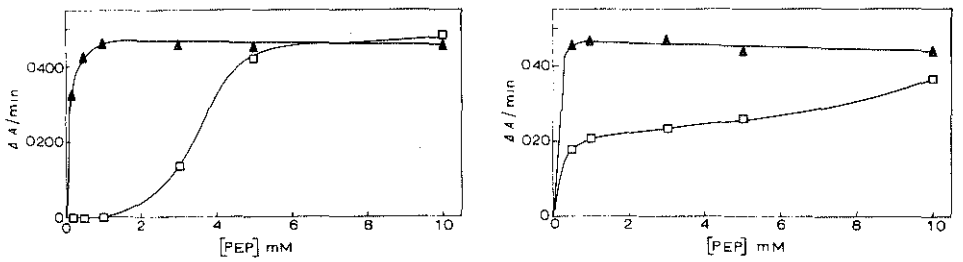

Fig. 2. The *v vs* [PEP] plot of pyruvate kinase activity in the parenchymal cell homogenate in the absence and presence of Fru-1,6-P_2 (0.5 mM). □—□, without Fru-1,6-P_2; ▲—▲, with Fru-1,6-P_2.

Fig. 3. The *v vs* [PEP] plot of pyruvate kinase activity in the Kupffer cell homogenate in the absence and presence of Fru-1,6-P_2 (0.5 mM). □—□, without Fru-1,6-P_2; ▲—▲, with Fru-1,6-P_2.

Fig. 3 shows the pyruvate kinase activity versus [PEP] of the Kupffer cell homogenate. This plot is completely different from the one obtained with parenchymal cells (Fig. 2). The activity increment at 1 mM PEP, due to 0.5 mM Fru-1,6-P_2, in Fig. 3 is 2.2 fold, which indicates that the M-type is enriched in the homogenate. We can calculate that the L/M ratio in this Kupffer cell preparation is 0.135 (compare the value of 1.56 in a crude liver homogenate), indicating a relative increase of the M-type contribution of at least 10 fold. The Fru-1,6-P_2 stimulations obtained with different preparations of crude liver, parenchymal cell and Kupffer cell homogenates are summarized in Table I.

TABLE I

RELATIVE ACTIVITIES OF L- AND M-TYPES OF PYRUVATE KINASE

For the measurement of activity see Fig. 1 and Materials and Methods.

Source of homogenate	*n*	*Fru-1,6-P_2 stimulation at 1 mM PEP*	*Ratio of activities* L-/M-type pyruvate kinase*
Whole rat liver	6	7.1	1.56
Parenchymal cells	6	∞	∞
Kupffer cells	6	1.7–2.4	0.07–0.16

* Fru-1,6-P_2 absent.

Since pronase and lysozyme are proteolytic enzymes, a possible criticism exists: these enzymes might preferentially digest one of the two types of pyruvate kinase. Therefore we incubated the isolated L- and M-types from rat liver with either lysozyme (0.05%) or pronase (0.25%). This concentration of lysozyme did neither affect the L- nor the M-type activity, even after 2 h incubation. That no preferential digestion by lysozyme occurred, could also be concluded from the fact that when citrate was used instead of lysozyme in the isolation of parenchymal cells, the same results were obtained. Pronase on the other hand affected the L-type in the presence of Fru-1,6-P_2 to the same extent as the M-type. When isolated L-type was incubated with pronase, the activation of the reaction was increased by the addition of Fru-1,6-P_2. Therefore, the activity ratio of L to M-type pyruvate kinase for Kupffer cells (as shown in Table I) may be overestimated. However, it is likely that during the isolation procedure, pyruvate kinase is protected from the added proteolytic enzymes by the cell membrane.

DISCUSSION

The data presented show that only the parenchymal cells of rat liver contain the L-type pyruvate kinase and it is highly suggestive that the Kupffer cells only contain the M-type. It is generally accepted that the L-type is involved in the regulation of glycolysis and that the enzyme can be switched off during gluconeogenesis. It is, therefore, likely that gluconeogenesis is also located solely in the parenchymal cells. Tanaka *et al.*[2] already in 1967 mentioned the occurrence of L- and M-type pyruvate kinase in liver and noticed a linkage between the L-type pyruvate kinase and glucokinase (EC 2.7.1.12). Their data also suggested a linkage between the M-type with hexokinase (EC 2.7.1.1). They concluded "that there may be two kinds of glycolysis pathway in the liver. One of these is catalysed by hexokinase and type M pyruvate kinase and might be called the basal pathway. The other is catalysed by glucokinase and type L pyruvate kinase and is a regulatory pathway. The rate of the basal pathway would not be influenced by dietary and hormonal conditions, and it would meet the minimum demands of the cell. The rate of the regulatory pathway would fluctuate in response to dietary and hormonal conditions and it would meet special demands of the cell". We agree with this parallelism, since Sapag-Hagar *et al.*[12] showed that glucokinase is present in parenchymal cells and hexokinase is virtually restricted to the non-parenchymal tissue. In the present paper it is shown that the L-type pyruvate kinase

is confined to the parenchymal cell. Therefore, it is clear now that the two pathways, distinguished by Tanaka *et al.*[2], are even localized in two different liver cell types. The parenchymal cell responds to the metabolic changes of the liver, whereas the Kupffer cells lack such a possibility.

NOTE ADDED IN PROOF (Received June 16th, 1972)

Recently, Crisp and Pogson[13] showed that also in mouse liver the L-type pyruvate kinase is confined to the parenchymal cell.

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DIFFERENCE SPECTRA, CATALASE- AND PEROXIDASE ACTIVITIES OF ISOLATED PARENCHYMAL AND NON-PARENCHYMAL CELLS FROM RAT LIVER

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SUMMARY. The reduced minus oxidized difference spectra from isolated parenchymal and non-parenchymal cells from rat liver indicate that the non-parenchymal cells contain a considerable amount of peroxidase. This interpretation is favoured by the more than 30 times higher specific activity of peroxidase (EC 1.11.1.7) in the non-parenchymal cells as compared to the parenchymal cells. The catalase (EC 1.11.1.6) activity in the non-parenchymal cells is 4 times lower than in the parenchymal cells. These results are consistent with an antimicrobial function of the non-parenchymal cells in liver.

The mammalian liver consists primarily of hepatocytes (parenchymal cells) and reticulo endothelial cells (Kupffer cells). As much as 35% of the liver cells are non-parenchymal cells representing 5 to 10% of the liver mass[1]. In an earlier paper[2] we have shown that parenchymal cells contain only L-type pyruvate kinase (EC 2.7.1.40) while the M_2-type was enriched in the non-parenchymal cells. From this distribution pattern we concluded that Kupffer cells do not significantly participate in gluconeogenesis. Histochemical studies have indicated that the main function of the Kupffer cells in the liver might be related to their phagocytic properties[3]. To obtain more information on the function and properties of the non-parenchymal cells, we separated intact parenchymal and non-parenchymal cells from rat liver and determined the difference spectra of these cell types. For the reason that the results were indicative for a large amount of peroxidase in the non-parenchymal cells, we also investigated the distribution of peroxidase and catalase activities between the different cell types. It is concluded that the biochemical data obtained are consistent with phagocytic and antimicrobial functions of the non-parenchymal cells in rat liver.

MATERIALS AND METHODS. Parenchymal cells were isolated from rat liver essentially according to the method of Berry and Friend[4], using collagenase (0.05%) and hyalurinidase (0.10%) as proteolytic enzymes in Hank's balanced salt solution. The cells were purified by differential centrifugation for 2 min at 45 g. The cells were resuspended in Hank's balanced salt solution and recentrifugated. This procedure was repeated 4 times. By centrifugation at this speed the non-parenchymal cells can be removed effectively from the parenchymal cells. The cells obtained, of which at least 90% excluded trypan-blue and were therefore considered to be intact, were not contaminated, as judged by light microscopy.

The non-parenchymal cells were isolated by two different methods both of which were based on a method described by Mills and Zucker-Franklin[5]. The first method was as described earlier[2]. In the second method a total cell suspension, obtained by the Berry and Friend[4] method, was incubated with 0.25% pronase for 1 h[6]. All parenchymal cells were destroyed after 1 h and the non-parenchymal cells were collected by centrifugation for 5 min at 600 g. The cells were resuspended in Hank's balanced salt solution and recentrifugated. This procedure was repeated 4 times. The non-parenchymal cells isolated by both methods did not differ in the assays and spectra mentioned under RESULTS.

Pyruvate kinase was assayed as described earlier[2]. Catalase was determined according to Bergmeyer[7] while peroxidase was measured with 3,3'-diamino benzidine (DAB) as hydrogen donor[8]. Difference spectra were run on a Perkin-Elmer 356 and on an Amino DW-2 spectrophotometer set for split-beam optics.

RESULTS

Fig. 1 shows the reduced minus oxidized spectrum of a homogenate of a typical parenchymal cell preparation. The interpretation of the maxima and minimum is indicated in the figure. The dotted line gives the succinate-reduced (antimycin A present) minus oxidized spectrum which allows the detection of cytochrome b. Fig. 2 gives the same kind of spectra for a homogenate of a typical non-parenchymal cell preparation. It can be seen that this difference spectrum differs markedly from that of the parenchymal cells (Fig. 1). Although the amount of protein present in the experiment of Fig. 2 is half of the amount used in Fig. 1, the peak in the 430 nm region is even higher. This high peak interferes with the determination of the mitochondrial cytochrome spectra. The succinate-reduced minus oxidized spectra show that at least the cytochrome b content of the different cell types is about equal. From these spectra we can conclude that the liver parenchymal cells show a normal cytochrome spectrum as for example is also found with

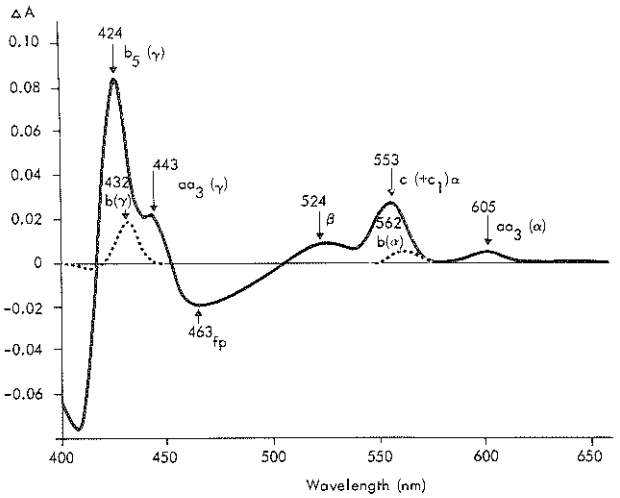

Fig. 1 Difference spectra of the parenchymal cell homogenate. —— reduced (with dithionite) minus oxidized (0.05% H_2O_2). ---- reduced (with succinate (10 mM) + antimycin A (4 µg)) minus oxidized (0.05% H_2O_2). The protein concentration with both spectra was 5.1 mg.

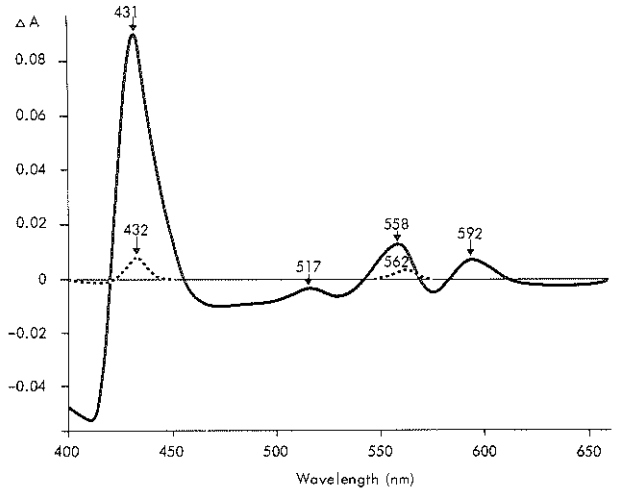

Fig. 2 Difference spectra of the non-parenchymal cell homogenate. —— reduced (with dithionite) minus oxidized (0.05% H_2O_2)).---- reduced (with succinate (10 mM) + antimycin A (4 µg)) minus oxidized (0.05% H_2O_2). The protein concentration with both spectra was 2.54 mg.

intestinal cells[9], while the non-parenchymal cell spectrum seems to be related to spectra obtained with leucocytes[10]. As the leucocyte spectrum is considered to be the consequence of the high peroxidase content of these cells[11], the distribution of the activities of peroxidase and catalase between the different cell types was determined. To ascertain the intactness of the cells pyruvate kinase was used as a marker because this enzyme is solely located in the cytoplasm and does not adhere to membranous structures (unpublished). Table I shows the pyruvate kinase activities of the liver homogenate, the parenchymal and non-parenchymal cells. In accordance with an earlier report[2], parenchymal cells do only contain the L-type pyruvate kinase while the activity on protein base further supports the intactness of the cells used. From the absence of Fru-1,6-P_2 activation it can be concluded that the non-parenchymal cells contain exclusively M_2-type pyruvate kinase. This is a further evaluation of earlier obtained results[2] in which the non-parenchymal cell fraction also contained a considerable amount of L-type pyruvate kinase probably due to the impurity of the earlier non-parenchymal cell preparations.

TABLE I

INTERCELLULAR DISTRIBUTION OF L- AND M_2-TYPE PYRUVATE KINASE

Source of homogenate	activity at 1 mM P-enolpyruvate (nmol min^{-1} mg^{-1} protein) ± SEM (n)	activity at 1 mM P-enolpyruvate + 0.5 mM Fru-1,6-P_2 (nmol min^{-1} mg^{-1} protein) ± SEM (n)
Whole rat liver	18.1 ± 2.0 (10)	124.4 ± 10.5 (10)
Parenchymal cells	11.8 ± 1.1 (8)	134.6 ± 16.3 (8)
Non-parenchymal cells	28.3 ± 4.4 (5)	27.6 ± 5.0 (5)

Table II shows the peroxidase and catalase activities in the different cell preparations. It can be seen that the non-parenchymal cells contain 30 times more peroxidase activity than the parenchymal cells, and that on the other hand catalase activity is more concentrated in the parenchymal cells. The reason for the relatively low catalase activity in the liver homogenate as compared to activity in the parenchymal cell homogenate cannot be explained at the moment but might be related to the results of Fujiwara et al.[12] who observed the same phenomenum with the enzyme collagenase.

TABLE II

INTERCELLULAR DISTRIBUTION OF PEROXIDASE AND CATALASE

Source of homogenate	peroxidase (μmol min^{-1} mg^{-1}) ± SEM	(n)	catalase (μmol min^{-1} mg^{-1}) ± SEM	(n)
Whole rat liver	7.0 ± 0.5	(4)	618 ± 92	(4)
Parenchymal cells	2.6 ± 0.3	(5)	1472 ± 315	(5)
Non-parenchymal cells	80.8 ± 12.7	(5)	368 ± 38	(5)

DISCUSSION

The results clearly demonstrate the heterogeneity of liver tissue. Conclusions drawn from work with total liver homogenates must be taken with caution when the different cellular contributions are not taken into account. The high specific activities obtained with the parenchymal cell homogenates, together with the trypan blue exclusion, clearly illustrate the usefulness of the reported cell preparation for cellular distribution studies. A quality test for the non-parenchymal cells is more difficult to assess although also for these cells the trypan blue exclusion is indicative for intactness.

From the distribution of the L- and M_2-type pyruvate kinases we can conclude that, at least in adult liver, the M_2-type is solely located in the non-parenchymal cells. Of course this does

not exclude an occurrence of this type in parenchymal cells under special conditions (fetal or regenerating liver cells[13]). However, under all conditions the M_2-type is the only type present in non-parenchymal cells which excludes a significantly contribution of these cell types to gluconeogenesis[2].

From histochemical studies it was concluded earlier that the Kupffer cells from rat liver contain peroxidase-like activity whereas the parenchymal cells were hardly positively stained[14,15]. The present paper shows that peroxidase is about 30 times more active in the homogenate of non-parenchymal cells as compared to the homogenate of parenchymal cells. For reason that this enzyme is involved in antimicrobial systems[16], the present data, together with the lower catalase activity, support an antimicrobial function of the non-parenchymal cells in liver.

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THE INFLUENCE OF GLUCOSE 1,6-DIPHOSPHATE ON THE ENZYMATIC ACTIVITY OF PYRUVATE KINASE

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SUMMARY

1. The influence of Glc-1,6-P_2 on hepatic and red blood cell pyruvate kinase (ATP: pyruvate phosphotransferase, EC 2.7.1.40) is quite similar to that of Fru-1,6-P_2. The hexose diphosphates can replace each other in stimulating pyruvate kinase; after maximal stimulation by one of the compounds, the other is not capable of further stimulation.

2. The regulatory role of Fru-1,6-P_2 on the activity of pyruvate kinase is discussed in view of the results obtained.

INTRODUCTION

A large number of studies have been devoted to the regulation of pyruvate kinase (ATP: pyruvate phosphotransferase, EC 2.7.1.40), one of the key enzymes in glycolysis. It was found that there are two types[1], a M (muscle) type and a L (liver) type. It turned out that the L type exhibits allosteric properties allowing regulatory function. The glycolytic intermediate Fru-1,6-P_2 is able to stimulate the activity of the L type and changes the S-shaped relationship between substrate concentration and velocity into a normal hyperbolic one[2]. The [Fru-1,6-P_2] of the liver fluctuates with the nutritional state and, therefore, it was assumed that Fru-1,6-P_2 has a regulatory influence on pyruvate kinase. Furthermore, it was found that alanine inhibits the hepatic pyruvate kinase reaction[3], which can be of importance during gluconeogenesis. In our studies[4,5] on hepatic and erythrocytic pyruvate kinase it was found that the phosphorylated hexoses are able to stimulate the pyruvate kinase reaction and to overcome the ATP inhibition. In this study we compare the influences of Glc-1,6-P_2 and Fru-1,6-P_2 on the enzymatic reaction.

MATERIALS AND METHODS

Wistar rats, maintained on a normal diet and water *ad libitum*, were decapitated and the livers removed. Liver homogenates were prepared in 0.15 M NaCl containing

Abbreviation: PEP, phosphoenolpyruvate.

TABLE I

THE EFFECT OF GLC-1,6-P_2 (0.2 mM) AND FRU-1,6-P_2 (0.2 mM) ON THE ACTIVITY OF HEPATIC PYRUVATE KINASE IN (0.25 M) TRIS–HCl BUFFER (pH 7.5) WITH [PEP] = 0.5 mM AND [ADP] = 0.3 mM

Addition	*Activity (%)*
None	100
Glc-1,6-P_2	218
Fru-1,6-P_2	228

1 mM mercaptoethanol and centrifuged for 60 min at 100 000 × *g*. From the supernatant, the partially purified L type preparations were obtained according to the method of PASSERON *et al.*[6]. The preparations were finally dissolved in 0.25 M Tris–HCl buffer (pH 7.5) containing 1 mM mercaptoethanol. Red blood cell pyruvate kinase was isolated from human erythrocytes according the isolation procedure described previously[5]. The enzymatic activity was assayed by following the decrease of absorbance at 340 nm in the reaction coupled with lactate dehydrogenase as described by BÜCHER AND PFLEIDERER[7]. The activities shown are initial reaction rates.

ADP, PEP (phosphoenolpyruvate), NADH, Fru-1,6-P_2 and Glc-1,6-P_2 were obtained from Boehringer (Mannheim, Germany). Glc-1,6-P_2 was free of Fru-1,6-P_2, when analysed by the method of BÜCHER AND HOHORST[8]. All other reagents were of analytical grade purity.

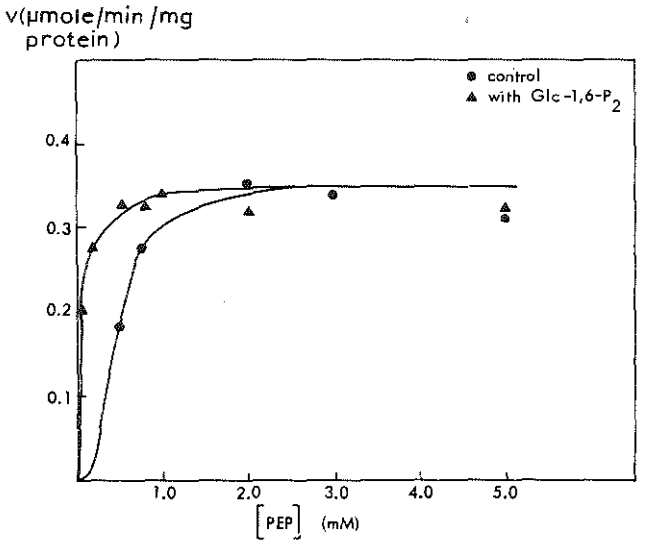

Fig. 1. The *v versus* [PEP] plot for hepatic pyruvate kinase at [ADP] = 0.3 mM, in the absence (●—●) and presence of 0.2 mM Glc-1,6-P_2 (▲—▲).

RESULTS

Table I shows that Glc-1,6-P_2 is able to stimulate the hepatic pyruvate kinase reaction. This stimulation is about equal to the stimulation obtained with Fru-1,6-P_2 (Table I), at least under these conditions.

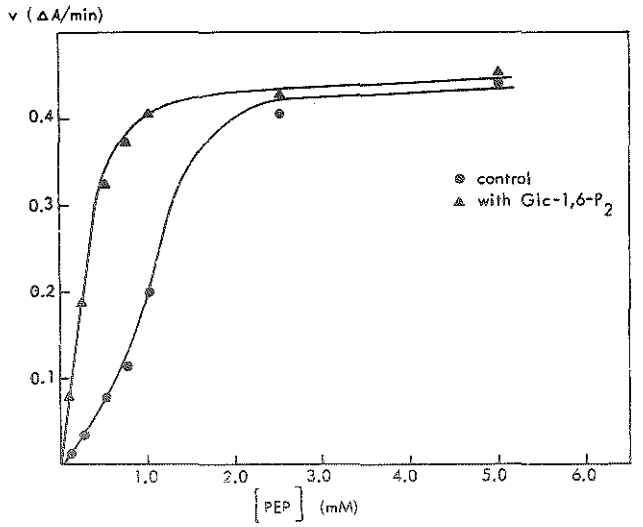

Fig. 2. The *v versus* [PEP] plot for red blood cell pyruvate kinase at [ADP] = 2 mM, in the absence (●—●) and presence of 0.2 mM Glc-1,6-P_2 (▲—▲), 20 μg of protein added.

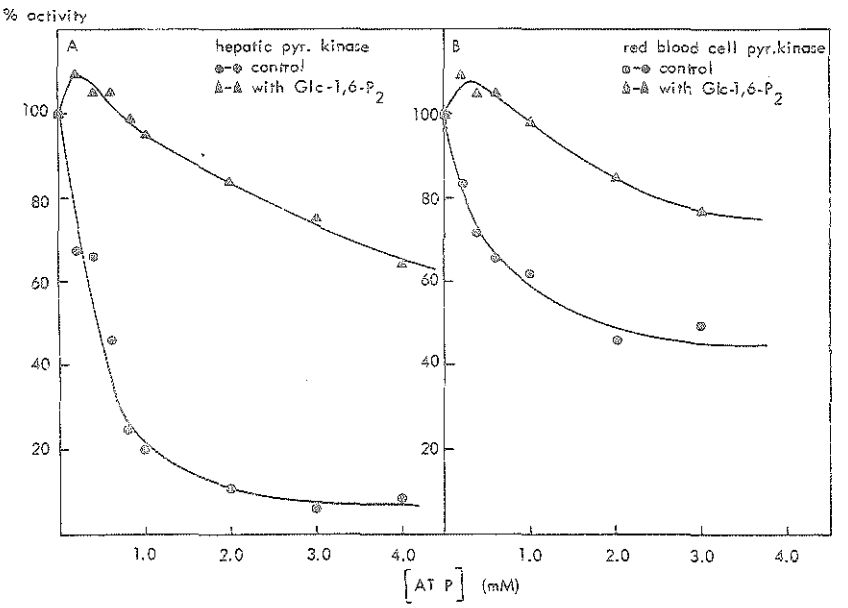

Fig. 3A. The ATP inhibition plots for hepatic pyruvate kinase at [PEP] = 0.5 mM and [ADP] = 0.5 mM. ●—●, control; ▲—▲, with Glc-1,6-P_2 (0.2 mM).

Fig. 3B. The ATP inhibition plot for red blood cell pyruvate kinase at [PEP] = 0.5 mM and [ADP] = 0.5 mM; ●—●, control; ▲—▲, with Glc-1,6-P_2 (0.2 mM), 20 μg protein added.

Glc-1,6-P_2, not only stimulates the pyruvate kinase reaction, but is also able to transform the S-shaped curve into a normal hyperbolic one (Fig. 1). These results are quite similar to the results obtained with the ligand Fru-1,6-P_2 (refs. 1, 2). At pH 5.9 the effects of Fru-1,6-P_2 disappear. The same results were obtained with Glc-1,6-P_2; no stimulation occurs at all. Fig. 2 shows the influence of Glc-1,6-P_2 on the red blood cell pyruvate kinase reaction at pH 7.6. These results are similar to the results obtained with the hepatic pyruvate kinase.

Similar to Fru-1,6-P_2, Glc-1,6-P_2 is able to reverse the ATP inhibition on the pyruvate kinase reaction (Fig. 3A). This is also the case with red blood cell pyruvate kinase (Fig. 3B).

It is clear from these data that the effects of Glc-1,6-P_2 and Fru-1,6-P_2 are quite similar. In liver both ligands have a synergistic effect only at low ($<50\ \mu M$) concentration (Fig. 4). Fig. 5 shows that with the red blood cell pyruvate kinase at

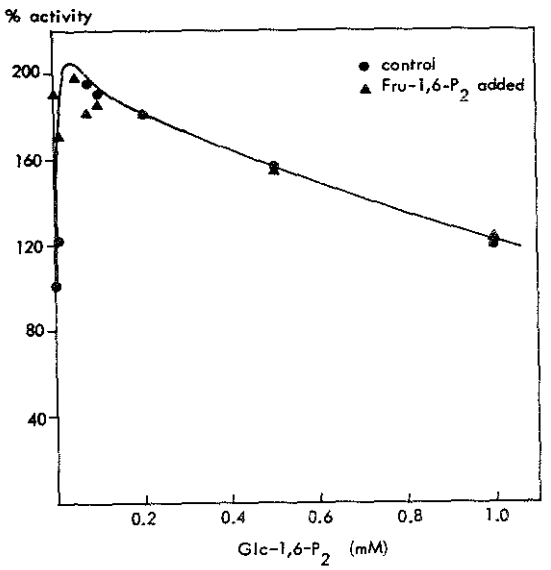

Fig. 4. The *v versus* [Glc-1,6-P_2] for hepatic pyruvate kinase in the presence and absence of Fru-1,6-P_2; ●—●, control; ▲—▲, with Fru-1,6-P_2 (0.2 mM), [PEP] = 0.5 mM and [ADP] = 0.3 mM.

500 μM, Glc-1,6-P_2 Fru-1,6-P_2, does not further stimulate, but that in the absence of Fru-1,6-P_2 half-maximal stimulation by Glc-1,6-P_2 is obtained at 60 μM.

At a [Fru-1,6-P_2] of 50 μM the hepatic pyruvate kinase is practically fully stimulated; however, at 10 μM the stimulation is already about 90% of the maximal obtainable. For Glc-1,6-P_2 the concentration necessary for full stimulation is about 50 μM; at a concentration of 10 μM the stimulation is about 60% of maximal obtained. For the red blood cell pyruvate kinase the situation is somewhat different. At 10 μM of Fru-1,6-P_2 the reaction is fully stimulated but for Glu-1,6-P_2 the pyruvate kinase reaction is still not fully stimulated at a concentration of 200 μM.

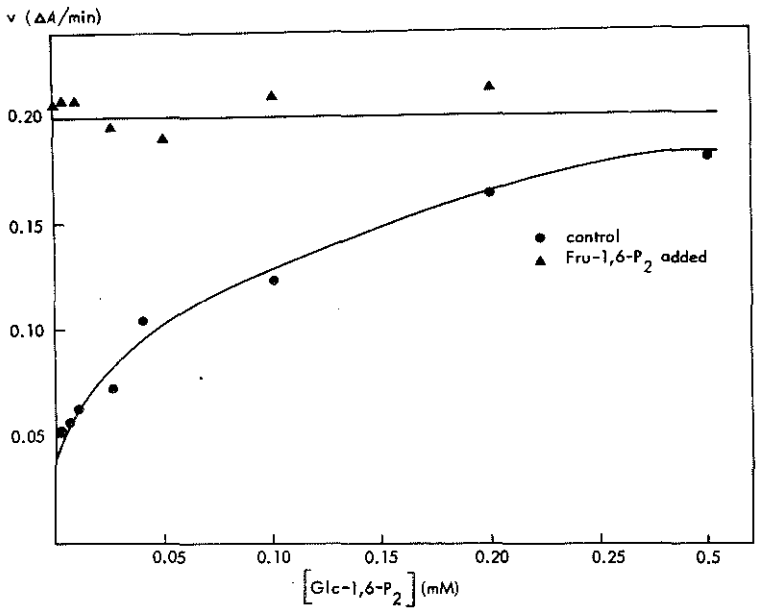

Fig. 5. The *v versus* [Glc-1,6-P_2] for red blood cell pyruvate kinase in the absence and presence of Fru-1,6-P_2; ●—●, control; ▲—▲, with Fru-1,6-P_2 (0.5 mM). [PEP] = 0.5 mM and [ADP] = 0.5 mM, 20 μg of protein added.

DISCUSSION

From the data presented it is clear that the effects of Fru-1,6-P_2 and Glc-1,6-P_2 are quite similar. The concentration of Fru-1,6-P_2 in the liver fluctuates during feeding and fasting (5–20 μM)[9]. However, the [Glc-1,6,P_2] remains constant at about 15 μM[10]. At this concentration of Glc-1,6-P_2 the hepatic pyruvate kinase is more than 80% stimulated, while at 5 μM Fru-1,6-P_2 pyruvate kinase is already stimulated for 50%. This means that Fru-1,6-P_2 cannot have any regulatory function on hepatic pyruvate kinase. For red blood cell pyruvate kinase it was shown that Fru-1,6-P_2 has already a fully stimulating effect at 10 μM, while for Glc-1,6-P_2 the concentration has to be more than 200 μM. However, the [Glc-1,6-P_2] in the red blood cell is 300 μM[11]. According to BARTLETT[11], the red blood cell [Fru-1,6-P_2] is 200 μM. With these concentrations the pyruvate kinase in the erythrocyte is fully stimulated. This means that the enzyme is in the R state[5]. For the red blood cell it is rather doubtfull if a regulation of pyruvate kinase is necessary. The rate of glycolysis is governed by the activities of hexokinase (ATP: D-hexose 6-phosphotransferase, EC 2.7.1.1) and phosphofructokinase (ATP: D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11), in which the concentrations of adenine nucleotides also play an important role[1,5,12,13].

Although there will be some wasting of ATP during the gluconeogenesis due to cycling[9], the pyruvate kinase reaction must be inhibited in order to increase the PEP concentration which is necessary for the gluconeogenesis. However, this regulation cannot be done by a fluctuating Fru-1,6-P_2 concentration of the liver.

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TWO INTERCONVERTIBLE FORMS OF L-TYPE PYRUVATE KINASE FROM RAT LIVER

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(Received July 24th, 1972)

SUMMARY

1. Reduced L-type pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40) from rat liver can be converted into an oxidized form by incubation with oxidized mercaptoethanol and oxidized glutathione. This interconversion can be completely reversed by incubation with reduced mercaptoethanol.

2. The kinetic and allosteric properties of the reduced and oxidized forms are described.

3. The results are discussed in view of a possible regulation of the enzyme.

INTRODUCTION

Pyruvate kinase (ATP: pyruvate phosphotransferase, EC 2.7.1.40) catalyses the last step of glycolysis. Its activity is important in the regulation of the dynamic balance between gluconeogenesis and glycolysis in the liver. In the liver two types of pyruvate kinase are present[1]. The L-type pyruvate kinase shows an allosteric response to one of its substrates phosphoenolpyruvate (PEP). Its activity is influenced by Fru-1,6-P_2, Glc-1,6-P_2 and other phosphorylated hexoses[2–5]. These compounds increase the apparent affinity for the substrate PEP. On the other hand ATP and alanine act as allosteric inhibitors[3,6]. It has been generally accepted that the L-type pyruvate kinase, localized in the rat liver hepatocytes[7], must be inhibited during gluconeogenesis in order to increase the [PEP] necessary for gluconeogenesis. In a previous paper[4] we pointed out that the L-type pyruvate kinase cannot be regulated by a fluctuating [Fru-1,6-P_2] in the liver. The ATP concentration *in vivo* only fluctuates between 2 and 3 mM (refs 8 and 9) and, therefore, some authors[6,10] assumed that during gluconeogenesis alanine is the most important inhibitor of pyruvate kinase activity. However, during gluconeogenesis the [alanine] in the liver is lowered (65%) due to an increased flux into the gluconeogenic pathway[11]. These properties would lead to an uninhibited enzyme under gluconeogenic conditions, which is rather

Abbreviation: PEP, phosphoenolpyruvate.

unlikely. Another possibility is that the reduction state of the enzyme plays an important role in its regulation. We investigated the influence of sulfhydryl compounds on the activity of the enzyme, after preliminary studies with coenzyme A (containing glutathione) indicated an influence of thiol groups on the allosteric behaviour of pyruvate kinase.

MATERIALS AND METHODS

Pyruvate kinase type L was isolated from rat liver according to the isolation procedure described earlier[4], except that during this procedure 1 mM mercaptoethanol was omitted and the final preparation was dissolved in 0.25 M Tris–HCl pH 8.0. Pyruvate kinase activity was assayed by following the decrease in absorbance at 340 nm in a coupled reaction with lactate dehydrogenase at 23 °C according to Valentine and Tanaka[12]. The triethanol–HCl buffer (0.4 M, pH 7.5) was replaced by Tris–HCl buffer (0.25 M, pH 8.0). Oxidized mercaptoethanol was prepared by bubbling O_2 through a 100-mM solution of reduced mercaptoethanol for 24 h. Oxidized L-type pyruvate kinase was prepared by incubating the enzyme for 6 h at 5 °C with 1 mM oxidized mercaptoethanol or 2.5 mM oxidized glutathione.

ADP, PEP, NADH, Fru-1,6-P_2, Glc-1,6-P_2 and oxidized glutathione were obtained from Boehringer (Mannheim, Germany). Glc-1,6-P_2 was free of Fru-1,6-P_2, when analysed by the method of Bücher and Hohorst[13]. Reduced glutathione was obtained from Sigma. All other reagents were of analytical grade purity.

RESULTS

Fig. 1 shows the *v vs* [PEP] plot at [ADP] = 0.5 mM for the freshly prepared pyruvate kinase (L-type) and the oxidized enzyme (preincubated with oxidized mercaptoethanol; compare Materials and Methods). In the presence of Fru-1,6-P_2

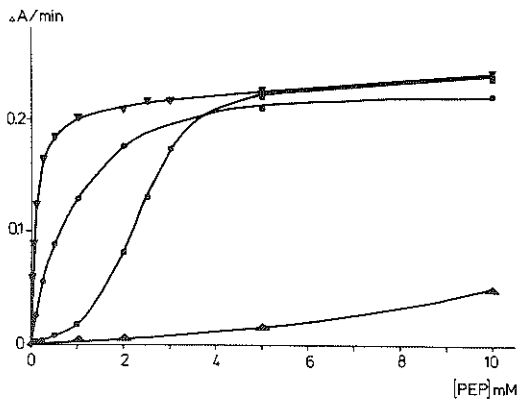

Fig. 1. The *v vs* [PEP] plot of the freshly isolated and oxidized L-type pyruvate kinase at [ADP] = 0.5 mM measured in the presence and absence of Fru-1,6-P_2 (0.5 mM). ■—■, the activity of the freshly isolated L-type pyruvate kinase; ▼—▼, the activity of the freshly isolated L-type pyruvate kinase measured in the presence of Fru-1,6-P_2; ▲—▲, the activity of the oxidized L-type pyruvate kinase (by oxidized mercaptoethanol); ●—●, the activity of the oxidized L-type pyruvate kinase (by oxidized mercaptoethanol) measured in the presence of Fru-1,6-P_2.

the freshly prepared enzyme gives an apparent K_m value of 0.1 mM for PEP, whereas in the absence of Fru-1,6-P_2 a $K_{0,5}$ value of 2.5 mM was obtained. Oxidation of the enzyme leads to a marked change in these activity curves. In the presence of Fru-1,6-P_2 the K_m value for PEP is increased to 0.7 mM and the $K_{0,5}$ value in the absence of Fru-1,6-P_2 was not measurable. This means that at least under our test conditions the oxidized enzyme cannot reach its maximal activity in the absence of Fru-1,6-P_2. The addition of Fru-1,6-P_2 stimulates the enzymatic activity to the same value as obtained with the freshly prepared enzyme, indicating that no loss of maximal activity occurred after oxidation of the enzyme.

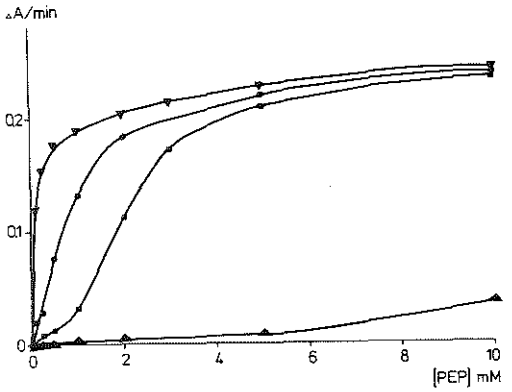

Fig. 2. The *v vs* [PEP] plot of the oxidized L-type pyruvate kinase (by oxidized glutathione) and the oxidized L-type pyruvate kinase after incubation for 1 h at 10 °C with 1 mM mercaptoethanol (reduced enzyme) measured in the presence and absence of Fru-1,6-P_2; ▲—▲, the activity of the oxidized L-type pyruvate kinase (by oxidized glutathione); ●—●, the activity of the oxidized L-type pyruvate kinase (by oxidized glutathione) measured in the presence of Fru-1,6-P_2; ■—■, the activity of the reduced L-type pyruvate kinase; ▼—▼, the activity of the reduced L-type pyruvate kinase measured in the presence of Fru-1,6-P_2.

Fig. 2 shows that the L-type pyruvate kinase can also be oxidized by incubation with oxidized glutathione In the absence and presence of Fru-1,6-P_2 the same pattern was obtained as in Fig. 1. Fig. 2 also shows that oxidation of the enzyme is a reversible process. By incubating the oxidized enzyme with 1 mM reduced mercaptoethanol for 1 h at 10 °C (reduced enzyme) the same kinetic data were obtained as with the freshly isolated enzyme. The $K_{0,5}$ value in the absence of Fru-1,6-P_2 becomes again 2 mM and the apparent K_m for PEP in the presence of Fru-1,6-P_2 is again lowered to 0.1 mM. The same curves were obtained when the enzyme was oxidized by using oxidized mercaptoethanol and incubated again with reduced mercaptoethanol. When the oxidized enzyme was incubated with reduced glutathione (5 mM), intermediate curves were obtained (Fig. 3).

From Figs 1, 2 and 3 we can conclude that there exist at least two forms of pyruvate kinase with different kinetic properties, which can be interconverted, probably by oxidation and reduction of the –SH groups of the enzyme.

The increase in K_m value for PEP in the presence of Fru-1,6-P_2, obtained with the oxidized enzyme as compared with the reduced enzyme, might be of physiological importance. Therefore the enzymatic activity of the oxidized and reduced

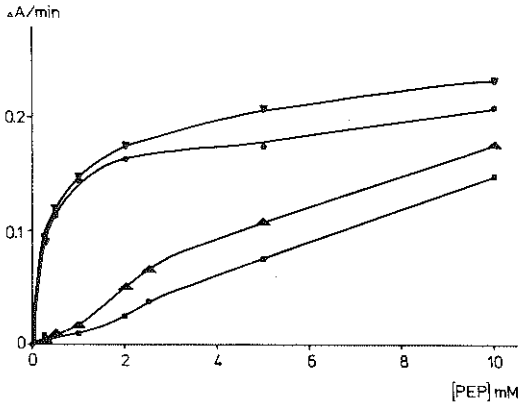

Fig. 3. The *v vs* [PEP] plot of the oxidized L-type pyruvate kinase after incubation for 1 h at 10 °C with 5 mM reduced glutathione measured in the presence and absence of Fru-1,6-P_2. ■—■, the activity of the oxidized L-type (by oxidized mercaptoethanol) incubated with reduced glutathione; ●—●, the activity of the oxidized L-type (by oxidized mercaptoethanol) which has been incubated with reduced glutathione measured in the presence of Fru-1,6-P_2; ▲—▲, the activity of the oxidized L-type (by oxidized glutathione) incubated with reduced glutathione; ▼—▼, the activity of the oxidized L-type (by oxidized glutathione) incubated with reduced glutathione measured in the presence of Fru-1,6-P_2.

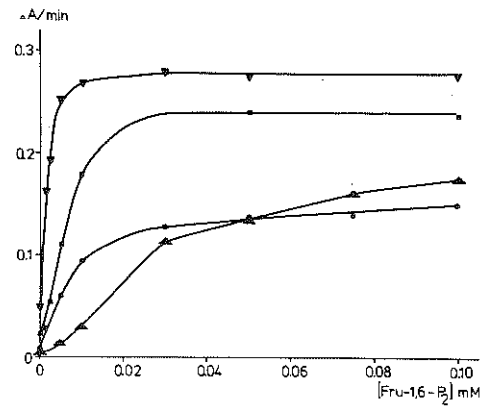

Fig. 4. The *v vs* [Fru-1,6-P_2] plot of the oxidized L-type pyruvate kinase and the oxidized L-type after incubation for 1 h at 10 °C with 5 mM reduced glutathione or 1 mM mercaptoethanol at [PEP] = 1 mM. ●—●, oxidized L-type pyruvate kinase (by oxidized mercaptoethanol); ▲—▲, oxidized L-type pyruvate kinase (by oxidized glutathione); ■—■, oxidized L-type pyruvate kinase (by oxidized glutathione) after incubation with reduced glutathione; ▼—▼, oxidized L-type pyruvate kinase (by oxidized mercaptoethanol) after incubation with reduced mercaptoethanol.

enzymes as a function of the [Fru-1,6-P_2] was studied. Fig. 4 shows that the reduced enzyme is already fully activated at 5 μM Fru-1,6-P_2, while the oxidized enzyme reaches this maximal activity at a concentration of about 30 μM Fru-1,6-P_2. Fig. 4 also shows the difference in maximal activities of the reduced and oxidized enzymes at [PEP] = 1 mM and [ADP] = 0.5 mM. When the oxidized enzyme is incubated with reduced mercaptoethanol, the same curve is obtained as with the freshly prepared enzyme. Incubation of the oxidized enzyme with reduced glutathione gives

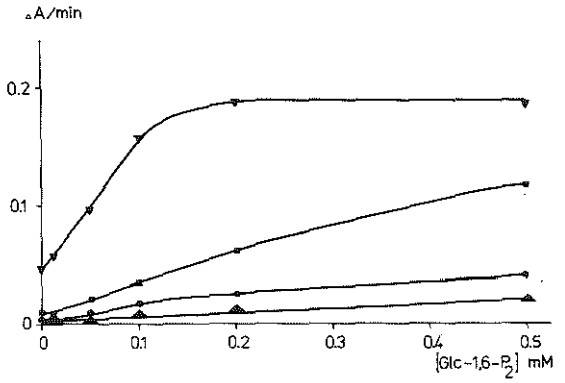

Fig. 5. The *v vs* [Glc-1,6-P_2] plot of the oxidized L-type pyruvate kinase and the oxidized L-type after incubation for 1 h at 10 °C with 5 mM reduced glutathione or 1 mM mercaptoethanol at [PEP] = 1 mM. ●—●, oxidized L-type pyruvate kinase (by oxidized mercaptoethanol); ▲—▲, oxidized L-type pyruvate kinase (by oxidized glutathione); ■—■, oxidized L-type pyruvate kinase (by oxidized glutathione) after incubation with reduced glutathione; ▼—▼, oxidized L-type pyruvate kinase (by oxidized mercaptoethanol) after incubation with reduced mercaptoethanol.

an intermediate curve; when this enzyme is further incubated with reduced mercaptoethanol, the same curve is obtained as with the freshly prepared enzyme.

Fig. 5 shows that the oxidized enzyme is rather insensitive to Glc-1,6-P_2 stimulation. When the oxidized enzyme was incubated with reduced glutathione for 1 h, a more marked stimulation by Glc-1,6-P_2 is found. Incubation with (reduced) mercaptoethanol for 1 h gives the same stimulation with Glc-1,6-P_2 as it is found with the freshly isolated enzyme. From Figs 4 and 5 the conclusion can be drawn that the oxidized enzyme is rather insensitive to Glc-1,6-P_2, whereas Fru-1,6-P_2 is still able to stimulate the enzyme, although the concentration necessary for maximal stimulation has increased from 5 μM with the reduced enzyme to 30 μM for the oxidized enzyme.

DISCUSSION

From the data presented it is clear that the L-type pyruvate kinase can exist in two forms with different kinetic properties. The two forms can be interconverted by incubation with oxidized or reduced mercaptoethanol or glutathione. Since these compounds affect the –SH groups of proteins, it seems likely that thiol groups are involved in the interconversion. When glutathione (5 mM) was used to reduce the oxidized enzyme, the results obtained were not identical with those obtained with mercaptoethanol (1 mM) as a reducing agent. This might be due to incomplete reduction of the enzyme with glutathione, suggesting at least two different types of –SH groups in the enzyme, involved in the interconversion.

For pyruvate formiate lyase from *Escherichia coli*[14,15] and also for xanthine oxidase (EC 1.2.3.2) from rat liver[16–18] an enzyme regulation by the oxidation and reduction of thiol groups has been shown and it is also likely that these interconversions are enzyme-catalysed. Also the fructose-1,6-diphosphatase (EC 3.1.3.11) activity from rabbit liver seems to be influenced by SH reagents[19–21]. For plants the re-

dox state of the SH groups of a number of enzymes is a general kind of control[22]. The existence of such an enzyme-catalysed interconversion for rat liver pyruvate kinase cannot be concluded from the presented data. The *in vitro* oxidation is a rather slow process (for full oxidation 6 h are required, data not shown) and subsequent reduction takes 1 h. These data are obtained with the partially purified enzyme. By analogy with other enzymes, it is tempting to speculate the existence of an *in vivo* enzyme-catalysed interconversion. One condition for physiological regulation is fullfilled in that the interconversion is reversible.

The difference in kinetic properties between the oxidized and reduced enzymes makes regulation of the enzymatic activity possible. In the oxidized enzyme the interaction of the PEP binding sites is raised (Figs 1 and 2) and Fru-1,6-P_2 is still able to stimulate the reaction. However the [Fru-1,6-P_2] needed for full activity is approximately 6 times higher than for the reduced enzyme and exceeds the liver [Fru-1,6-P_2] (ref. 23). This decrease in affinity for PEP and Fru-1,6-P_2 can be important during fasting, when PEP is needed for gluconeogenesis. In this view the markedly decreased influence of Glc-1,6-P_2 (of which the concentration in the liver remains constant during fasting and feeding, at least in the mouse[24]) on the oxidized enzyme is of great importance. These properties can make the interconversion of the two forms of pyruvate kinase (L-type) of physiological importance, if a system exists which can catalyse these interconversions more rapidly than under our *in vitro* conditions.

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ON THE REGULATION AND ALLOSTERIC MODEL OF L-TYPE PYRUVATE KINASE FROM RAT LIVER

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SUMMARY

1. The influence of P_i and the phosphorylated hexoses Fru-1,6-P_2, Fru-6-*P*, Fru-1-*P*, Glc-1,6-P_2, Glc-6-*P*, Glc-1-*P* and Gal-1-*P* on L-type pyruvate kinase from rat liver has been investigated at physiological ADP, *P*-enolpyruvate, GSH, ATP and alanine concentrations. At 0.1 mM *P*-enolpyruvate and in the presence of 2 mM MgATP + 1 mM alanine the enzyme is inactive and the only effective activator in a physiological concentration range is Fru-1,6-P_2.

2. The phosphorylated hexoses were used as a probe for the conformation of the enzyme. A comparison of the effects of MgATP and alanine on the "affinities" * for the hexoses reveals the following differences. (a) MgATP (2 mM) and alanine (1 mM) increase the concentration of Glc-1,6-P_2 and Glc-6-*P* necessary for half-maximal activation to the same extent; however, MgATP and alanine introduce a different cooperative interaction towards Glc-1,6-P_2 and Glc-6-*P* at low *P*-enolpyruvate concentrations. (b) MgATP (2 mM) has less influence on the "affinities" for Fru-1,6-P_2 and Fru-6-*P* as compared to alanine (1 mM). (c) Enzyme modification by oxidation of the thiol groups increases the effect of MgATP on the "affinity" for Glc-1,6-P_2, whereas the influence of alanine is decreased. These results reveal the conclusion that MgATP and alanine introduce a different conformation, probably by binding at different sites. The results are difficult to explain by the $R \rightleftharpoons T$ equilibrium model of Monod, Wyman and Changeux and indicate that sequential conformation changes are involved in the allosteric transitions of the enzyme.

INTRODUCTION

Pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40) catalyses the last step of glycolysis. Its activity is important in the regulation of the dynamic balance between gluconeogenesis and glycolysis in the liver. In the liver two types of pyruvate kinase are present [1]. The M_2-type is located in the non-parenchymal cells [2, 3] and although this type possesses regulatory properties [4–8], its presence in a cell seems to exclude gluconeogenesis. The L-type is located in the parenchymal

* With "affinity" is meant the relative ability of a compound to activate the enzyme; this ability is concluded from the concentration of activator necessary for half maximal activation.

cells [2, 3] and responds to diet and hormones [9]. Although the L-type pyruvate kinase possesses many regulatory properties, the mechanism to inactivate the enzyme under gluconeogenic conditions is still uncertain [10–13]. From the effect of the allosteric activators P_i, phosphorylated hexoses [10] and glucose-1,6-diphosphate (Glc-1,6-P_2) [11] on the L-type pyruvate kinase it was concluded that the presence of these compounds in rat liver will result in a fully active enzyme under both glycolytic and gluconeogenic conditions. It was concluded that in the liver Glc-1,6-P_2, of which the concentration is quite constant, makes the regulation of the pyruvate kinase activity by Fru-1,6-P_2 impossible [11]. However, these conclusions were based on experiments in which 0.5 mM *P*-enolpyruvate was used and in which effectors, such as alanine and ATP were absent. Recent studies on pyruvate kinase from yeast [14] and erythrocytes [15] have shown that the affinity* of the enzyme for Fru-1,6-P_2 is increased by an increment in the *P*-enolpyruvate concentration. This finding made it important to reinvestigate the affinity of pyruvate kinase for P_i and the phosphorylated hexoses at a more physiological *P*-enolpyruvate concentration (0.1 mM) to see to what extent the relatively high *P*-enolpyruvate concentration, which has been employed earlier, influences the measured affinities for these compounds. The affinity of L-type pyruvate kinase for Fru-1,6-P_2 is also influenced by MgATP and alanine [12]. For the reason that these compounds are both present in liver we investigated also the influence of P_i and the phosphorylated hexoses in the presence of the physiological concentrations of MgATP (2 mM) and alanine (1 mM). Under these conditions it can be concluded to what extent P_i and the phosphorylated hexoses contribute to the overall activity of pyruvate kinase in vivo.

Our earlier studies with L-type pyruvate kinase from rat liver [16] and erythrocytes [17] have shown that the kinetic properties of the L-type are highly dependent upon the reduction state of the thiol groups, properties also found by Kutzbach and co-workers [13, 18]. For this reason we isolated and assayed the enzyme in the presence of the physiological reductant reduced glutathione (GSH).

In the course of this investigation we became aware of the value of P_i and the phosphorylated hexoses as probes for the conformation of the enzyme. The similarity in structure of these compounds and their similar kinetic behaviour [10, 11] has led to the conclusion that these compounds bind at the same activator site. A change in conformation of this site by an effector will influence the affinity for P_i and the phosphorylated hexoses (this will be reflected in the concentration required for half maximal activation) and their way of binding (reflected in the Hill coefficient). In this way we can compare the effects of MgATP and alanine on the conformation of this activation site while also the effect of increasing *P*-enolpyruvate concentration can be determined.

MATERIALS AND METHODS

Wistar rats, maintained on a normal diet and water ad libitum, were decapitated and the livers were removed. Liver homogenates were prepared in 0.15 M NaCl containing 5 mM reduced glutathione (GSH) and centrifuged for 60 min at 100 000 × *g*. From the supernatant, the partially purified L-type preparation was obtained according to the method of Passeron et al. [19]. The preparations were dissolved in 0.25 M Tris–HCl buffer (pH 7.5) containing 5 mM GSH. The enzymatic activity was assayed

following the decrease in absorbance at 340 nm, at room temperature (22 °C) in a reaction mixture (3.0 ml) containing, unless indicated otherwise, 25 mM Tris–HCl buffer (pH 7.5), 0.1 mM KCl, 1 mM ADP, 0.12 mM NADH, 0.1 mg lactate dehydrogenase, 20 mM $MgCl_2$, 0.1 mM *P*-enolpyruvate and 5 mM GSH.

The applied Mg^{2+} and K^+ concentrations are rather high, as compared to the free concentration found in liver [20]. Therefore the reported experiments at 0.1 mM *P*-enolpyruvate were repeated at a free Mg^{2+} concentration of 1 mM (calculated by a method based on that of Morrison and co-workers [21, 22], which will be described in detail (Van Berkel, Th. J. C., in preparation)). The K^+ concentration was lowered from 100 to 25 mM. These changes in ion concentrations did not change the obtained affinities for the phosphorylated hexoses.

ADP, *P*-enolpyruvate, NADH, P_i and the phosphorylated hexoses were obtained from Boehringer (Mannheim, Germany). The phosphorylated hexoses were analysed for the presence of Fru-1,6-P_2 by the method of Bücher and Hohorst [23]. Only Fru-6-*P* contained about 0.03% Fru-1,6-P_2, which contamination was removed enzymatically by the addition of aldolase, followed by deproteinization with trichloroacetic acid.

Control assays were performed with P_i or the phosphorylated hexoses used, in the absence of *P*-enolpyruvate. When MgATP or alanine or both were present, corresponding blanks were performed in the presence of these compounds. In all cases there was no change of NADH absorption. Duplicates of the reported experiments were run with twice the amount of lactate dehydrogenase to exclude possible effects on this enzyme reaction. Doubling the amount of lactate dehydrogenase did not influence the results obtained.

RESULTS

Affinity for P_i and the phosphorylated hexoses at 0.1 mM P-enolpyruvate and physiological effector concentrations

In preliminary studies on the effect of phosphorylated hexoses at 0.1 mM *P*-enolpyruvate, the L-type pyruvate kinase was isolated in the absence of a thiol group reductant. The obtained affinity of the enzyme for Fru-1,6-P_2 was markedly decreased, as compared to the enzyme isolated in the presence of 1 mM mercaptoethanol (data not shown). The purpose of this study was to verify if the phosphorylated hexoses can influence the pyruvate kinase activity under physiological conditions. Therefore we isolated the enzyme in the presence of the physiological reductant GSH (5 mM) [24].

Fig. 1 shows the influence of Glc-1,6-P_2 at 0.1 mM *P*-enolpyruvate on the activity of L-type pyruvate kinase under various conditions. At 0.1 mM *P*-enolpyruvate the concentration of Glc-1,6-P_2, necessary for half-maximal activation, is 120 μM. In the presence of 2 mM MgATP or 1 mM alanine the Glc-1,6-P_2 concentration, necessary for half-maximal activation, is increased to about 300 μM, whereas in the presence of both alanine + MgATP the enzyme activity is hardly influenced by the applied Glc-1,6-P_2 concentrations. Since the Glc-1,6-P_2 concentration in liver is about 15 μM [25], we can conclude that Glc-1,6-P_2 is not able to activate pyruvate kinase in the presence of the physiological concentrations of alanine and MgATP.

Glc-6-*P* is somewhat less effective at a *P*-enolpyruvate concn of 0.1 mM as

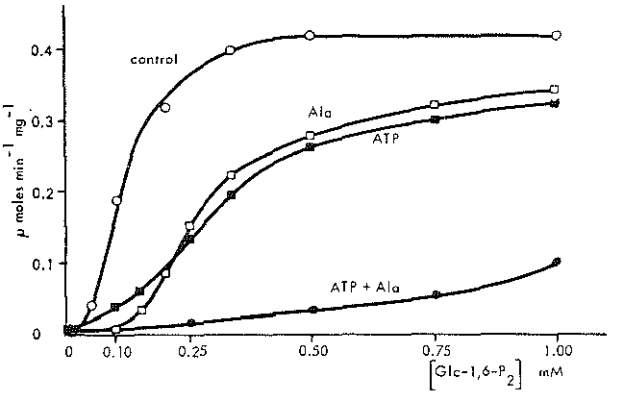

Fig. 1. The activity vs Glc-1,6-P_2 concn plot for L-type pyruvate kinase at *p*-enolpyruvate concn = 0.1 mM, ADP concn = 1 mM and 5 mM GSH. ○—○, control curve; □—□, in the presence of 1 mM alanine; ■—■, in the presence of 2 mM MgATP; ●—●, in the presence of 1 mM alanine + 2 mM MgATP.

compared to Glc-1,6-P_2 (see Table I). The effects of 2 mM MgATP and 1 mM alanine on the affinity for Glc-6-*P* are comparable (figure not shown). In the presence of both inhibitors also the influence of Glc-6-*P* has almost disappeared, which seems to exclude a role of Glc-6-*P* in the regulation of pyruvate kinase [26].

Furthermore the following phosphorylated hexoses, Glc-1-*P*, Fru-1-*P*, Fru-6-*P*

TABLE I

THE AFFINITY OF L-PYRUVATE KINASE FROM RAT LIVER FOR THE PHOSPHORYLATED HEXOSES AND P_i AT SEVERAL *P*-ENOLPYRUVATE CONCENTRATIONS

The values are the concentrations of hexoses (μM) and P_i (mM) necessary for half maximal activation. The corresponding Hill values are calculated. The *P*-enolpyruvate concentrations used are indicated.

	Control	*n* Value	+alanine (1 mM)	*n* value	+MgATP (2 mM)	*n* value	Alanine (1 mM) + MgATP (2 mM)	*n* value
P-Enolpyruvate concn = 0.1 mM								
Fru-1,6-P_2	1.2	2.6	3.2	2.6	1.8	2.8	4.5	3.9
Fru-6-*P*	No effect		No effect		No effect		No effect	
Glc-1,6-P_2	120	2.7	290	3.3	310	2.0	>1000	
Glc-6-*P*	190	2.7	590	2.4	590	1.9	>1500	
P_i	No effect		No effect		No effect		No effect	
P-Enolpyruvate concn = 0.25 mM								
Fru-1,6-P_2	1.2	2.1	3.6	2.9	2.0	2.8	5.0	3.0
Fru-6-*P*	>1500		No effect		No effect		No effect	
Glc-1,6-P_2	70	2.0	210	2.9	230	2.1	600	3.0
Glc-6-*P*	70	2.1	200	2.9	230	2.2	600	3.0
P_i	>30		No effect		No effect		No effect	
P-Enolpyruvate concn = 0.50 mM								
Fru-1,6-P_2	0.4	1.1	2.1	1.9	1.5	2.3	4.5	2.3
Fru-6-*P*	900		No effect		1500		No effect	
Glc-1,6-P_2	50	1.6	170	2.2	170	2.1	500	3.1
Glc-6-*P*	50	1.5	170	1.9	170	2.0	500	3.2
P_i	16	3.2	>30		>30		No effect	

and Gal-1-*P* were tested in a concentration range between 0 and 1.5 mM and for P_i between 0 and 30 mM. Under the applied conditions (identical as for Figs 1 and 2) these compounds did not influence the pyruvate kinase activity, even in the absence of MgATP or alanine. These obtained data, at 0.1 mM *P*-enolpyruvate and physiological effector concentrations, make it unlikely that P_i and the phosphorylated hexoses (except Fru-1,6-P_2) are able to influence the pyruvate kinase activity in vivo.

Fig. 2 shows that under the applied conditions the only activator in a physiological concentration range will be Fru-1,6-P_2. In accordance with other reports [12,

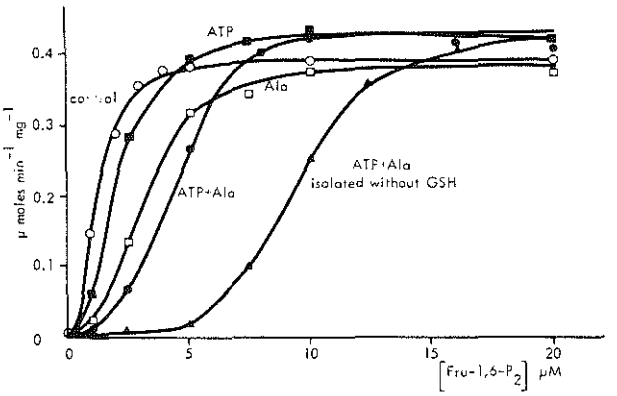

Fig. 2. The activity vs Fru-1,6-P_2 concn plot for L-type pyruvate kinase at *p*-enolpyruvate = 0.1 mM, ADP concn = 1 mM and 5 mM GSH. ○—○, control curve; □—□, in the presence of 1 mM alanine; ■—■, in the presence of 2 mM MgATP; ●—●, in the presence of 1 mM alanine + 2 mM MgATP; ▲—▲, in the presence of 1 mM alanine + 2 mM MgATP, the enzyme was isolated and assayed in the absence of GSH.

13] it can be observed that the affinity of the enzyme for Fru-1,6-P_2 is high even in the presence of both MgATP and alanine. This figure shows also that the affinity for Fru-1,6-P_2 of the enzyme, isolated in the presence of GSH, is more influenced by 1 mM alanine as compared with 2 mM MgATP. This figure shows further that the enzyme isolated in the absence of the physiological reductant has a markedly lower affinity for Fru-1,6-P_2 and is practically inactive at 5 μM Fru-1,6-P_2.

The phosphorylated hexoses and P_i as a probe for the conformation of the enzyme

As already mentioned in Introduction, the phosphorylated hexoses and P_i can be used as a probe for the conformation of the activator site. From the similarity of the structure of these compounds and from data reported elsewhere [10, 11] it can be concluded that these compounds bind on the same activator site. The use of these compounds has the advantage over fluorescent dyes that their influence is measured as enzymatic activity, which excludes a non-specific reaction. Furthermore, the affinity for these compounds can be measured accurately, which is an advantage over the relative insensitivity of the fluorescent probe methods [28].

Table I shows the *n* values calculated from the Hill plots (not shown) and the corresponding concentrations of phosphorylated hexoses necessary for half-maximal activation. At a *P*-enolpyruvate concentration of 0.1 mM the enzyme has a higher

affinity for Glc-1,6-P_2 than for Glc-6-*P*, both in the absence and presence of MgATP and/or alanine. From the obtained *n* values we can conclude that the activators bind in a cooperative way. 1 mM alanine or 2 mM MgATP increase the Glc-1,6-P_2 concentration necessary for half-maximal activation to the same extent. However, the *n* value in the presence of alanine is significantly higher as in the presence of MgATP which means that the conformational state of the enzyme in the presence of alanine differs from that with MgATP. The same phenomenon is observed with Glc-6-*P* as activator, whereas with Fru-1,6-P_2 as activator no significant difference is observed.

At 0.25 mM *P*-enolpyruvate the difference in "affinity" between Glc-1,6-P_2 and Glc-6-*P* has disappeared (middle of Table I). This indicates that by increasing the *P*-enolpyruvate concentration not only the affinity for Glc-1,6-P_2 and Glc-6-*P* increases, but that there is also a change in specificity. The activator site can no longer discriminate between Glc-1,6-P_2 and Glc-6-*P*. Such behaviour cannot be explained by a simple shift of the R ⇌ T equilibrium to the R state and indicate that *P*-enolpyruvate changes the conformation of the activator site in such a way that the phosphate on the C_1 place of the glucose molecule does no longer influence the effector binding. At 0.25 mM *P*-enolpyruvate the cooperativity toward Fru-1,6-P_2, Glc-1,6-P_2 and Glc-6-*P* is decreased as compared with 0.10 mM *P*-enolpyruvate. Also at this *P*-enolpyruvate concentration the cooperativity for Glc-1,6-P_2 and Glc-6-*P* in the presence of alanine is higher than in the presence of MgATP.

A further increase in *P*-enolpyruvate concentration to 0.5 mM results in a higher affinity for the phosphorylated hexoses and P_i. The binding of Fru-1,6-P_2 is no longer cooperative, while the cooperativity towards Glc-1,6-P_2 and Glc-6-*P* is further decreased. The cooperativity in the presence of alanine is also decreased, while this does not occur in the presence of MgATP. This leads to a disappearance of the difference in *n* values observed at 0.25 and 0.1 mM *P*-enolpyruvate. A comparison of the effects of MgATP and alanine on the affinity for the phosphorylated hexoses reveals the following differences. (a) 2 mM MgATP increases the concentration of Glc-1,6-P_2 and Glc-6-*P* necessary for half-maximal activation to the same extent as 1 mM alanine; the influence of MgATP on the affinity for Fru-1,6-P_2 and Fru-6-*P*, however, is less when compared to the influence of alanine. (b) Although MgATP and alanine increase the concentrations of Glc-1,6-P_2 and Glc-6-*P*, necessary for half-maximal activation to the same extent, they introduce a different cooperative interaction towards these activators at 0.25 and 0.10 mM *P*-enolpyruvate. These differences indicate that MgATP and alanine introduce a different conformation, probably by binding to different sites. The evidence for binding to different sites is presented in Fig. 3. In this figure is plotted the influence of Glc-1,6-P_2 on the enzyme, isolated in the absence of a thiol group reductant. The influence of alanine on the affinity for Glc-1,6-P_2 is decreased, as compared to Fig. 1 (half maximal activation at 190 μM instead of 290 μM), whereas the effect of MgATP on the contrary is increased. Incubation of this enzyme with 5 mM GSH at 10 °C for 1 h converts the enzyme into a form with the same kinetic properties as reported in Fig. 1, suggesting that the modification is the consequence of oxidation of thiol groups. This difference in response to modification of the enzyme strengthens the earlier statement that MgATP and alanine bind to different allosteric sites.

These results can also explain the discrepancies described in literature between the sensitivities for MgATP and alanine [12, 29] of L-type pyruvate kinase, which

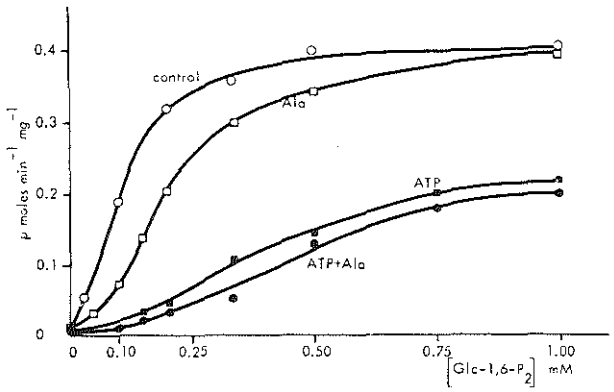

Fig. 3. The activity vs Glc-1,6-P_2 concn plot for L-type pyruvate kinase at *p*-enolpyruvate = 0.1 mM and ADP concn = 1 mM. The enzyme was isolated and assayed in the absence of GSH. ○—○, control curve; □—□, in the presence of 1 mM alanine; ■—■, in the presence of 2 mM MgATP; ●—●, in the presence of 1 mM alanine + 2 mM MgATP.

has been extensively discussed by Seubert and Schoner [30] in a recent review. Schoner et al. [29] purified the enzyme in the absence of a thiol group reductant and found the enzyme more sensitive to MgATP than to alanine, while the opposite was found by Llorente et al. [12] with crude enzyme extracts. It seems likely that in the enzyme, as isolated by Schoner et al. [29], thiol groups are oxidized during the isolation procedure, which would explain their kinetic properties (cf. Fig. 3). With the enzyme, isolated according to Llorente et al. [12], such a modification would not occur.

DISCUSSION

The influence of P_i and the phosphorylated hexoses on L-type pyruvate kinase from rat liver has led to the conclusion that the presence of these compounds in liver results in an active enzyme under both glycolytic and gluconeogenic conditions [10,11]. Our data show that the affinity of L-type pyruvate kinase for P_i and the phosphorylated hexoses is increased by raising the *P*-enolpyruvate concentration. The affinities for P_i and the phosphorylated hexoses were tested earlier [10, 11] at 0.5 mM *P*-enolpyruvate. Lowering of the *P*-enolpyruvate concentration to an in vivo value [32] results in lower affinities for P_i and the phosphorylated hexoses, which are further decreased, when the inhibitors MgATP and/or alanine are also present. From the affinities, obtained at physiological concentrations of *P*-enolpyruvate, MgATP and alanine it can be concluded that P_i and the phosphorylated hexoses (except Fru-1,6-P_2) in their physiological concentration range [25, 26] will not be able to activate pyruvate kinase. These findings reduce the number of possible regulatory legends to a great extent.

The extrapolation of in vitro data to the in vivo regulation of an enzyme needs some assumptions. However, it has been shown for the L-type from yeast, which is very similar to the L-type from rat liver, that its properties in situ are comparable to data obtained in vitro [32, 33]. For reason that the L-type pyruvate kinase is inactive in the presence of physiological concentrations of *P*-enolpyruvate, MgATP and

alanine it seems reasonable to conclude that in the liver cell the enzymatic activity will be dependent upon the Fru-1,6-P_2 concentration. However, as a consequence of the high affinity for Fru-1,6-P_2, the enzyme will be active even in the presence of the lowest reported Fru-1,6-P_2 concentration for liver. For this reason Sols and Marco [27] proposed that under fasting conditions the free Fru-1,6-P_2 concentration in liver will be lower than 5 μM due to binding to aldolase. To our opinion two other additional possibilities exist:

(A) A change in the enzyme itself can influence its affinity for Fru-1,6-P_2 as is shown in Fig. 2 and cf. [13] and [16]. Since feeding influences the GSH/GSSG ratio in liver (10 under feeding and 4 under fasting conditions; unpublished), the enzyme was isolated in the presence of the various ratio's of GSH and GSSG. However, the affinity for Fru-1,6-P_2 was found to be equal to the affinity obtained for the enzyme isolated in the presence of 5 mM GSH alone. Further investigations are therefore required to see if such an enzyme modification can occur within the cell.

(B) This possibility is based on the heterogeneity of the liver. 35% of the liver cells is of non-parenchymal origin. These cells contribute to about 10% of the liver mass [34]. Under glycolytic conditions it is reasonable to assume that Fru-1,6-P_2 is equally divided between the different cell types. The Fru-1,6-P_2 concentration under this condition is 30 μM, in which the non-parenchymal cells will contribute 3 nmoles/g and the parenchymal cells 27 nmoles/g. Under fasting conditions, gluconeogenesis is introduced in the parenchymal cells and not in the non-parenchymal cells [2, 3]. The Fru-1,6-P_2 concentration under these conditions is 5 μM of which 3 nmoles/g are located in the non-parenchymal cells and the other 2 nmoles/g are contributed by the parenchymal cells. Since these cells occupy 90% of the liver mass, the Fru-1,6-P_2 concentration in these cells will be 2.2 μM. How much the above mentioned three possibilities mutually contribute to the overall regulation of pyruvate kinase by Fru-1,6-P_2, cannot be said and deserve more experimentation in which isolated parenchymal cells may be a useful object.

The use of phosphorylated hexoses as a probe for the conformation of the enzyme makes it clear that the two-state model of Monod et al. [35] cannot explain our results with the phosphorylated hexoses. From the different behaviour of the enzyme towards the phosphorylated hexoses in the presence of ATP or alanine we can conclude that these inhibitors induce a different conformation. The different behaviour towards modification suggests that these different conformations are the consequence of binding at different allosteric centers. Recently Rozengurt et al. [36] showed that K^+ and Fru-1,6-P_2 introduce different conformation states rather than a unique R state as predicted by the model of Monod et al. [35]. Our results obtained in the presence of MgATP and alanine show that these inhibitors introduce a different T conformation and suggest that sequential conformation changes [28] are involved in the allosteric transitions of the enzyme.

ACKNOWLEDGEMENTS

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M-TYPE PYRUVATE KINASE OF LEUCOCYTES: AN ALLOSTERIC ENZYME

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SUMMARY

1. The influence of pH and amino acids on the activity of pyruvate kinase (ATP: pyruvate phosphotransferase, EC 2.7.1.40) from leucocytes were studied.

2. The data can be explained by the model of Monod, Wyman and Changeux. It is proposed that this model is also valid for the other various types of pyruvate kinase.

INTRODUCTION

It is generally accepted that the mammalian glycolytic enzyme pyruvate kinase (ATP: pyruvate phosphotransferase, EC 2.7.1.40) exists in two different forms[1]: L(liver) type and M(muscle) type. L-type pyruvate kinase is present in liver, erythrocytes and kidney[2] and the enzymatic activity of this form is allosterically modulated by phosphoenolpyruvate (PEP), K^+, Fru-1,6-P_2, Glc-1,6-P_2, phosphorylated hexoses, ATP and alanine[3–7]. The M-type is present in muscle, brain, heart, liver, kidney and leucocytes[2]. The M-type was called a non-allosteric enzyme as could be concluded from the Michaelis–Menten kinetics. From the differences in kinetic behaviour between the M- and L-type it was concluded that the metabolic changes in the cell affect the M-type pyruvate kinase less than the L-type. Recently Jiménez de Asúa *et al.*[8] showed that the M-types from liver and muscle can be inhibited by amino acids. From these inhibition studies they concluded that the M-type pyruvate kinase exists at least in two different forms. It was subsequently discovered by Sols (personal communication) that the glycolytic intermediate Fru-1,6-P_2 is able to reverse the inhibition of the liver M-type by alanine.

Since the M-type of pyruvate kinase is found only in Kupffer cells[9,10] and the M-type occurs also in the leucocytes[2], we intended to investigate more closely the kinetic behaviour of the leucocyte enzyme. This is of special interest in the question as to what extent the leucocyte enzymes are related to the Kupffer cell enzymes, in connection with the use of leucocyte enzymes in metabolic liver disease.

Abbreviation: PEP, phosphoenolpyruvate.

MATERIALS AND METHODS

Leucocytes were isolated according to the method described by Wyss *et al.*[11]. Finally, the cells were sonicated for 1 min at 21 kHz, and centrifuged for 10 min at 10 000 × g_{max}. In the supernatant, pyruvate kinase activity was assayed by following the decrease in absorbance at 340 nm in the coupled reaction with lactate dehydrogenase at room temperature, according to the method of Valentine and Tanaka[12]. The triethanol–HCl buffer (0.4 M, pH 7.5) was replaced by Tris–HCl buffer (0.25 M, pH 7.5 or 8.5 as indicated in the figures). For the assay at pH 5.9, a 0.1 M Tris–maleate buffer was used.

ADP, PEP, NADH and Fru-1,6-P_2 were obtained from Boehringer (Mannheim, Germany). L-Alanine was obtained from Merck (Darmstadt, Germany). L-Proline, L-tryptophan, L-phenylalanine, L-glutamate, DL-valine and L-threonine were obtained from British Drug Houses Ltd. All other reagents were of analytical grade purity.

RESULTS

Fig. 1 shows the dependence of the leucocyte pyruvate kinase activity on the PEP concentration at an ADP concentration of 2 mM and pH 7.5 in various conditions. The activity in the absence of alanine shows, in agreement with Campos *et al.*[13], normal Michaelis–Menten kinetics at this pH. From the Hill plot (insert Fig. 1) an n value of 1.1 can be calculated, suggesting no cooperative interaction between

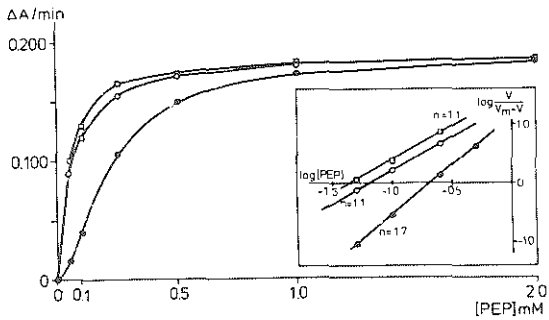

Fig. 1. ΔA *vs* [PEP] plot of M-type leucocyte pyruvate kinase at pH 7.5 with [ADP] = 2 mM in various conditions. □—□, control; ●—●, with 1 mM alanine; and ○—○, with 1 mM alanine and 0.5 mM Fru-1,6-P_2. Added protein, 84 μg.

the binding sites of PEP, at least under these conditions. In the presence of alanine (1 mM) a curve is obtained which is slightly sigmoidal and has the same V as in the absence of alanine. Under these conditions a Hill coefficient of 1.7 can be calculated. However, addition of Fru-1,6-P_2 (0.5 mM) converts the sigmoidal curve into a hyperbolic one (n = 1.1). From this plot it can be concluded that Fru-1,6-P_2 overcomes the alanine inhibition, which is accompanied by a loss of the cooperative interaction between the PEP binding sites. These properties resemble those of the L-type pyruvate kinase. Therefore we investigated the influence of pH, since pH affects the al-

losteric properties of the L-type pyruvate kinase. In Fig. 2, leucocyte pyruvate kinase activity *versus* PEP concentration at an ADP concentration of 2 mM and pH 8.5 is plotted. With the normal activity curve no significant cooperativity is found (n = 1.2). In the presence of alanine (1 mM) an n value of 1.8 is obtained, whereas the $K_{0.5}$ for PEP is increased from 0.2 mM at pH 7.5 to 0.75 mM at this pH. Fru-1,6-P_2 (0.5 mM) is still able to overcome the alanine inhibition and lowers the n value from 1.8 to 0.9. At pH 5.9 no inhibition of alanine (1 mM) could be observed (not shown) and the $K_{0.5}$ for PEP in the presence or absence of alanine (1 mM) is 0.05 mM. The same value is obtained when alanine (1 mM) *plus* Fru-1,6-P_2 (0.5 mM) are present.

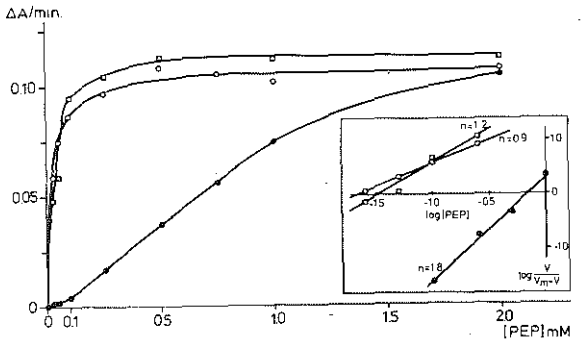

Fig. 2. ΔA *vs* [PEP] plot of M-type leucocyte pyruvate kinase at pH 8.5 with [ADP] = 2 mM in various conditions. □—□, control; ●—●, with 1 mM alanine; and ○—○, with 1 mM alanine and 0.5 mM Fru-1,6-P_2. Added protein, 60 μg.

Fig. 3 shows the influence of the alanine concentration at 0.5 mM PEP and ADP concentration of 2 mM at pH 7.5 on the pyruvate kinase activity before and after addition of 0.5 mM Fru-1,6-P_2. The curve obtained in the absence of Fru-1,6-P_2 is sigmoidal. Addition of Fru-1,6-P_2 overcomes the alanine inhibition, even at high concentrations of alanine (5–10 mM).

It is known that alanine does not inhibit the muscle M-type pyruvate kinase, but that phenylalanine inhibits this enzyme[8]. Fig. 4 shows the influence of phenylalanine on the leucocyte pyruvate kinase in the absence and presence of Fru-1,6-P_2.

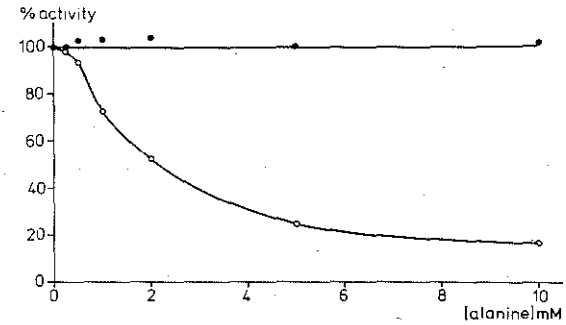

Fig. 3. The influence of alanine on the activity of M-type leucocyte pyruvate kinase at [PEP] = 0.5 mM, [ADP] = 2 mM and at pH 7.5. ○—○, control; and ●—●, the addition of Fru-1,6-P_2 (0.5 mM).

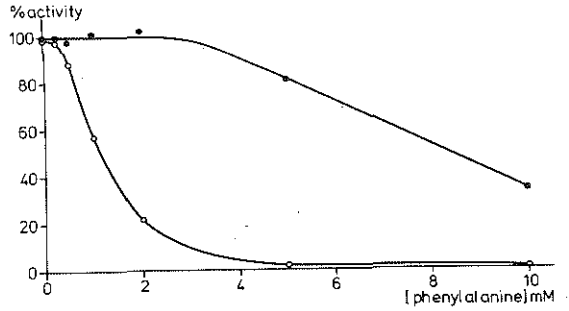

Fig. 4. The influence of phenylalanine on the activity of M-type leucocyte pyruvate kinase at [PEP] = 0.1 mM, [ADP] = 2 mM and at pH 7.5. ○—○, control; and ●—●, the addition of Fru-1,6-P_2 (0.5 mM).

In the absence of Fru-1,6-P_2 a sigmoidal curve is obtained, while Fru-1,6-P_2 can only overcome this inhibition completely up to 2 mM phenylalanine. At high phenylalanine concentration this restoration of activity is impaired.

In Table I the influences of various amino acids on the activity of the leucocyte pyruvate kinase are summarized. When these results are compared with the effects of amino acids on the activity of the liver and muscle M-type[8], it follows that the M-type of leucocytes is very similar to the liver M-type. With valine, threonine and proline, added in a final concentration of 1 mM, no inhibition is found and addition of Fru-1,6-P_2 has no effect. Increasing this concentration to 5 mM results in a striking inhibition, suggesting that these compounds act also as allosteric inhibitors, while the restoration of activity by Fru-1,6-P_2 is dependent on the amino acid used. With valine, proline and alanine the activity is fully restored, whereas with phenylalanine, threonine and tryptophan the activity is only restored to the extent of 81–86%.

TABLE I

THE INFLUENCE OF VARIOUS AMINO ACIDS ON THE LEUCOCYTE M-TYPE PYRUVATE KINASE AT [PEP] = 0.1 mM, [ADP] = 2 mM and pH 7.5 IN THE ABSENCE AND PRESENCE OF Fru-1,6-P_2 (0.5 mM).

Addition	*Concentration (mM)*	*% of activity*	
		−Fru-1,6-P_2	*+Fru-1,6-P_2*
L-Phenylalanine	1	57	101
	5	2	81
L-Valine	1	100	105
	5	48	97
L-Proline	1	101	101
	5	41	100
L-Tryptophan	1	93	97
	5	34	86
L-Glutamate	1	107	102
	5	93	92
L-Alanine	1	38	102
	5	20	99
L-Threonine	1	100	98
	5	65	86

DISCUSSION

The similarity in inhibition by amino acids of the activity of the M-type of leucocytes and the M-type of liver pyruvate kinase makes it very likely that these types are quite similar. Moreover, Fru-1,6-P_2 is able to overcome the amino acid inhibition of the M-type of leucocytes, as has also been shown for the liver M-type pyruvate kinase (A. Sols, personal communication). We have shown previously[9] that the M-type of liver is confined to the Kupffer cells. The similarities described here between leucocyte and Kupffer cell pyruvate kinase support the common reticulo endothelial origin of these cell types, as suggested earlier (*cf.* ref. 14).

From the presented kinetic data it must be concluded that the M-type of leucocytes can show cooperative interaction between the binding sites for PEP. The curves obtained in the presence of the allosteric inhibitor alanine are sigmoidal and are very similar to the normal activity curves obtained with the L-types of liver[5,15] or erythrocytes[16]. At pH 5.9 the latter enzymes obey Michaelis–Menten kinetics with respect to PEP and cannot be activated by Fru-1,6-P_2. At pH 7.5 these enzymes have a sigmoidal response to PEP and this response is transformed into a normal hyperbolic relationship by the addition of Fru-1,6-P_2[15,16]. At pH 8.5 these effects are more pronounced[8]. The results obtained with the M-type of leucocytes are quite similar, but the presence of alanine (1 mM) is required. The inhibition curves of alanine and of phenylalanine show that these compounds act as allosteric inhibitors. When 5–10 mM phenylalanine is present, Fru-1,6-P_2 is not able to overcome the inhibition, which makes it clear that phenylalanine and Fru-1,6-P_2 act as allosteric antagonists. These properties are in complete agreement with the two-state R⇌T model of Monod *et al.*[17], applied originally to the allosteric L-type liver pyruvate kinase by Rozengurt *et al.*[15] and extended to human erythrocyte L-type pyruvate kinase by Staal *et al.*[16].

For the leucocyte enzyme we can state that this enzyme is normally in the R state, while alanine and phenylalanine are able to convert this R form into the T form. The effect of Fru-1,6-P_2 can be explained by a simple reconversion of the T state to the R state. It is likely that this model is also valid for the liver M-type pyruvate kinase, whereas the allosteric inhibition by phenylalanine on the activity of muscle M-type pyruvate kinase[18] indicates that this model may even be extended to the muscle M-type pyruvate kinase. Probably, the differences in kinetic behaviour of the various isoenzymes of pyruvate kinase are the result of a different allosteric constant (L). This L value is different from zero for the various L-types and zero for the various M-types, at least at physiological pH and in the absence of modifiers. It is likely that this difference in L value is the consequence of structural differences between the various pyruvate kinase isoenzymes.

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SOME KINETIC PROPERTIES OF THE ALLOSTERIC M-TYPE PYRUVATE KINASE FROM RAT LIVER; INFLUENCE OF pH AND THE NATURE OF AMINO ACID INHIBITION

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SUMMARY

1. The influence of the pH on the activity of the M-type pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40) from rat liver has been studied. The $K_{0.5}$ for the substrate phosphoenolpyruvate was pH dependent and above pH 7.25 sigmoidal curves have been obtained. Fructose-1,6-diphosphate was able to convert these curves into a hyperbolic relationship.

2. It was found that alanine acts as an allosteric inhibitor. The inhibition could be fully abolished by the addition of fructose-1,6-diphosphate. The alanine-inhibition is dependent on the pH and on the phosphoenolpyruvate concentration.

3. It is concluded that most of the properties can be explained by the model of Monod, Wyman and Changeux.

INTRODUCTION

It is generally accepted that the mammalian glycolytic enzyme pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40) exists in two different forms[1]: L(liver) type and M(muscle) type. The L-type pyruvate kinase is present in liver, erythrocytes and kidney[2] and the enzymatic activity of this form is allosterically modulated by phosphoenolpyruvate (PEP), K^+, fructose-1,6-diphosphate (Fru-1,6-P_2), glucose-1,6-diphosphate, phosphorylated hexoses, ATP and alanine[3–7]. The M-type is present in muscle, brain, heart, liver, kidney and leucocytes[2,8]. The M-type was called a non-allosteric enzyme, as could be concluded from the Michaelis–Menten kinetics. Recently Jiménez de Asúa *et al.*[9] showed that the M-types from liver and muscle can be inhibited by amino acids. From these inhibition studies they concluded that there are at least two different forms of M-type pyruvate kinase. From our studies with M-type pyruvate kinase from leucocytes[8] it was concluded that this

Abbreviations: PEP, phosphoenolpyruvate; Fru-1,6-P_2, fructose-1,6-diphosphate.

enzyme shows cooperative interaction towards PEP in the presence of the inhibitors alanine and phenylalanine, Fru-1,6-P_2 completely reversed this inhibition and normal Michaelis–Menten kinetics were obtained. On the basis of the similarity in inhibition by amino acids of the M-type pyruvate kinase of leucocytes and the M-type of liver, it was suggested that these M-types are quite similar. However Jiménez de Asúa *et al.*[9] were not able to show cooperative interaction between the PEP binding sites in the presence of amino acids. Therefore, we studied the nature of the amino acid inhibition of this enzyme at different pH values, especially in connection with the earlier proposed R ⇌ T model for the M-type pyruvate kinase[8].

For comparison with the properties of the L-type pyruvate kinase from rat liver, we also isolated L-type by $(NH_4)_2SO_4$ precipitation of the cytosol between 20 and 45% saturation. The M-type, on the other hand, is isolated in the 55–70% $(NH_4)_2SO_4$ fraction. The properties obtained with the L-type were found to be similar to the results obtained by Rozengurt *et al.*[10] (compare also Van Berkel *et al.*[11]). This makes it possible to compare our results obtained with the M-type with the properties of the L-type described by Rozengurt *et al.*[10].

MATERIALS AND METHODS

Type L pyruvate kinase was isolated from rat liver according to the isolation procedure described earlier[5]. Type M pyruvate kinase was isolated by the method of Passeron *et al.*[12], except that during this procedure 1 mM mercaptoethanol was omitted and the final $(NH_4)_2SO_4$ precipitation (between 55 and 70%) was dissolved in 0.25 M Tris–HCl of pH 7.25, 7.5, 7.75, 8.0 or 8.5, as indicated in the legends. The purification was about 10 to 12-fold. For the assay at pH 5.9 or 6.5 the enzyme was dissolved in 0.1 M Tris–maleate buffer of the corresponding pH.

Pyruvate kinase was assayed by following the decrease in absorbance at 340 nm in the coupled reaction with lactate dehydrogenase at room temperature according to Valentine and Tanaka[13]. The triethanol–HCl buffer (0.4 M, pH 7.5) was replaced by Tris–HCl buffer (0.25 M, pH 7.25, 7.5, 7.75, 8.0 or 8.5, as indicated in the figures). For the assay at pH 5.9 and 6.5 a 0.1 M Tris–maleate buffer was used.

ADP, ATP, PEP, NADH, Fru-1,6-P_2 and oxidized glutathione were obtained from Boehringer (Mannheim, Germany). L-Alanine was obtained from Merck (Darmstadt, Germany). Reduced glutathione was obtained from Sigma Chemical Co. All other reagents were of analytical grade purity.

RESULTS

Fig. 1 shows the dependence of the liver M-type pyruvate kinase on the PEP concentration at an ADP concentration of 2 mM at pH 7.5 and pH 8.0 before and after the addition of Fru-1,6-P_2. The activity at pH 7.5 shows only a slight sigmoidal response on increasing the PEP concentration ($n = 1.3$), suggesting that the cooperative interaction between the PEP binding sites at this pH, at least under these conditions, is very slight. With the addition of Fru-1,6-P_2 we only find a significant increase of the activity at very low PEP concentrations (up to 0.05 mM). Fru-1,6-P_2 lowers the n value, calculated from the Hill plot (insert Fig. 1), from 1.3 to 1.0. At pH 8.0 the relation between the activity and the PEP concentration is clearly

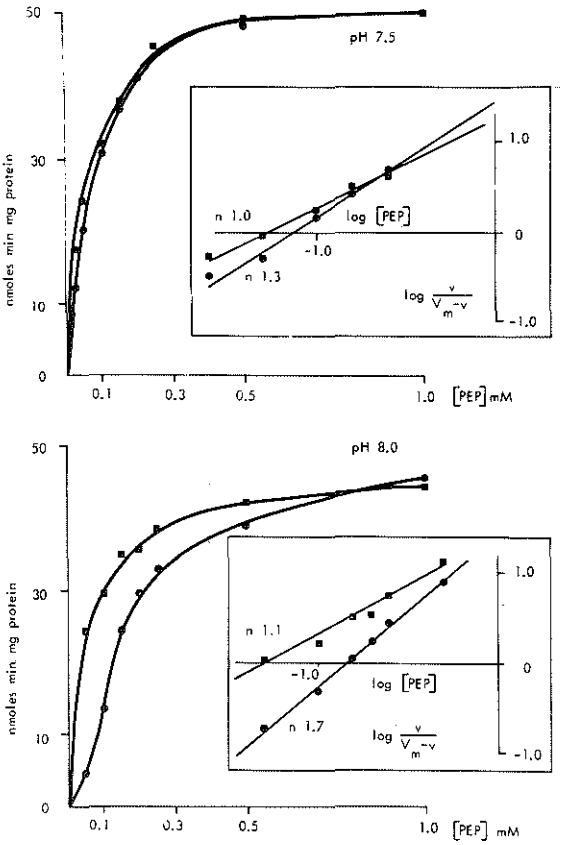

Fig. 1. M-type pyruvate kinase activity *vs* PEP concentration at pH 7.5 and pH 8.0 with 2 mM ADP. ●—●, control; ■—■, with 0.5 mM Fru-1,6-P_2. The inserts are the Hill plots of the values obtained. The calculated Hill coefficients (n) are indicated.

sigmoidal. The n value is raised from 1.3 at pH 7.5 till 1.7 at pH 8.0. Also the $K_{0.5}$ value for PEP has markedly increased from 0.06 mM at pH 7.5 to 0.14 mM at pH 8.0. At this pH Fru-1,6-P_2 markedly stimulates the enzymatic activity at low PEP concentrations and lowers the n value to $n = 1.1$, which allows the conclusion that

TABLE I

EFFECT OF pH ON THE PEP SATURATION CURVE OF THE M-TYPE PYRUVATE KINASE FROM RAT LIVER

Assay conditions as indicated in the legend to Fig. 1. n_H signifies Hill coefficient.

pH	$K_{0.5}$ *(mM)*	n_H
5.9	0.05	1.13
6.5	0.06	1.14
7.25	0.07	1.13
7.5	0.06	1.28
7.75	0.11	1.38
8.0	0.14	1.69
8.5	0.14	1.73

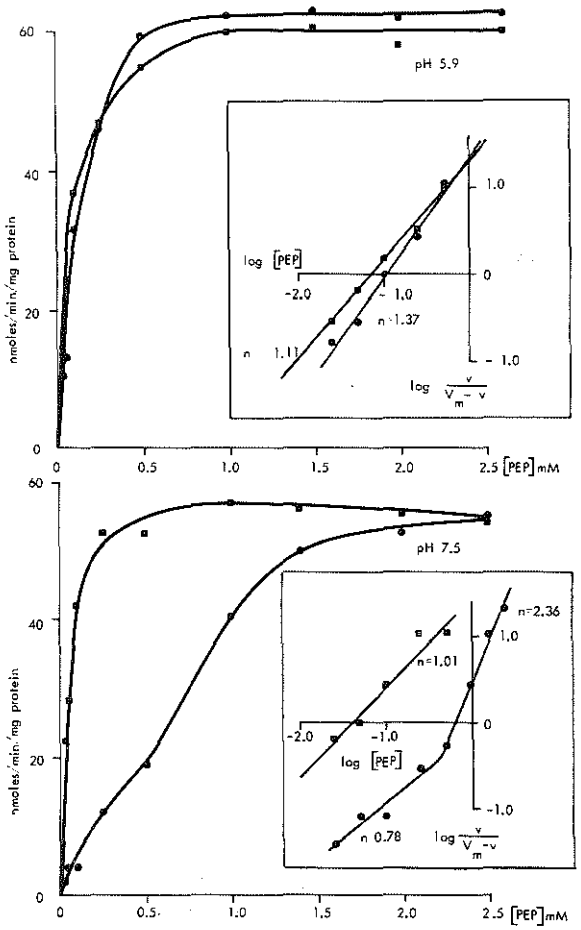

Fig. 2. M-type pyruvate kinase activity *vs* PEP concentration at pH 5.9 and pH 7.5 with 2 mM ADP. ●—●, 1 mM alanine present; ■—■, both 1 mM alanine and 0.5 mM Fru-1,6-P_2 present. The inserts are the Hill plots of the values obtained. The calculated Hill coefficients (n) are indicated.

the decrease in the $K_{0.5}$ value is accompanied by a loss of cooperative interaction between the PEP binding sites. Since alterations in the pH may play a regulatory role *in vivo*, we investigated the effect of the pH on the allosteric behaviour of the enzyme more closely. Table I shows that no cooperativity is observed when the pH is equal to or less than 7.25. However, when the pH is raised from 7.25 to 8.0 the cooperativity increases, and this is accompanied by an increase in the $K_{0.5}$ value. By plotting the p$K_{0.5}$ against the pH, according to Dixon and Webb[14], a pK_a value for the enzyme of about 7.5 is calculated. Similar calculations for the L-type pyruvate kinase from rat liver lead to a pK_a value of 6.9 (ref. 10). According to Wieker and Hess[15] this method of Dixon and Webb[14] can lead to erroneous results with allosteric enzymes, if the dissociation constants are not sufficiently separated from each other. However, this objection is not valid in this case, because both with L- and M-type only one pK_a value is found. Therefore, we can compare the pK_a value found for the M-type with the value found by Rozengurt *et al.*[10] for the L-type.

It has been reported that the alanine inhibition of the M-type pyruvate kinase from rat liver was pH independent[9]. Since the alanine inhibition of the M-type from leucocytes was found to be pH dependent[8], we investigated the influence of pH on the alanine inhibition of our preparation of the M-type from liver. Fig. 2 shows the activity as a function of the PEP concentration in the presence of 1 mM alanine before and after the addition of Fru-1,6-P_2 at pH 5.9 and pH 7.5. At pH 5.9 in the presence of 1 mM alanine the $K_{0.5}$ for PEP has hardly increased and the n value obtained is 1.37. With the addition of Fru-1,6-P_2 the n value has decreased to 1.11, suggesting that the small degree of cooperativity is lost. At pH 7.5 the alanine inhibition is more pronounced and alanine (1 mM) increases the $K_{0.5}$ value for PEP from 0.06 mM to 0.70 mM. Also at this pH Fru-1,6-P_2 counteracts the cooperativity between the PEP binding sites. At low PEP concentration (up to 0.25 mM) in the presence of 1 mM alanine a n value of 0.78 is found, which might indicate negative cooperativity[16]. This n value < 1.0 at low PEP concentration was quite reproducible. With four different preparations at pH 7.5 we found n values between 0.7 and 0.8. At higher concentrations of PEP the n value is about 2.4, which indicates a positive cooperativity. Table II summarizes the $K_{0.5}$ and n values for PEP in the

TABLE II

EFFECT OF pH ON THE PEP SATURATION CURVE OF THE M-TYPE PYRUVATE KINASE FROM RAT LIVER IN THE PRESENCE OF 1 mM ALANINE

Assay conditions as indicated in the legend to Fig. 2. n_{HI} signifies Hill coefficient at low PEP concentrations. n_{HII} signifies Hill coefficient at higher PEP concentrations (*cf.* Fig. 2).

pH	$K_{0.5}$ *(mM)*	n_{HI}	n_{HII}
5.9	0.07	—	1.37
6.5	0.15	0.89	1.89
7.25	0.72	0.80	2.56
7.5	0.68	0.78	2.36
7.75	1.65	0.55	2.45
8.0	1.70	0.53	2.60
8.5	1.76	0.59	2.58

presence of 1 mM alanine at different pH values. From this table it can be concluded that also in the presence of 1 mM alanine the $K_{0.5}$ for PEP rapidly increases between pH 7.5 and pH 8.0, suggesting that the same ionizing group as obtained in the absence of 1 mM alanine influences the activity. A further increase in pH does not alter the $K_{0.5}$ value, as is also the case when alanine is absent (*cf.* Table I).

Fig. 3 shows the influence of alanine at 0.1 mM PEP at pH 7.5 and pH 5.9. At pH 7.5 the shape of the alanine inhibition curve is hyperbolic, which is in agreement with Jiménez de Asúa *et al.*[9]. At alanine concentrations up to 2.5 mM, Fru-1,6-P_2 is able to restore the activity completely. At higher concentrations of alanine the restoration of activity is incomplete. When the reaction is carried out at pH 5.9, the plot obtained is sigmoidal. When the n value is measured according to the method of Jensen and Nester[17], a value of 2.01 is obtained. At this pH, the activity is completely restored with the addition of Fru-1,6-P_2. From this plot it can also be concluded, that alanine inhibition is pH dependent, which is at variance with the conclusion reached by Jiménez de Asúa *et al.*[9]. It appears that the pH dependence is

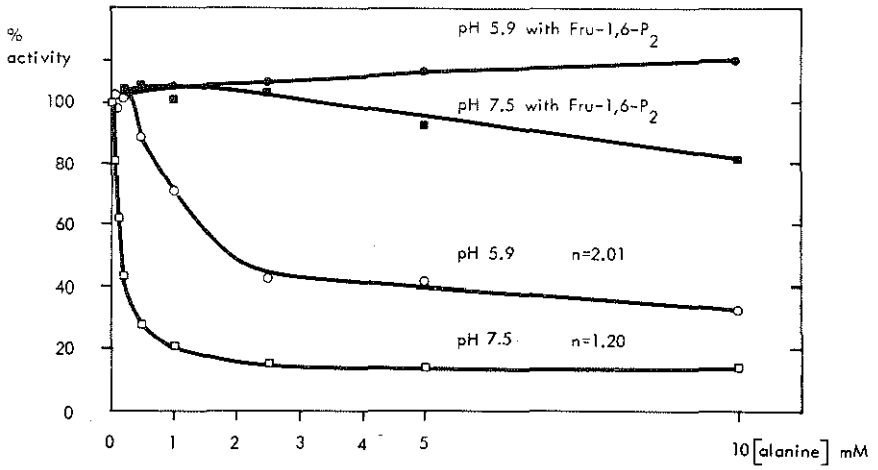

Fig. 3. The influence of alanine on the activity of liver M-type pyruvate kinase at 0.1 mM PEP 2 mM ADP and pH 5.9 or 7.5. □—□, reaction at pH 7.5; ■—■, reaction at pH 7.5 in the presence of 0.5 mM Fru-1,6-P_2; ○—○, reaction at pH 5.9; ●—●, reaction at pH 5.9 in the presence of 0.5 mM Fru-1,6-P_2. The calculated Hill coefficients (n) are indicated.

influenced by the concentration of alanine used. At 0.2 mM alanine and pH 7.5 an inhibition of 57% is found whereas 0.2 mM alanine is not inhibitory at all at pH 5.9.

In Fig. 4 the alanine inhibition curve at 1 mM PEP is shown. It can be observed that the alanine inhibition curve at 1 mM PEP and pH 7.5 is sigmoidal. A n value of 2.35 is found. When we compare this with the n value of 1.20 obtained at 0.1 mM PEP, we can conclude that there is a antagonistic interaction between the PEP-and the alanine binding sites. At pH 5.9 and 1 mM PEP alanine does not inhibit the enzymatic activity up to concentrations of 5 mM. Only at 10 mM alanine a small

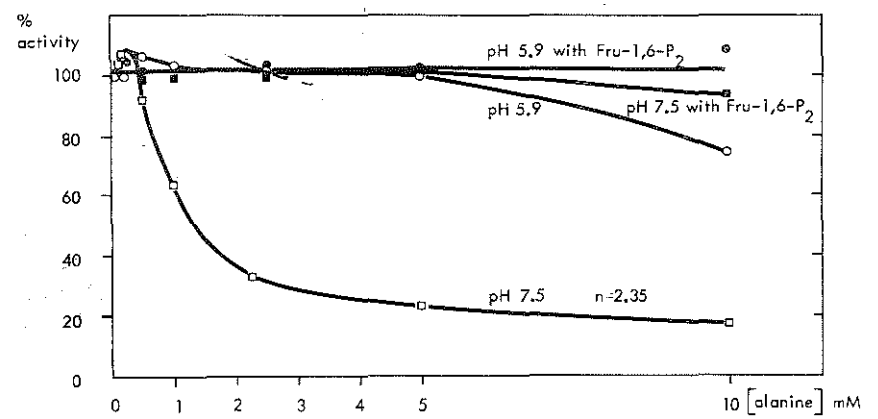

Fig. 4. The influence of alanine on the activity of liver M-type pyruvate kinase at 1.0 mM PEP, 2 mM ADP and pH 5.9 or pH 7.5. □—□, reaction at pH 7.5; ■—■, reaction at pH 7.5 in the presence of 0.5 mM Fru-1,6-P_2; ○—○, reaction at pH 5.9; ●—●, reaction at pH 5.9 in the presence of 0.5 mM Fru-1,6-P_2. The calculated Hill coefficient (n) is indicated.

inhibition can be observed. Both at pH 7.5 and pH 5.9 the restoration of activity by the addition of Fru-1,6-P_2 is complete.

These results are in contrast with the results of Imamura *et al.*[18], who found that with the highly purified enzyme there was no restoration of the activity when they added Fru-1,6-P_2 to the alanine-inhibited enzyme. From their results they concluded that the mechanism of inhibition by alanine for the L- and M-type is different. However, with the freshly isolated M-type the effect of alanine on the pyruvate kinase activity is quite similar to the results described by Schoner *et al.*[19] for the L-type. Since ATP affects the L-type pyruvate kinase activity in the same way as alanine[7,19], we also investigated the influence of pH on the ATP inhibition with the M-type. Fig. 5 shows the action of ATP at a PEP concentration of 0.1 mM and this can be compared with Fig. 3. A comparison of both figures shows that

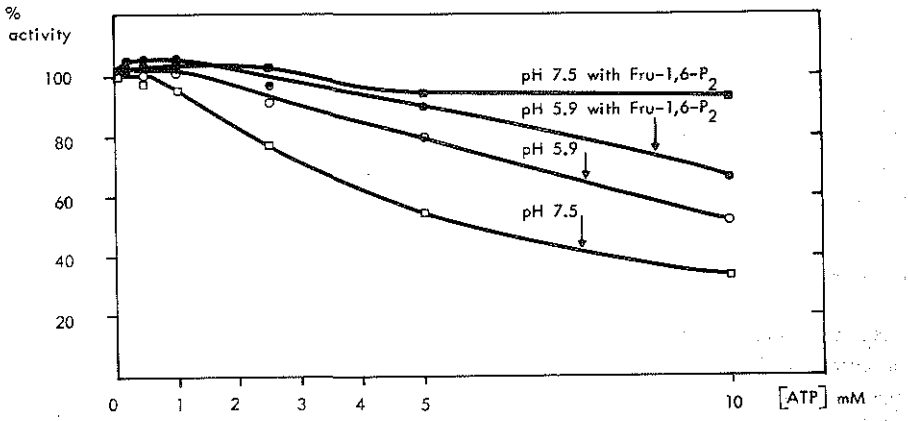

Fig. 5. The influence of ATP on the activity of liver M-type pyruvate kinase at 0.1 mM PEP, 2 mM ADP and pH 5.9 or pH 7.5. □—□, reaction at pH 7.5; ■—■, reaction at pH 7.5 in the presence of 0.5 mM Fru-1,6-P_2. ○—○, reaction at pH 5.9; ●—●, reaction at pH 5.9 in the presence of 0.5 mM Fru-1,6-P_2.

the mechanism of ATP inhibition differs from that of alanine. The ATP inhibition is slightly pH dependent and the restoration of activity by the addition of Fru-1,6-P_2 is only partial, especially at pH 5.9.

Because Imamura *et al.*[18] observed no restoration of activity with their purified M-type pyruvate kinase from rat liver when Fru-1,6-P_2 was added to the alanine-inhibited enzyme, we investigated some possibilities for these differences. As reported earlier[11], the L-type pyruvate kinase from rat liver can exist in two forms which can be reversibly interconverted by sulphydril reagents. Such an interconversion could also be important for the M-type. Therefore, the effect of oxidizing reagents on this M-type from liver was studied. By incubating the M-type at 4 °C overnight in the presence of 2.5 mM oxidized glutathione, an enzyme with the same V as the freshly prepared M-type was obtained. However, the $K_{0.5}$ for PEP is raised and the alanine inhibition is not affected by the addition of Fru-1,6-P_2 (Table III). In contrast to the results obtained with the L-type from rat liver[11], the oxidation of thiol groups is not reversible. Neither reduced glutathione, nor mercaptoethanol was able to convert this "oxidized" enzyme to the freshly prepared form. There is a possibility that our

TABLE III

COMPARISON OF KINETIC PARAMETERS OF THE FRESHLY PREPARED-AND "OXIDIZED" M-TYPE PYRUVATE KINASE FROM RAT LIVER

The assay is performed at pH 8.0; further conditions as indicated in the legend to Fig. 1.

	Freshly prepared enzyme	*"Oxidized" enzyme*
$K_{0.5}$ for PEP (mM)	0.14	0.30
K_m for PEP (mM) in the presence of 0.5 mM Fru-1,6-P_2	0.03	0.23
$K_{0.5}$ for PEP (mM) in the presence of 1 mM alanine	1.7	1.7
Reversal of alanine (1 mM) inhibition by Fru-1,6-P_2 (0.5 mM)	Complete	None

partially purified preparation of the M-type is also affected by the isolation procedure. At pH 8.0, however, it is possible to measure the $K_{0.5}$ of the M-type for PEP in a crude liver homogenate and a value of 0.15 mM has been obtained. This value agrees with the value of 0.14 mM obtained with the partially purified M-type, which suggests that our M-type preparation is identical with the enzyme present in the crude liver homogenate.

DISCUSSION

By comparing the inhibition of the pyruvate kinase activities of the M-type of leucocytes and the M-type of liver by amino acids it was concluded[8] that these M-types were similar. However, the data of Imamura *et al.*[18] suggested that there were differences. They found that, in contrast to the leucocyte enzyme, Fru-1,6-P_2 was not able to overcome the alanine inhibition. Moreover, Jiménez de Asúa *et al.*[9] were not able to show cooperative interaction between the binding sites for PEP in the presence of alanine and showed a hyperbolic inhibition curve by the amino acids alanine and phenylalanine. With the leucocyte enzyme, however, the alanine inhibition was of an allosteric nature and alanine induced cooperative interactions between the PEP binding sites[8]. Moreover, the alanine inhibition was pH dependent, whereas for the liver M-type only a slight pH dependence has been reported[9].

From the presented kinetic data, obtained with the freshly prepared enzyme, it must be concluded that at the higher pH values the M-type of liver can show cooperative interaction between the binding sites for PEP. This interaction is abolished in the presence of the positive effector Fru-1,6-P_2. The presence of 1 mM alanine can raise the $K_{0.5}$ value for PEP and also under these conditions Fru-1,6-P_2 transforms the obtained curve into a hyperbolic relationship towards PEP concentration. It is clear that the allosteric properties are markedly influenced by the pH. At a pH lower than 7.5, hyperbolic curves are obtained; Fru-1,6-P_2 does not affect the activity. An increase in the pH to 8.0 introduces cooperative interactions and the $K_{0.5}$ value for PEP increases markedly. Above pH 8.0 a further increase in pH has no further affect on the allosteric properties. These effects are qualitatively quite similar to the results obtained with the L-type. There are, however, quantitative differences: (1) The pK_a, possibly associated with the cooperative interaction for

PEP, is about 6.9 for the L-type, whereas for the M-type a value of about 7.5 can be calculated; (2) The Hill coefficient of the L-type increases from 1.0 to 3.0, when the pH is raised from 5.9 to 8.35. The increase of the n value is accompanied by an increase in $K_{0.5}$ for PEP from 0.3 to 2.3 mM. The Hill coefficient of the M-type raises only till 1.7, which means that the homotropic cooperative interaction of PEP with M-type pyruvate kinase is lower than with L-type pyruvate kinase. Also the increase in $K_{0.5}$ from the M-type for PEP varies only from 0.06 mM to 0.14 mM. This shows that the M-type has a higher affinity for PEP than the L-type.

The influence of the pH on the alanine inhibition seems to be of the same nature as that found with the L-type[19]. At pH 7.5 the inhibition is more pronounced than at pH 5.9. PEP is able to overcome the alanine inhibition and Fru-1,6-P_2 acts as an allosteric antagonist. These properties are in complete agreement with the two-state R $\rightleftharpoons$ T model of Monod *et al.*[20] applied originally to the allosteric L-type liver pyruvate kinase by Rozengurt *et al.*[10], which we have extended to the M-type pyruvate kinase by our earlier studies on the leucocyte enzyme[8]. There are, however, discrepancies as is also the case with the L-type pyruvate kinase[21,22]. One of the main discrepancies is the effect of ATP on the M-type pyruvate kinase. As has been reported earlier[9,18] the ATP inhibition can be partly reversed by Mg^{2+}. From our data we can also conclude that the ATP inhibition is different from the alanine inhibition, which can be an unique property for the M-type. The n values observed in the presence of alanine at low PEP concentrations are also difficult to interprete on the basis of the R $\rightleftharpoons$ T model at this moment. n values lower than unity have been shown earlier with yeast pyruvate kinase by Haeckel *et al.*[21], who observed n values of 0.7 at low ADP and K^+ concentrations. With the L-type from liver at low PEP concentrations such values have also been found[22]. We cannot conclude at the moment, whether these low n values are a consequence of intramolecular interactions between the binding sites for PEP (*cf.* ref. 16) or of the existence of two interconvertible forms of M-type pyruvate kinase, as has been suggested by Imamura *et al.*[18]. These two forms should then possess different affinities for alanine or PEP. However, we were not able to show such interconversions by thiol reagents, which makes it likely that, if such forms exist, the interconversion cannot be due to oxidation or reduction of the –SH groups.

The fact that the allosteric nature has not been detected earlier except by Imamura *et al.*[18], may be due to the fact that most authors[23,24] test the enzymatic activity at about pH 7.4. Indeed, at this pH it is very difficult to detect the allosteric interaction, because Fru-1,6-P_2 stimulates the enzyme only at very low PEP concentrations (less than 0.05 mM). Furthermore, high purification of the enzyme leads to a great loss of activity[9,18,23] and can lead to a modification of the enzyme. Some alteration of the thiol groups may be involved in this modification, as can be concluded from the properties of the "oxidized" enzyme. Comparison of the M-types of liver and leucocytes leads to the conclusion that these M-types are very similar. However, some differences are obvious. We do not observe a clear allosteric behaviour towards the substrate PEP with the leucocyte enzyme and in the whole pH range from 5.9 to 8.5 a $K_{0.5}$ value of 0.05 mM has been observed. Higher concentrations of alanine are needed to inhibit the leucocyte enzyme to the same extent as the M-type from rat liver and the $K_{0.5}$ value for PEP in the presence of 1 mM alanine at pH 7.5 is 0.2 mM for the leucocyte enzyme, whereas with the M-type from liver a

value of 0.68 is obtained. It is likely that these differences are due to a different allosteric constant (L).

The metabolic implication of the possibility to regulate the M-type pyruvate kinase is not obvious. In liver the M-type pyruvate kinase is located in the non-parenchymal cells[24,25] and little is known about regulation in these cell types. An important common property between non-parenchymal cells from rat liver and leucocytes is the high rate of phagocytosis of both cell types. During phagocytosis carbohydrate metabolism is highly activated[26] and allosteric stimulation of pyruvate kinase activity may be part of this activation process.

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SOME KINETIC PROPERTIES OF M_2-TYPE PYRUVATE KINASE FROM RAT LIVER AT PHYSIOLOGICAL Mg^{2+} CONCENTRATION

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SUMMARY

1. The influence of the free Mg^{2+} concentration on the kinetic parameters of M_2-type pyruvate kinase (ATP: pyruvate phosphotransferase, EC 2.7.1.40) from rat liver, isolated in the presence of the physiological free Mg^{2+} concentration, is investigated.

2. At 1 mM Mg^{2+}_{free} and pH 7.5 the affinity of the enzyme for the substrate phosphoenolpyruvate is decreased as compared to 10 mM Mg^{2+}_{free}, whereas the affinity for the second substrate ADP does not change. The decrease in affinity for phosphoenolpyruvate at low free Mg^{2+} concentrations appears to be the consequence of the presence of a low affinity form of the enzyme.

3. It is shown that the ATP inhibition is mainly due to Mg^{2+} binding and that it is probably not of physiological importance.

4. The enzyme possesses a high affinity for Fru-1,6-P_2 (half maximal activation at 1 μM) which is decreased by the addition of alanine. The affinity of the enzyme for Fru-1,6-P_2 at 0.1 mM phosphoenolpyruvate is not influenced by the free Mg^{2+} concentration. In the presence of alanine the affinity for Fru-1,6-P_2 is increased by increasing the phosphoenolpyruvate concentration.

5. It is concluded that at physiological concentrations of alanine, Mg^{2+}, ADP and phosphoenolpyruvate the M_2-type pyruvate kinase activity is mainly dependent upon the Fru-1,6-P_2 concentration.

INTRODUCTION

Recent data have shown that there are at least three non-interconvertible rat pyruvate kinases [1–3]. The L-type pyruvate kinase is present in liver, erythrocytes and kidney [1] and the enzymatic activity of this form is allosterically modulated by phosphoenolpyruvate, K^+, fructose-1,6-diphosphate (Fru-1,6-P_2), glucose-1,6-diphosphate (Glc-1,6-P_2), phosphorylated hexoses, ATP and alanine [4–8]. The presence of this form of pyruvate kinase in a cell seems to be obligatory for gluconeogenesis. The M_1-type is present in muscle and brain [9] and possesses fewer regulatory properties. The third type of pyruvate kinase, called M_2-type by Imamura and

from the Hill plot (see insert Fig. 1). The obtained $K_{0.5}$ at 10 mM Mg^{2+}_{free} is 0.16 mM while in previous experiments with 23 mM total Mg^{2+} a $K_{0.5}$ value for *P*-enolpyruvate of 0.14 mM was obtained. Lowering of the Mg^{2+}_{free} concentration to 1 mM results in an increase of the $K_{0.5}$ value for *P*-enolpyruvate to 0.34 mM while the V is only slightly lowered. A further decrease in the Mg^{2+}_{free} concentration to 0.5 mM potentiates these effects and the $K_{0.5}$ value for *P*-enolpyruvate becomes 0.45 mM. From the Hill plot (insert Fig. 1) it can be observed that the breakpoints of the lines drop to lower log $v/V-v$ values when the free Mg^{2+} concentration is lowered. This indicates that at the lower free Mg^{2+} concentration the relative contribution of the enzymatic activity with the lower n value to the overall activity is less. For reason that the lowering of the free Mg^{2+} concentration is accompanied by a lowering of the $MgADP^-$ concentration it might be possible that the $MgADP^-$ concentration is responsible for the reported effects. Therefore, we increased the total ADP and total Mg^{2+} concentrations at 0.5 mM free Mg^{2+} in such a way that the same $MgADP^-$ concentrations were present both with 1 and 10 mM free Mg^{2+}. Although the V was raised, the $K_{0.5}$ for *P*-enolpyruvate in both cases was 0.45 mM (not shown) which indicates that the shift in the $K_{0.5}$ for *P*-enolpyruvate is indeed due to the decrease in the Mg^{2+}_{free} concentration. Furthermore we plotted the values obtained in Fig. 1 as a function of the free *P*-enolpyruvate and MgPEP concentrations (not shown). When the activity is plotted against the MgPEP concentration the curves are abnormal, especially in the presence of Fru-1,6-P_2, which makes it unlikely that MgPEP is the true substrate. Activity plots against the free *P*-enolpyruvate concentration yields curves qualitatively the same to those in Fig. 1, although the differences in $K_{0.5}$ for free *P*-enolpyruvate at different Mg^{2+}_{free} concentrations are larger. However, to allow comparison with data reported in literature, we decided to plot the activity against the total *P*-enolpyruvate concentration.

In Fig. 2 is plotted the pyruvate kinase activity under the same circumstances as for Fig. 1 except that 0.5 mM Fru-1,6-P_2 is present. This figure shows that in the presence of Fru-1,6-P_2 the curves have a biphasic pattern. To investigate this pheno-

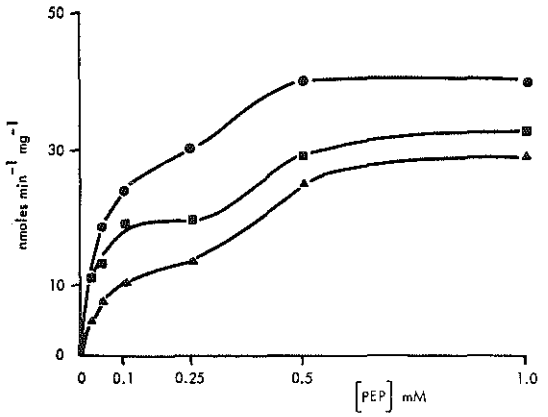


Fig. 2. Influence of the Mg^{2+}_{free} concentration on the *P*-enolpyruvate saturation curve of the M_2-type pyruvate kinase at pH 8.0 in the presence of 0.5 mM Fru-1,6-P_2. ●—●, 10 mM Mg^{2+}_{free}; ■—■, 1 mM Mg^{2+}_{free}; ▲—▲, 0.5 mM Mg^{2+}_{free}.

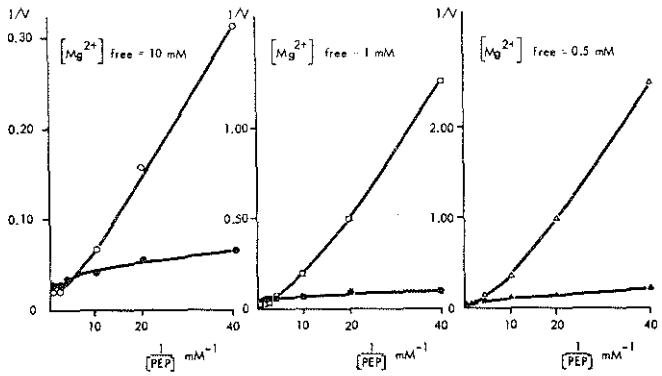


Fig. 3. The $1/v$ vs $1/[PEP]$ plot of the M_2-type pyruvate kinase activity at pH 8.0 tested in the absence and presence of Fru-1,6-P_2 (0.5 mM) at different Mg^{2+}_{free} concentrations. ○—○, 10 mM Mg^{2+}_{free} present; ●—●, 10 mM Mg^{2+}_{free} and 0.5 mM Fru-1,6-P_2 present; □—□, 1 mM Mg^{2+}_{free} present; ■—■, 1 mM Mg^{2+}_{free} and 0.5 mM Fru-1,6-P_2 present; △—△, 0.5 mM Mg^{2+}_{free} present; ▲—▲, 0.5 mM Mg^{2+}_{free} and 0.5 mM Fru-1,6-P_2 present.

menon more closely the $1/v$ vs $1/[PEP]$ plot was made (Fig. 3) from the values shown in Figs 1 and 2. In the presence of Fru-1,6-P_2 (0.5 mM) the $1/v$ vs $1/[PEP]$ curve has two components: a straight part at low *P*-enolpyruvate concentrations and a concave upward part at high *P*-enolpyruvate concentrations. This indicates that at low *P*-enolpyruvate concentrations in the presence of Fru-1,6-P_2 the enzyme follows Michaelis–Menten kinetics whereas the concave upward part is indicative for an allosteric response. By extrapolation of the straight part an apparent K_m for the enzyme at low *P*-enolpyruvate concentrations of 0.05 mM can be calculated irrespective of the Mg^{2+}_{free} concentration used. In the absence of Fru-1,6-P_2 the upward concave curves are characterized by two different n values (see Hill plot Fig. 1). This figure 3 shows clearly that only the curve obtained at low *P*-enolpyruvate concentrations, and characterized by a low n value, is stimulated by Fru-1,6-P_2. The latter addition converts the upward concave curve into a straight line.

It seems likely that these results reflect the presence of two forms of the enzyme with different affinities for *P*-enolpyruvate. From results [13] reported earlier it was concluded that we could not discriminate between this possibility and the presence of negative cooperativity [13, 23]. The results obtained at different Mg^{2+}_{free} concentrations allow the conclusion that the Mg^{2+}_{free} concentration influences the ratio between two different forms of the enzyme. The Hill plot (insert Fig. 1) shows that the n values for the form active at low *P*-enolpyruvate concentrations decreases by lowering the Mg^{2+}_{free} concentration. However, when the data are replotted by applying the V belonging to this form this decrease disappears and both at 1 and 0.5 mM Mg^{2+}_{free} a n value of 1.7 is obtained. This result indicates that the Mg^{2+}_{free} concentration regulates the relative amounts of the two forms without influencing the allosteric behaviour of the form active at low *P*-enolpyruvate concentrations.

Fig. 4 shows that the Mg^{2+}_{free} concentration does not change the apparent K_m for ADP. Both at 10 and 1 mM Mg^{2+}_{free} the enzyme follows Michaelis–Menten kinetics with an apparent K_m for ADP of 0.31 mM at 10 mM Mg^{2+}_{free} and 0.33 mM at 1 mM Mg^{2+}_{free}. Determination of the apparent K_m values from the plot $1/v$ vs $1/[MgADP]$

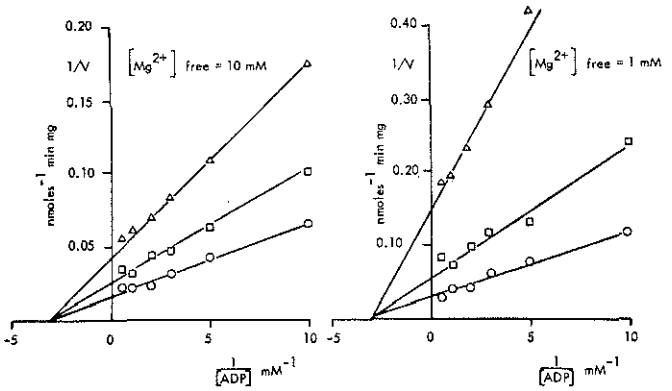


Fig. 4. The $1/v$ vs 1/ADP plot of the M_2-type pyruvate kinase activity at pH 8.0 tested at 10 and 1 mM Mg^{2+}_{free} at different *P*-enolpyruvate concentrations. △—△, 0.1 mM *P*-enolpyruvate; □—□, 0.25 mM *P*-enolpyruvate; ○—○, 0.5 mM *P*-enolpyruvate. The applied Mg^{2+}_{free} concentrations are indicated in the figure.

yields values of 0.30 and o.27 mM, respectively. These values are in good agreement with the values reported earlier for M_2-type from liver [24] and other tissues [3, 10, 14].

The afore-mentioned experiments were performed at pH 8.0 to exclude the presence of MgHADP and $HADP^{2-}$. At pH 7.5 the effect of the Mg^{2+}_{free} concentration is qualitatively similar to that obtained at pH 8.0 (Table I). At 10 mM Mg^{2+}_{free} a slight sigmoidal response towards increasing *P*-enolpyruvate concentrations is obtained with a n value of 1.4 (not shown). At 1 mM Mg^{2+}_{free} the response is a curve characterized by two n values of 1.1 and 2.2, while the same phenomenon occurs at 0.25 mM Mg^{2+}_{free}. Also at this pH a replotting of the activities correlating with the lower n value, by applying the corresponding V, indicates that the n value observed at 10 mM Mg^{2+}_{free} remains constant by lowering the Mg^{2+}_{free} concentration. Table I summarizes the $K_{0.5}$ values observed both in the absence and presence of Fru-1,6-P_2. It can be seen

TABLE I

EFFECT OF THE Mg^{2+}_{free} CONCENTRATION ON THE $K_{0.5}$ VALUE FOR *P*-ENOLPYRUVATE IN THE ABSENCE AND PRESENCE OF FRU-1,6-P_2 (0.5 mM)

Assay conditions are indicated in the legends to Figs 1 and 2. The $K_{0.5}$ in the presence of Fru-1,6-P_2 is calculated from the straight part of the $1/v$–1/[PEP] curve (see Fig. 3).

Free Mg^{2+} concentrations	pH	$K_{0.5}$ (mM)	$K_{0.5}$ (mM) of the high affinity form measured in the presence of Fru-1,6-P_2
10	5.9	0.05	0.08
1	5.9	0.05	0.07
10	7.5	0.06	0.05
1	7.5	0.28	0.06
0.5	7.5	0.32	0.06
0.25	7.5	0.35	0.06
10	8.0	0.16	0.04
1	8.0	0.34	0.04
0.5	8.0	0.45	0.06

that at pH 5.9 the same $K_{0.5}$ value is obtained for 1 and 10 mM Mg^{2+}_{free}. At increasing pH values the effect of Mg^{2+} on the $K_{0.5}$ is enforced. Further investigations however are needed to see if the effect of the pH on the affinity for *P*-enolpyruvate is due to a proton effect on the equilibrium of the two interconcertible forms or on the affinity of the low affinity form for *P*-enolpyruvate.

Although the observed activity of pyruvate kinase was linear in time during the assay it was stated [1, 10, 14] that slow transitions may be involved in the B⇌A equilibrium. Imamura and Tanaka [1] showed that pre-incubation with Fru-1,6-P_2 is able to change the biphasic *P*-enolpyruvate dependency in the presence of Fru-1,6-P_2 into normal curves. We have shown earlier that by incubation of M_2-type pyruvate kinase with oxidized glutathione the ability of Fru-1,6-P_2 to relieve the alanine inhibition is lost [13] and higher $K_{0.5}$ values for *P*-enolpyruvate are obtained also at high Mg^{2+} concentrations. The same result is obtained after prolonged dialysis (3 h) of the enzyme in the absence of Mg^{2+}. If the dialysed enzyme was incubated with Mg^{2+} (10 mM) + Fru-1,6-P_2 (0.5 M) for 30 min the original B form is restored. These data indicate that after a prolonged stay of the enzyme in the A form the conversion to the B form is a slow transition. Under these conditions also at high Mg^{2+}_{free} concentrations the properties of the A form are obtained.

Inhibitory action of alanine and ATP

The inhibitory actions of alanine and ATP on the M_2-type pyruvate kinase have been noticed by many authors [1–3, 12–14, 18]. Earlier we reported that alanine acts as an allosteric inhibitor [12, 13] and that the ATP inhibition differs from the amino acid inhibition. Imamura and Tanaka [1] reported that the ATP inhibition can be partly reversed by Mg^{2+}. However, the nature of the ATP inhibition has not been studied in detail. For reason, that this inhibition can be of regulatory importance we studied the effect of ATP at low free Mg^{2+} concentrations. Furthermore we compared this effect with the inhibition by alanine. Fig. 5 shows the pyruvate kinase activity at increasing *P*-enolpyruvate concentrations at 1 mM free Mg^{2+} in the presence of 2 mM

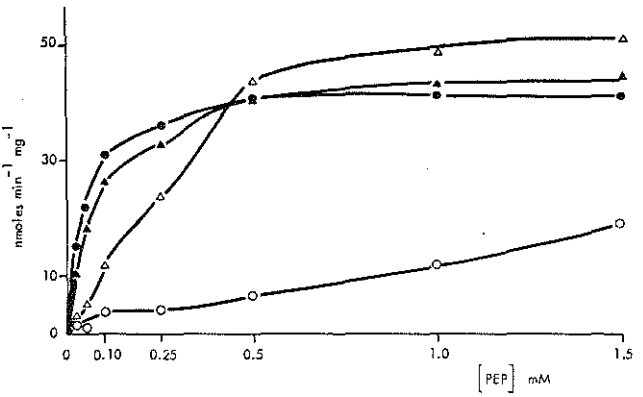


Fig. 5. M_2-type pyruvate kinase activity vs *P*-enolpyruvate concentration in the presence of 2 mM MgATP or 1 mM alanine at 1 mM Mg^{2+}_{free}. △—△, 2 mM MgATP present; ▲—▲, 2 mM MgATP and 0.5 mM Fru-1,6-P_2 present; ○—○, 1 mM alanine; ●—●, 1 mM alanine and 0.5 mM Fru-1,6-P_2 present.

MgATP or 1 mM alanine. In the presence of 2 mM MgATP the obtained $K_{0.5}$ for *P*-enolpyruvate of 0.30 mM indicates that MgATP in the physological concentration does not change the affinity for *P*-enolpyruvate. This is in contrast to the effect of 1 mM alanine, which is a strong inhibitor under the applied conditions. Under both conditions, Fru-1,6-P_2 (0.5 mM) is able to activate the enzyme and the obtained $K_{0.5}$ values in the presence of Fru-1,6-P_2 are simular whether ATP or alanine were present or not.

To differentiate between the effect of ATP on the Mg^{2+} concentration and a direct inhibitory action on the enzyme we plotted in Fig. 6 the influence of ATP at different Mg^{2+} concentrations while the effect of increasing EDTA concentrations is plotted in the same figure. It can be seen that the inhibitory action of ATP is mainly

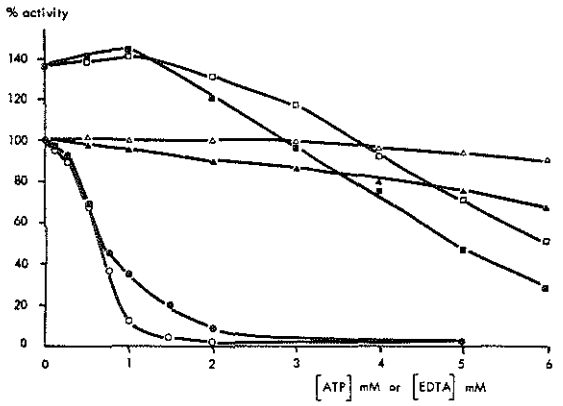


Fig. 6. The influence of ATP and EDTA on the activity of M_2-type pyruvate kinase at 0.5 mM PEP and pH 8.0. Open symbols are the added EDTA concentrations and the closed symbols the added ATP concentrations. ○—○, EDTA and 1 mM total Mg^{2+}; ●—●, ATP and 1 mM total Mg^{2+}; □—□, EDTA and 5 mM total Mg^{2+}; ■—■, ATP and 5 mM total Mg^{2+}; △—△, EDTA and 1 mM Mg^{2+}_{free}; ▲—▲, ATP and 1 mM Mg^{2+}_{free}. The 100% value is the activity at 0.5 mM *P*-enolpyruvate and total 1 mM Mg^{2+}. For 1 mM Mg^{2+}_{free} the 100% value is the activity at 0.5 mM *P*-enolpyruvate and 1 mM Mg^{2+}_{free}.

the consequence of Mg^{2+} binding. At 1 mM total Mg^{2+} the inhibitory action of ATP can be completely explained by Mg^{2+} binding. EDTA used in the same concentration is under the applied condition even more inhibitory, which is probably due to its higher affinity for Mg^{2+}, as compared with ATP. At 5 mM total Mg^{2+}, inhibition of the enzymatic activity is found at higher ATP concentrations while the data obtained at 1 mM Mg^{2+}_{free} allow the conclusion that under this probably physiological condition [15] the ATP inhibition is very small and does not seem to be of regulatory importance.

Affinity for Fru-1,6-P_2 and effect of other phosphorylated hexoses

It is well-known that Fru-1,6-P_2 activates the L-type pyruvate kinase from liver [8], erythrocytes [25] and yeast [26] in the micromolar range. For the M_2-type pyruvate kinase from liver Carbonell et al. [2] showed that 10 μM Fru-1,6-P_2 can overcome the alanine inhibition completely. Fig. 7 shows that the enzymatic form present at high Mg^{2+} concentrations shows a high affinity for Fru-1,6-P_2. At 0.1 mM

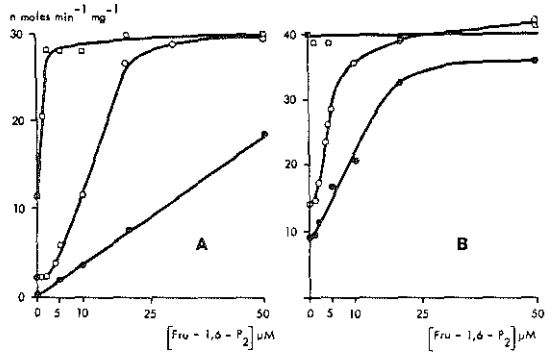


Fig. 7. (A and B) The affinity of M_2-type pyruvate kinase for Fru-1,6-P_2 in the absence and presence of alanine at: A, 0.1 mM *P*-enolpyruvate and B, 0.5 mM *P*-enolpyruvate, pH 8.0, and total Mg^{2+} concentration of 23 mM. □—□, control; ○—○; 1 mM alanine present; ●—●, 5 mM alanine present.

P-enolpyruvate full activation of the enzyme occurs already at 2 μM Fru-1,6-P_2. Addition of 1 mM alanine lowers the affinity of the enzyme for Fru-1,6-P_2 and for half maximal activation a concentration of 13 μM Fru-1,6-P_2 is needed. In the presence of 5 mM alanine the affinity for Fru-1,6-P_2 is even lower. Fig. 7B shows that at a higher *P*-enolpyruvate concentration (0.5 mM) the effect of alanine on the affinity of the enzyme for Fru-1,6-P_2 is less. This effect of alanine and *P*-enolpyruvate on the affinity of M_2-type pyruvate kinase for Fru-1,6-P_2 is very similar to that obtained with the L-type. This indicates that at high Mg^{2+} concentrations the experimental data can be explained by the earlier proposed R⇌T model [13] for M_2-type pyruvate kinase.

Fig. 8 shows that a lowering of the Mg^{2+}_{free} concentration to its physiological level of 1 mM does not change the affinity of the enzyme for Fru-1,6-P_2 at 0.1 mM *P*-enolpyruvate. The concentrations of Fru-1,6-P_2 necessary for half maximal activation measured at high free Mg^{2+} (10 mM) and low free Mg^{2+} (1 mM) are comparable and this is also the case in the presence of 1 mM alanine.

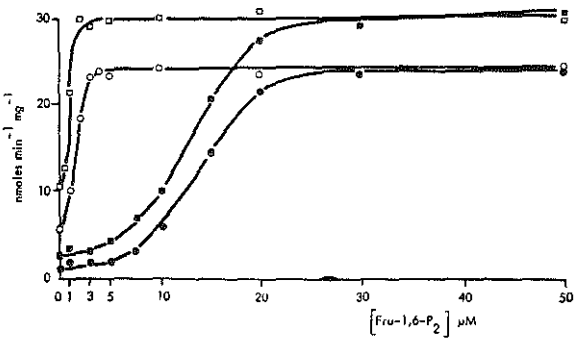


Fig. 8. The influence of the Mg^{2+}_{free} concentration on the affinity of M_2-type pyruvate kinase for Fru-1,6-P_2 in the absence and presence of 1 mM alanine at pH 8.0 and 0.1 mM PEP. □—□, 10 mM Mg^{2+}_{free}; ○—○, 1 mM Mg^{2+}_{free}; ■—■, 10 mM Mg^{2+}_{free} and 1 mM alanine present; ●—●, 1 mM Mg^{2+}_{free} and 1 mM alanine present.

TABLE II

EFFECT OF P_i AND THE PHOSPHORYLATED HEXOSES ON THE ACTIVITY OF M_2-TYPE PYRUVATE KINASE

Addition (0.5 mM)	Relative activity* (%)	Relative activity in the presence of 1 mM alanine (%)
None	28	12
Glc-1-*P*	24	10
Glc-6-*P*	28	12
Glc-1,6-P_2	61	14
Gal-1-*P*	24	10
Fru-1-*P*	27	15
Fru-6-*P*	26	10
Fru-1,6-P_2	100	111
P_i (15 mM)	25	11

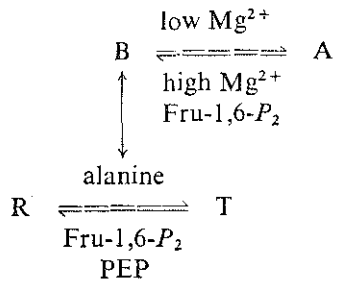
* The 100% value is the activity at 0.1 mM *P*-enolpyruvate in the presence of Fru-1,6-P_2 (0.5 mM) pH 8.0, and 1 mM Mg^{2+}_{free}.

The L-type pyruvate kinase from rat liver is not only activated by Fru-1,6-P_2 but also by P_i and phosphorylated hexoses [6, 7]. Since these latter compounds may also contribute to the "in vivo" regulation of the enzyme, we investigated the effect of P_i and phosphorylated hexoses on the M_2-type (Table II). The only effective compound besides Fru-1,6-P_2 is Glc-1,6-P_2. However, in the presence of alanine (1 mM) (a physiological concentration for liver [33]) the effect of Glc-1,6-P_2 has disappeared, which allows the conclusion that these compounds besides Fru-1,6-P_2 do not play a regulatory role in vivo.

DISCUSSION

The kinetic behaviour of M_2-type pyruvate kinase of rat liver towards *P*-enolpyruvate at low free Mg^{2+} concentrations can be completely explained by the presence of two interconvertible forms with different affinities for the substrate *P*-enolpyruvate. Pogson [10] has shown with the pyruvate kinase from adipose tissue that the presence of EDTA in the extraction buffer modifies the enzyme, resulting in an enzyme with a completely different kinetic behaviour (called by Pogson Pyk-A). The B form of the enzyme, isolated in the absence of EDTA, shows a high affinity for *P*-enolpyruvate (K_m for *P*-enolpyruvate 0.06 mM) whereas with the A form a $K_{0.5}$ of 0.6 mM has been obtained. Recently Walker and Potter [14] have shown that rat liver cells, cultured in the absence of glucose, contain this A form whereas in the presence of glucose the B form of M_2-type pyruvate kinase was obtained. The data presented make it clear that isolation of M_2-type pyruvate from normal liver in the absence of an unphysiological metal ion chelator yields an enzyme which exists in interconvertible forms at physiological Mg^{2+} concentrations. From the data presented (Fig. 1) it can be concluded that the relative contribution of the two forms to the overall pyruvate kinase activity is regulated by the free Mg^{2+} concentration. The B form of M_2-type pyruvate kinase which is present at high Mg^{2+} concentrations shows a high affinity for *P*-enolpyruvate and it was shown earlier [13] that its properties can be explained on the basis of the R⇌T model of Monod et al. [27]. In this model Fru-

1,6-P_2 and *P*-enolpyruvate should favour the R state whereas alanine and the other inhibitory amino acids possess a higher affinity for the T state. This paper shows that the B form of M_2-type pyruvate kinase has a high affinity for Fru-1,6-P_2. The effect of alanine on the affinity for Fru-1,6-P_2 at different *P*-enolpyruvate concentrations (Figs 7A and 7B) is in complete agreement with this earlier proposed R⇌T model of Monod et al. [27]. Lowering of the Mg^{2+}_{free} concentration introduces the A form, which has less affinity for *P*-enolpyruvate. The following scheme might help to explain the observed kinetic data



The A form of the enzyme is characterized by a lower affinity for the substrate *P*-enolpyruvate while this form is not sensitive to Fru-1,6-P_2. However, Fru-1,6-P_2 can convert the A form into the B form which is potentiated by high Mg^{2+} concentrations. A prolonged stay of the enzyme in the A form makes the A to B concersion more difficult and longer incubation times are needed. The occurrence of the interconvertible forms of M_2-type pyruvate kinase might also explain the variations in kinetic behaviour described in the literature. Imamura and Tanaka [1] described for the highly purified enzyme that Fru-1,6-P_2 was not able to restore the alanine inhibition, a property which we found after a prolonged incubation of the enzyme in the A form. Furthermore Costa et al. [28] recently described a new isoenzyme of pyruvate kinase in rat kidney cortex. The described kinetic properties of this enzyme are very similar to the properties of the A form of M_2-type pyruvate kinase. Since the authors purified this enzyme in the presence of 5 mM EDTA, it seems likely that the A form of M_2-type pyruvate kinase is responsible for their results.

The given scheme also explains the difference in behaviour of Fru-1,6-P_2 and Mg^{2+}. Besides an effect of Fru-1,6-P_2 on the A⇐B transition, this phosphorylated hexose is an allosteric activator of the B form, which explains the stimulation also at higher Mg^{2+} concentrations. Recently a similar kind of enzyme regulation has been shown for phosphofructokinase [29, 30] and it will be interesting to see if this kind of regulation can also account for other enzymes.

Whether Mg^{2+} itself can act as a regulatory modulator of the M_2-type pyruvate kinase in liver seems not likely, as Veloso et al. [15] have shown that the free Mg^{2+} concentration in liver is nearly constant. From the kinetic data obtained in vitro it is rather speculative to draw conclusions about the regulation of the enzyme in vivo. This remark may account for every enzyme, but is of special importance for the M_2-type from liver as this type is not uniformly localized in this tissue [31, 32]. However, applying the physiological concentrations of Mg^{2+}, *P*-enolpyruvate and alanine (for these compounds there exists no large differences between various tissues [15, 33] the

activity of the M_2-type pyruvate kinase would be low, unless the enzyme is activated by Fru-1,6-P_2 (Figs 5 and 8). An distinction between the L-type pyruvate kinase and the M_2-type is the difference in ATP inhibition. With the L-type the influence of ATP is comparable with that of alanine [8], whereas with the M_2-type ATP inhibition under physiological conditions seems negliglible (Figs 5 and 6). Whether this difference in ATP inhibition is related with the regulation of gluconeogenesis, for which the presence of L-type seems obligatory, needs further investigation.

The physiological meaning of the B⇌A equilibrium is not completely clear at the moment. As the Mg^{2+}_{free} concentration for liver, brain and kidney [15] is rather constant the B⇌A equilibrium will be dependent upon the Fru-1,6-P_2 concentration. This can explain the A form found in cells cultured in the absence of glucose. Under these conditions the Fru-1,6-P_2 will be low. However, for liver and also for leucocytes this will imply that the equilibrium will be on the B side because these cells have easily available glucose present. Preliminary studies with M_2-type from different cell types indicate that this is also the case with M_2-type from thrombocytes, whereas for rat small intestine the results are indivative for the fact that this enzyme occurs in vivo mainly in the A form (van Berkel, Th. J. C., de Jonge, H. R., Koster, J. F. and Hülsmann, W. C. unpublished). The M_2-type pyruvate kinase is widely distributed in glycolytic tissues [9] and the B⇌A equilibrium may provide an additional property of the enzyme to accommodate its properties to the specific requirements of the several tissues.

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KINETIC EVIDENCE FOR THE PRESENCE OF TWO FORMS OF M_2-TYPE PYRUVATE KINASE IN RAT SMALL INTESTINE

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SUMMARY. Some kinetic properties of pyruvate kinase from rat small intestine have been investigated. The relative insensitivity of the enzyme to ATP inhibition and the amino acid inhibition pattern allows the conclusion that intestinal pyruvate kinase belongs to the M_2-type. The pyruvate kinase activity as a function of the phosphoenol pyruvate concentration is characterized by two different n values. The activity correlating with the low n value is stimulated by Fru-1,6-P_2, whereas the activity at higher phosphoenol pyruvate concentrations is not influenced by this glycolytic intermediate. These results, together with the partial relief of the amino acid inhibition by Fru-1,6-P_2, show that two forms of the enzyme are present with different kinetic properties. The metabolic implication of the kinetic properties of pyruvate kinase for rat small intestine is discussed.

Recent work has demonstrated that there are at least three non-interconvertible types of pyruvate kinase[1-3] (ATP: pyruvate phosphotransferase, EC 2.7.1.40) in rat tissues. The L-type is present in liver, erythrocytes and kidney[1], the M_1-type in muscle and brain[2], while the third type of pyruvate kinase, called M_2-type by Imamura et al.[1], is the most widely distributed type. The occurrence of M_2-type pyruvate kinase has now been clearly shown in adipose tissue[4], kidney[2,3], leucocytes[5], liver[1,6] and hepatomas[1,7] Pogson[4] has shown that this type can occur in two interconvertible forms, called Pyk-A and Pyk-B. Isolation of pyruvate kinase from adipose tissue in the absence of EDTA yields the high-affinity form (Pyk-B), while the low-affinity form (Pyk-A) is obtained by isolation in the presence of EDTA. Although this condition is rather unphysiological, we were able to show that in the liver at 1 mM Mg^{2+}_{free} (a physiological condition for liver) these two forms of the M_2-type are in equilibrium[8]. This indicates that the presence of these two forms may be of physiological importance. From incubation experiments it was also shown that a prolonged storage of

the enzyme in the low-affinity form makes its conversion to the high-affinity form more difficult[8]. This makes it possible to conclude that when pyruvate kinase is isolated from a tissue under conditions which favour the B-form, that the detection of the A-form indicates its presence in vivo. This paper shows that this is indeed the case for pyruvate kinase from rat small intestine.

MATERIALS AND METHODS. Villous- and crypt cell suspensions originating from the duodenal, ileal and jejunal parts of rat small intestine were harvested separately according to the high-frequency vibration technique of Harrison and Webster[9] in a medium containing 0.01 M Tris-HCl buffer (pH 7.5), 0.13 M NaCl and 5 mM EDTA. Cells and cell sheets were collected by centrifugation for 15 sec at 800 g_{max}. The cells were washed two times with the isolation medium except that EDTA was omitted. Homogenates were prepared in a medium containing 0.5 M Tris-HCl (pH 7.5) + 1 mM $Mg\ Cl_2$, by Polytran treatment as described earlier by de Jonge[10]. A more heterogeneous cell population originating from the whole length of the small intestine was prepared by gently scraping off the intestinal mucosa with a glass slide. Homogenization medium and technique were similar to those mentioned above.

Pyruvate kinase was assayed as described earlier[6]. The assay mixture contained: 25 mM Tris HCl pH 7.5, 225 mM KCl, 1 mM ADP, 0.12 mM NADH, 23 mM $MgCl_2$, 0.1-0.2 mg lactate dehydrogenase and [PEP] as indicated in the legends to the figures. Duplicates were run with twice the amount of lactate dehydrogenase to exclude possible effects on this enzyme reaction.

RESULTS

In preliminary experiments we isolated villus- and crypt cells from the duodenal, ileal and jejunal parts of rat small intestine to investigate whether the isoenzyme patterns in the fast-dividing crypt and the non-proliferative villus cells would be similar and also whether differences along the length of the small intestine would occur. In these experiments (not shown) no significant differences in kinetic properties between the various celltypes were observed and the same activity dependence from the PEP concentration was found as shown in Fig. 1 for the intestinal scrapings isolated in the absence of EDTA. Scrapings obtained in the absence of EDTA followed by isolation of the cell fraction in the presence of 1 mM Mg^{2+} results in a kinetic pattern characterized by two different n values (see Hill plot insert Fig. 1). Addition of Fru-1,6-P_2 (0.5 mM) only stimulates the pyruvate kinase activity corresponding with the n value of 1.2, whereas the activity at higher PEP concentration is not influenced. This result indicates

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Abbreviation: phosphoenol pyruvate PEP

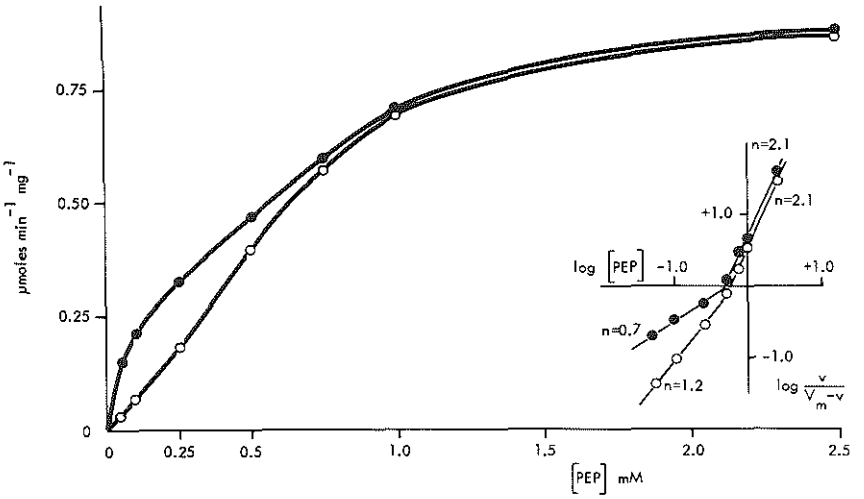

Fig. 1 Rat small intestine pyruvate kinase activity as a function of the PEP concentration.
0-0, activity in the absence of Fru-1,6-P_2; ●-●, 0.5 mM Fru-1,6-P_2 present. The insert is the Hill plot of the values obtained.

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that a Fru-1,6-P_2 sensitive and an insensitive form of pyruvate kinase may be detected in small intestine. To clarify the nature of this phenomenon we studied the effect of alanine and ATP on the pyruvate kinase activity. Fig. 2 shows that in the presence of 1 mM alanine a strong inhibition of the pyruvate kinase activity is obtained while addition of Fru-1,6-P_2 to this inhibited enzyme does only relieve the alanine inhibition at low PEP concentrations. In comparison with 1 mM alanine, ATP (2 mM) is a weak inhibitor of intestinal pyruvate kinase (Fig. 3). This relative insensitivity of the intestinal pyruvate kinase activity for ATP indicates that intestinal pyruvate kinase is of the M_2-type. This conclusion is further strenghtened by the amino acid inhibition pattern (TABLE I) which is similar to that of the M_2-type from liver[11] and leucocytes[5] and differs from those of the L- and M_1-types[2]. Fig. 4 shows the influence of increasing alanine concentrations on the enzymatic activity. It can be seen that the presence of 0.5 mM alanine results already in a 70% inhibition of the activity at 1 mM PEP, whereas the influence of increasing ATP concentrations (Fig. 5) illustrates that ATP is far less effective as inhibitor. Fru-1,6-P_2 (0.5 mM) stimulates the alanine-inhibited enzyme at the lower PEP concentration only at higher alanine concentrations.

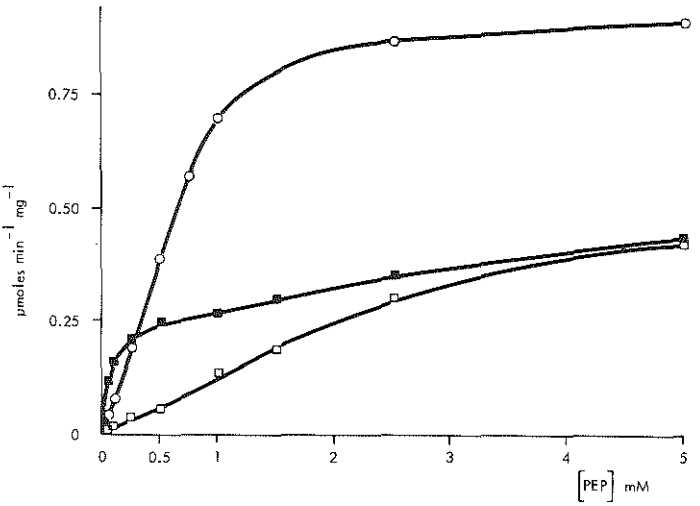

Fig. 2 Rat small intestine pyruvate kinase activity as a function of the PEP concentration in the presence of alanine. 0-0, control; □ -□, 1 mM alanine present; ■- ■, 1 mM alanine + 0.5 mM Fru-1,6-P_2 present.

TABLE I

THE INFLUENCE OF VARIOUS AMINO ACIDS ON INTESTINAL PYRUVATE KINASE

Addition	Concentration (mM)	% of activity - Fru-1,6-P_2	% of activity + Fru-1,6-P_2 (0.5 mM)
L-Phenylalanine	1	18	39
	5	8	33
L-Valine	1	57	68
	5	22	39
L-Proline	1	75	77
	5	32	47
L-Tryptophan	1	50	59
	5	20	32
L-Glutamate	1	98	91
	5	77	81
L-Alanine	1	21	41
	5	12	38
L-Threonine	1	48	59
	5	22	42
L-Cysteïne	1	24	42
	5	16	37
L-Histidine	1	83	88
	5	62	72

The 100% value is the activity at [PEP] = 1.0 mM. Further conditions are indicated in the materials and methods section.

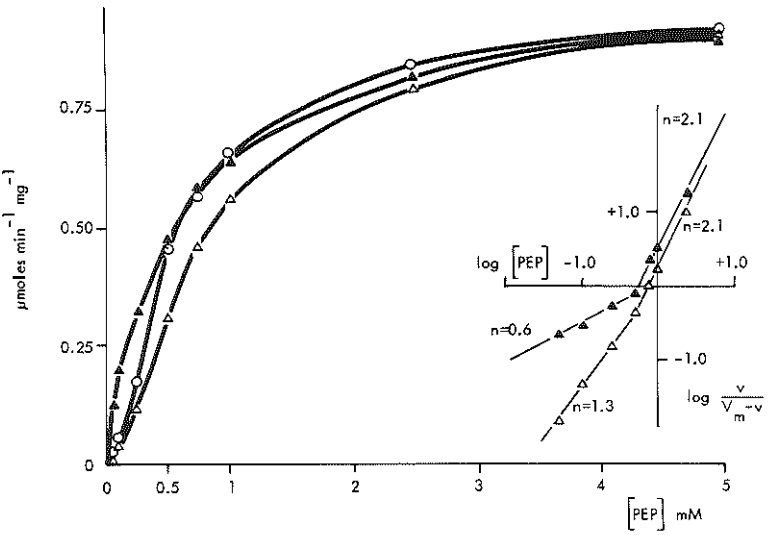

Fig. 3 Rat small intestine pyruvate kinase activity as a function of the PEP concentration in the presence of 2 mM ATP.
0-0, control; Δ-Δ, 2 mM ATP present; ▲-▲, 2 mM ATP + 0.5 mM Fru-1,6-P_2 present. The insert is the Hill plot of the values obtained.

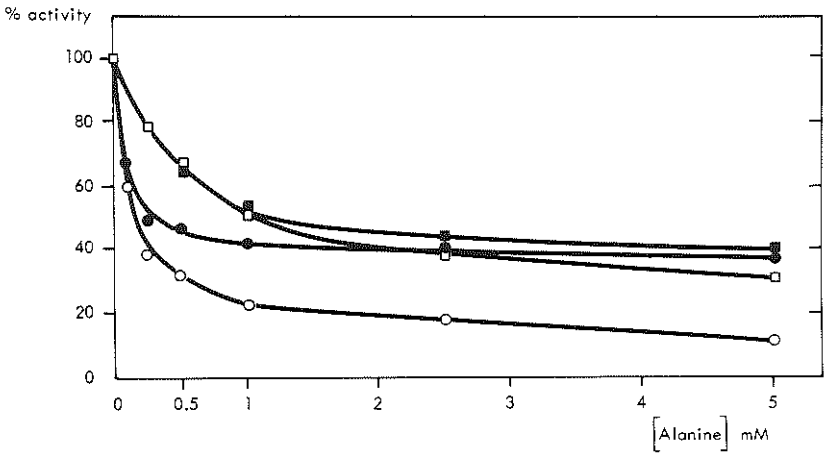

Fig. 4 Influence of alanine on rat small intestine pyruvate kinase activity at two PEP concentrations in the absence and presence of Fru-1,6-P_2 (0.5 mM).
0-0, 1 mM PEP present; ●-●, 1 mM PEP + 0.5 mM Fru-1,6-P_2 present; □-□, 5 mM PEP present; ■-■, 5 mM PEP + 0.5 mM Fru-1,6-P_2 present. The 100% value is the activity in the absence of alanine.

DISCUSSION

From the sensitivity of the intestinal pyruvate kinase activity towards ATP and alanine and from the amino acid inhibition pattern we can conclude that intestinal pyruvate kinase has to be

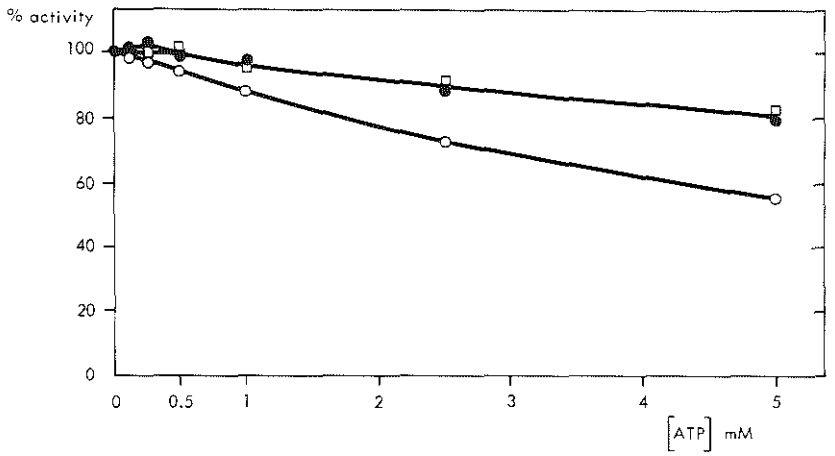

Fig. 5 Influence of ATP on rat small intestine pyruvate kinase activity at two PEP concentrations in the absence and presence of Fru-1,6-P_2 (0.5 mM).
O-O, 1 mM PEP present; ●-●, 1 mM PEP + 0.5 mM Fru-1,6-P_2 present; □-□, 5 mM PEP present and 5 mM PEP + 0.5 mM Fru-1,6-P_2 present. The 100% value is the activity in the absence of ATP.

classified as M_2-type. This finding is consistent with the absence of gluconeogenesis in this tissue[12]. Recently we have shown for the liver that this type of pyruvate kinase at physiological Mg^{2+}_{free} concentrations can occur in two forms[8]. The so-called Pyk-B form[4] has a high affinity for the substrate PEP and is inhibited by amino acids, an inhibition which is completely relieved by the addition of Fru-1,6-P_2[5-6]. At lower Mg^{2+}_{free} concentrations the Pyk-A form of the enzyme is formed which has less affinity for PEP. This form is more sensitive to amino acid inhibition and this inhibition is not abolished by the addition of Fru-1,6-P_2[8]. The kinetic behaviour of pyruvate kinase from rat small intestine can be explained completely by the presence of both forms. Fig. 1 shows that the form with high affinity for PEP is sensitive to Fru-1,6-P_2, whereas the low affinity form is not. Fru-1,6-P_2 relieves only the alanine inhibition of the high affinity form (Fig. 2). Fig. 4 shows that especially at higher alanine concentrations Fru-1,6-P_2 relieves the inhibition, indicating that the form which is less sensitive to alanine (Pyk-B) can be influenced by Fru-1,6-P_2. For reason that intestinal pyruvate kinase was isolated and assayed under conditions which favour formation of the Pyk-B form it can be concluded that "in vivo" in the small

intestine M_2-type pyruvate kinase is at least partially present in the Pyk-A form.

We have discussed earlier (ref. 8) that the B⇌A equilibrium may provide an additionalproperty for the most widely distributed type of pyruvate kinase to accommodate its properties to the specific requirements of the several tissues. As rat small intestine is the first tissue for which it is shown that a considerable amount of Pyk-A type is present "in vivo" one might speculate about its physiological function. As the major difference between B and A type is the amino acid sensitivity, the presence of A type might indicate that glycolysis will be inhibited when a protein-rich diet is ingested. Preliminary experiments with isolated intestinal cells indeed indicate that glycolysis is inhibited in the presence of alanine (H.R. de Jonge, unpublished results). A similar inhibitory effect of amino acids from the diet on the glycolytic rate "in vivo" may be important because it will induce a glucose sparing effect, which can be of importance during limited carbohydrate uptake. Experiments are in progress to investigate if the relative amounts of Pyk-B and Pyk-A in intestine can be regulated by the diet.

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ON THE MOLECULAR BASIS OF PYRUVATE KINASE DEFICIENCY

I. PRIMARY DEFECT OR CONSEQUENCE OF INCREASED GLUTATHIONE DISULFIDE CONCENTRATION

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SUMMARY

1. Human erythrocyte pyruvate kinase (ATP:pyruvate phosphotransferase EC 2.7.1.40) can be converted into an oxidized form by incubation with oxidized glutathione. The oxidized enzyme can be reduced again by incubation with mercaptoethanol, with reduced glutathione a partial reduction of the enzyme is obtained.

2. The oxidized enzyme shows a lower affinity for the substrate phosphoenolpyruvate and for the allosteric effector fructose 1,6-diphosphate. The thermolability of the oxidized enzyme is markedly increased, compared with the freshly isolated or reduced enzyme.

3. The data obtained with the oxidized enzyme are discussed in relation to the data obtained with pyruvate kinase from pyruvate kinase-deficient patients. It is concluded that erythrocyte pyruvate kinase deficiency can be a consequence of an increased oxidized glutathione concentration in the red blood cell.

INTRODUCTION

Erythrocyte pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40) deficiency is a well-known cause of hemolytic anaemia. The deficiency comprises a heterogeneous group of disorders characterized by both quantitative and qualitative enzyme abnormalities[1]. Cases of hemolytic anemia attributed to pyruvate kinase abnormalities have been found with markedly decreased[2], moderately diminished[3–6], normal[3,7] or even increased[8] enzyme activity. The majority of the cases showed a decreased affinity for the substrate phosphoenolpyruvate[6,7,9–12], although also normal[3] or increased[13,14] affinities for phosphoenolpyruvate have been described. Most of the cases showed a normal or even increased glycolytic rate as can be concluded from the glucose consumption and lactate production[1]. The hemolytic anemia has been ascribed to the lowered ATP level found with the patients[1]. However, also

normal[15] or even increased ATP levels[16] have been reported. These data suggest that pyruvate kinase deficiency is a very heterogeneous molecular disorder and the lack of relation between the enzymatic activity and the degree of hemolysis suggested to us that the alteration in the pyruvate kinase activity might be a secondary defect. Reports have been published of cases with pyruvate kinase deficiency, which are combined with a decreased activity of glutathione reductase[17–19]. This prompted us to study the effect of oxidized glutathione on the kinetic behavior of the erythrocyte pyruvate kinase. Also the influence of oxidized glutathione on the stability of the enzyme is investigated, because it has been reported that the most common property in pyruvate kinase deficiency is the increased heat lability of the enzyme. The effect of oxidized glutathione is of special interest because the link between alterations in erythrocyte pyruvate kinase and the GSH/GSSG ratio might explain the increased hemolysis found in pyruvate kinase-deficient patients[20].

MATERIALS AND METHODS

Pyruvate kinase from erythrocytes, obtained from healthy volunteers (aged 20–35 years) was purified up to Stage 4 as described by Staal *et al.*[21], except that during the isolation procedure mercaptoethanol was omitted. The final $(NH_4)_2SO_4$ precipitate was dissolved in 0.25 M Tris–HCl, pH 8.0. Pyruvate kinase activity was assayed by following the decrease in absorbance at 340 nm in a coupled reaction with lactate dehydrogenase at 23 °C according to Valentine and Tanaka[22]. The triethanol–HCl buffer (0.4 M, pH 7.5) was replaced by Tris–HCl buffer (0.25 M, pH 8.0). Routinely about 0.08 mg enzyme was included in the assay mixture. The enzyme was oxidized by incubation with 2.5 mM oxidized glutathione at 4 °C for the time indicated in the legends to the figures. For the thermostability test 0.5 ml of the enzyme, containing precisely 2.0 mg/ml protein, was incubated at 53 °C. After the times indicated in Fig. 5 samples were taken and immediately assayed for pyruvate kinase activity at 5 mM phosphoenolpyruvate in the presence of 0.5 mM fructose 1,6-diphosphate (Fru-1,6-P_2).

ADP, phosphoenolpyruvate, NADH, Fru-1,6-P_2 and oxidized glutathione were obtained from Boehringer (Mannheim, Germany). Reduced glutathione was obtained from Sigma. All other reagents were of analytical grade purity.

RESULTS

Fig. 1 shows the v *vs* [phosphoenolpyruvate] plot at [ADP] = 2 mM in the presence or absence of 0.5 mM Fru-1,6-P_2 for the freshly prepared erythrocyte pyruvate kinase and the enzyme, which has been incubated with 2.5 mM oxidized glutathione for 8 h. With the freshly prepared enzyme we obtained in the absence of Fru-1,6-P_2 a $K_{0.5}$ value of 0.75 mM. Addition of 0.5 mM Fru-1,6-P_2 converted the sigmoidal curve into a hyperbolic one and the apparent K_m was now 0.15 mM. The enzyme which has been incubated with 2.5 mM oxidized glutathione showed a marked change in these activity curves. After an 8-h incubation the apparent K_m in the presence of Fru-1,6-P_2 was raised to 0.75 mM, and the $K_{0.5}$ in the absence of Fru-1,6-P_2 was about 5 mM. It could be observed that in the presence of Fru-1,6-P_2 the same maximal activity was measured, indicating that no loss of maximal activity occurred

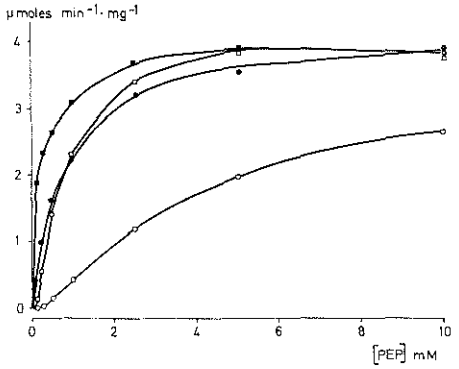

Fig. 1. The activity *vs* [phosphoenolpyruvate] plot of freshly isolated and partly oxidized human erythrocyte pyruvate kinase at [ADP] = 2 mM measured in the presence and absence of Fru-1,6-P_2 (0.5 mM). □—□, the activity of the freshly isolated erythrocyte pyruvate kinase; ■—■, the activity of the freshly isolated erythrocyte pyruvate kinase in the presence of Fru-1,6-P_2; ○—○, the activity of the erythrocyte pyruvate kinase after an 8-h incubation with 2.5 mM GSSG; ●—●, the activity of the erythrocyte pyruvate kinase after an 8-h incubation with 2.5 mM GSSG measured in the presence of Fru-1,6-P_2.

after partial oxidation of the enzyme. Prolonged oxidation caused a further decrease in the affinity of the enzyme for the substrate phosphoenolpyruvate both in the absence and presence of Fru-1,6-P_2 (Fig. 2). After a 24-h incubation at 4 °C in the presence of 2.5 mM oxidized glutathione the $K_{0.5}$ in the presence of 0.5 mM Fru-1,6-P_2 was increased to 1 mM, whereas the $K_{0.5}$ value in the absence of Fru-1,6-P_2 was higher than 10 mM. Fig. 2 also shows that oxidation of the erythrocyte pyruvate kinase is a reversible process. When the oxidized enzyme was incubated for 1 h at 10 °C with 1 mM mercaptoethanol the same kinetic data were obtained as with the freshly isolated enzyme. The $K_{0.5}$ in the absence of Fru-1,6-P_2 was lowered again

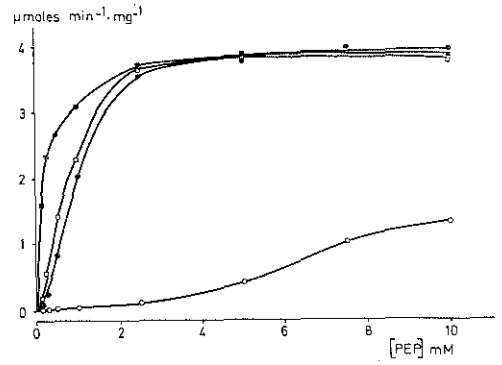

Fig. 2. The activity *vs* [phosphoenolpyruvate] plot of the oxidized erythrocyte pyruvate kinase and the oxidized erythrocyte pyruvate kinase after incubation for 1 h at 10 °C with 1 mM mercaptoethanol (reduced enzyme) measured in the presence and absence of Fru-1,6-P_2 at [ADP] = 2 mM. ○—○, the activity of the erythrocyte pyruvate kinase after a 24-h incubation with 2.5 mM GSSG; ●—●, the activity of the erythrocyte pyruvate kinase after a 24-h incubation with 2.5 mM GSSG measured in the presence of Fru-1,6-P_2; □—□, the activity of the reduced erythrocyte pyruvate kinase; ■—■, the activity of the reduced erythrocyte pyruvate kinase measured in the presence of Fru-1,6-P_2.

to 0.75 mM whereas in the presence of Fru-1,6-P_2 an apparent K_m value of 0.15 mM was obtained. Incubation of the enzyme without GSSG at 4 °C and pH 8.0 did not affect the enzyme, even if the incubation period was prolonged till 5 days. Also oxygen bubbling through the enzyme solution for 24 h did not result in an alteration of the enzyme.

Incubation of the oxidized enzyme with 5 mM reduced glutathione does not result in a complete restoration of the enzyme kinetics as shown in Fig. 3. After incubation of the oxidized enzyme with 5 mM reduced glutathione for 1 h the $K_{0.5}$ in the presence of 0.5 mM Fru-1,6-P_2 was 0.6 mM whereas in the absence of Fru-1,6-P_2 a $K_{0.5}$ value of about 8 mM was obtained. Extension of the incubation period with 5 mM GSH to 4 h did not influence the kinetic profile any further. However, if the partly-reduced enzyme (by 5 mM GSH) was treated for 1 h with 1 mM mercaptoethanol, the reduced enzyme was obtained (*cf.* Fig. 2).

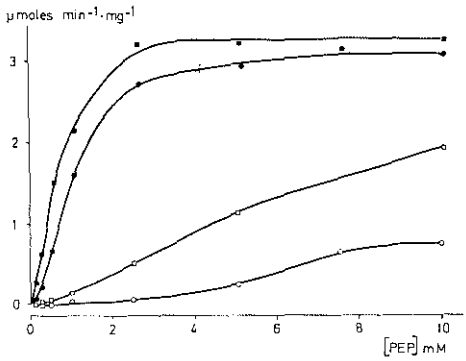

Fig. 3. The activity *vs* [phosphoenolpyruvate] plot of the oxidized erythrocyte pyruvate kinase and the oxidized erythrocyte pyruvate kinase after incubation for 1 h at 10 °C with 5 mM GSH measured in the presence and absence of Fru-1,6-P_2 at [ADP] = 2 mM; ○—○, the activity of the erythrocyte pyrvate kinase after a 24-h incubation with 2.5 mM GSSG; ●—●, the activity of the erythrocyte pyruvate kinase after a 24-h incubation with 2.5 mM GSSG measured in the presence of Fru-1,6-P_2; □—□, the activity of the oxidized erythrocyte pyruvate kinase which has been incubated with 5 mM GSH for 1 h at 10 °C; ■—■, the activity of the oxidized erythrocyte pyruvate kinase which has been incubated with 5 mM GSH for 1 h at 10 °C measured in the presence of Fru-1,6-P_2.

The activity of the oxidized erythrocyte pyruvate kinase as a function of the phosphoenolpyruvate concentration behaves in the same way as pyruvate kinase from patients with a lowered affinity for the substrate phosphoenolpyruvate. In most of these patients the affinity for Fru-1,6-P_2 is lowered[6,7,23]. Therefore, we investigated the activity of the normal and oxidized enzyme from control erythrocytes at increasing Fru-1,6-P_2 concentrations. Fig. 4 shows the pyruvate kinase activity of the freshly isolated, oxidized, partly reduced and reduced enzyme as a function of the [Fru-1,6-P_2] at 0.5 mM phosphoenolpyruvate. It can be seen that the oxidized enzyme showed a lower affinity for Fru-1,6-P_2 in comparison with the freshly isolated and reduced enzyme. The freshly isolated and reduced enzymes were already fully activated at 2 μM Fru-1,6-P_2 whereas with the oxidized enzyme full stimulation was obtained at 20 μM Fru-1,6-P_2. The curve obtained with the freshly isolated enzyme is identical with the activity curve obtained when the oxidized enzyme was incubated

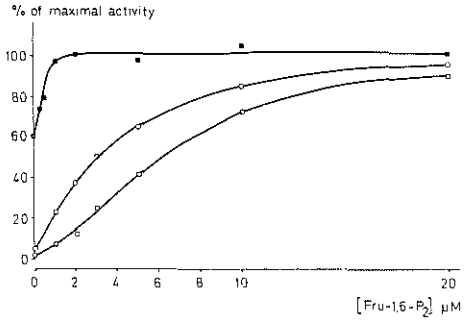

Fig. 4. The activity *vs* [Fru-1,6-P_2] plot of the freshly isolated, oxidized, partly reduced and reduced erythrocyte pyruvate kinase at [phosphoenolpyruvate] = 0.5 mM and [ADP] = 2 mM. ■—■, the activity of the freshly isolated erythrocyte pyruvate kinase and of the oxidized pyruvate kinase after incubation for 1 h at 10 °C with 1 mM mercaptoethanol (reduced enzyme); □—□, the activity of the erythrocyte pyruvate kinase after a 24-h incubation with 2.5 mM GSSG (oxidized enzyme); ○—○, the activity of the oxidized erythrocyte pyruvate kinase after incubation for 1 h at 10 °C with 5 mM GSH (partly reduced enzyme).

for 1 h with 1 mM mercaptoethanol at 10 °C. When the oxidized enzyme was incubated with 5 mM reduced glutathione an intermediate curve was obtained, which suggests that the oxidized enzyme cannot be reduced completely by GSH, at least in the concentration tested.

From Figs 1–4 we can conclude that the kinetics obtained with the enzyme which has been incubated with 2.5 mM GSSG are quite identical with the kinetics

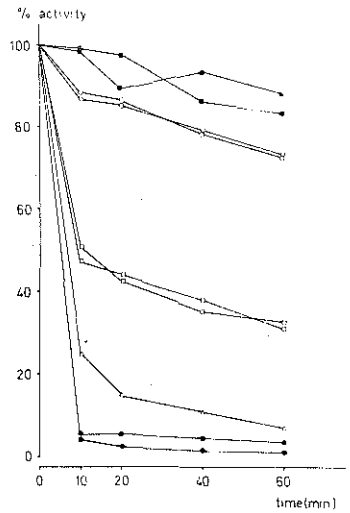

Fig. 5. The thermostability of the freshly isolated, partly oxidized, oxidized, partly reduced and reduced erythrocyte pyruvate kinase at 53 °C. The enzymatic activity was determined at [phosphoenolpyruvate] = 5 mM, [ADP] = 2 mM and [Fru-1,6-P_2] = 0.5 mM. ■—■, freshly isolated erythrocyte pyruvate kinase; □—□, erythrocyte pyruvate kinase which has been incubated for 4 h with 2.5 mM GSSG ($K_{0.5}$ for phosphoenolpyruvate about 3 mM); ●—●, erythrocyte pyruvate kinase which has been incubated for 24 h with 2.5 mM GSSG (oxidized enzyme); △—△, oxidized erythrocyte pyruvate kinase (24 h of oxidation with 2.5 mM GSSG) which has been incubated for 1 h with 5 mM GSH; ( ) - ( ), oxidized erythrocyte pyruvate kinase (24 h of oxidation with 2.5 mM GSSG) which has been incubated for 1 h with 1 mM mercaptoethanol.

obtained with most of the patients with the affected pyruvate kinase. It has been concluded earlier that the most common property of pyruvate kinase from pyruvate kinase-deficient patients is the increased lability of the enzyme at higher temperatures. Blume *et al.*[23] showed recently that the stability of the enzyme at 53 °C can serve as a useful tool for the diagnosis of pyruvate kinase deficiency. Therefore, the thermostability of the freshly isolated, partly oxidized, oxidised partly reduced and reduced enzyme at 53 °C was investigated (Fig. 5). Under the applied condition (see legend to Fig. 5) the freshly isolated enzyme showed, in accordance with Blume *et al.*[23], only a slight decrease in activity. The partly oxidized enzyme ($K_{0.5}$ for phosphoenolpyruvate about 3 mM) showed an increased thermolability whereas the oxidized enzyme ($K_{0.5}$ for phosphoenolpyruvate higher as 10 mM) lost, within 10 min, nearly all activity. When the oxidized enzyme was reduced with 5 mM GSH (*cf.* for the kinetics Figs 3 and 4) the thermolability was lowered, whereas reduction with 1 mM mercaptoethanol was able to restore the stability of the enzyme almost completely.

DISCUSSION

Since Valentine *et al.*[24] described in 1961 the first cases of non-spherocytic anemia with affected pyruvate kinase, more than 160 reports[1] about erythrocyte pyruvate kinase deficiency have been published. Since a quantitative correlation could neither be demonstrated between the pyruvate kinase activity and the clinical severity nor between the pyruvate kinase activity and the degree of hemolysis, it was suggested earlier that a lowered pyruvate kinase activity might not be the primary lesion causing hemolysis[25,26]. The heterogeneity observed in the kinetics of pyruvate kinase from pyruvate kinase-deficient patients and the fact that the glycolytic rate is not affected in these patients, supports the idea that an alteration in pyruvate kinase activity might be a secondary effect. It has been reported[20] that the GSH/GSSG ratio can play an important rôle in the hemolysis of the red blood cell. Therefore, the effects of oxidized glutathione on the kinetics and stability of the erythrocyte pyruvate kinase are of special interest.

The presented kinetic data obtained with the oxidized enzyme are quite identical with the kinetic data described for most of the patients: the decreased affinities for phosphoenolpyruvate and Fru-1,6-P_2 (refs 6, 7, 9–12 and 23). Also the thermostability of the oxidized enzyme and the enzyme from pyruvate kinase-deficient patients seems identical[23]. Incubation of the oxidized enzyme with reduced glutathione only influences the kinetics obtained with the oxidized enzyme to a small extent and also the influence on the thermostability is suboptimal. This suggests that it is not the GSH/GSSG ratio which is important for the pyruvate kinase enzyme but only the GSSG concentration. However, since we applied only one GSH/GSSG ratio, the definite answer has to await further experimentation. Further support for the rôle of glutathione as a causative factor in most cases of pyruvate kinase deficiency is the description of several patients with both glutathione reductase and pyruvate kinase deficiency[17–19]. Deficiency in glutathione reductase increases the GSSG concentration, which affects pyruvate kinase. Also with three of the four pyruvate kinase-deficient patients described earlier by Staal and Koster[13,14], the glutathione reductase activity is lowered to 50% of controls with the same amount of young erythrocytes. Preliminary experiments with pyruvate kinase from these

patients suggest indeed that –SH groups are involved in the obtained abnormal kinetics (van Berkel, Th. J. C., Staal, G. E. J., Koster, J. F. and Nijessen, J. G., in preparation).

The question remains whether the obtained abnormal kinetics in pyruvate kinase-deficient patients are a reflection of the *in vivo* situation or a consequence of oxidation of the enzyme during the isolation procedure. The fact that in hemolysates from pyruvate kinase-deficient patients the enzyme is also altered, suggests that oxidation can occur in vivo. This would be a further support for the hypothesis[27] that –SH groups can play a rôle in the regulation of the pyruvate kinase activity *in vivo*.

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ON THE MOLECULAR BASIS OF PYRUVATE KINASE DEFICIENCY

II. ROLE OF THIOL GROUPS IN PYRUVATE KINASE FROM PYRUVATE KINASE-DEFICIENT PATIENTS

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SUMMARY

1. Human erythrocyte pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40) from the class of pyruvate kinase-deficient patients, characterized by an increased affinity towards phosphoenolpyruvate and a loss of cooperative interaction towards this substrate, shows less affinity for the allosteric inhibitor ATP, when compared to pyruvate kinase from control persons. From the obtained kinetic data we can conclude that the loss of cooperativity towards phosphoenolpyruvate is a consequence of a shift in the R $\rightleftharpoons$ T equilibrium to the R state.

2. Incubation of pyruvate kinase, obtained from this class of pyruvate kinase-deficient patients with mercaptoethanol, changes the abnormal kinetics into normal kinetics, as can be concluded from the change in phosphoenolpyruvate dependency and ATP inhibition.

3. The effect of mercaptoethanol on the kinetics of pyruvate kinase from pyruvate kinase-deficient patients suggests that the alteration in the enzyme is a consequence of a modification of the –SH groups. It is suggested that pyruvate kinase deficiency is a secondary defect and that the process which causes the change in the –SH groups of pyruvate kinase, may also be responsible for the increased rate of haemolysis, found in these patients.

INTRODUCTION

Erythrocyte pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40) deficiency is a well-known cause of hemolytic anemia. The deficiency comprises a heterogeneous group of disorders, characterized by both quantitative and qualitative enzyme abnormalities [1, 2]. By a comparison of the clinical and biochemical data we earlier suggested [2], that alteration in the kinetics of erythrocyte pyruvate kinase from pyruvate kinase-deficient patients might be a secondary defect. It was shown [2] that the primary defect, responsible for the abnormal pyruvate kinase kinetics, might be the redox state of the thiol groups in the red blood cell. Glutathione disulfide as

oxidizing agent was able to oxidize the thiol groups of the isolated erythrocyte pyruvate kinase, which resulted in a decreased affinity for one of the substrates, phosphoenolpyruvate and the allosteric activator Fru-1,6-P_2; kinetic properties also found with pyruvate kinase from most of the pyruvate kinase-deficient patients. This report deals with the effect of the reducing compound mercaptoethanol on pyruvate kinase, from pyruvate kinase-deficient patients, characterized by an increased affinity for the substrate phosphoenolpyruvate and a loss of the cooperative interaction towards this substrate [3, 4]. It will be shown, that in the altered kinetic properties of this class of patients thiol groups are also involved.

MATERIALS AND METHODS

Pyruvate kinase from erythrocytes was purified up to stage 4, as described by Staal et al. [5], except that during the isolation procedure mercaptoethanol was omitted. The final $(NH_4)_2SO_4$ precipitate was dissolved in 0.2 M Tris–maleate buffer pH 8.2 and divided into two fractions. One enzyme fraction was incubated with 10 mM mercaptoethanol at 10 °C for 1 h, whereas the other was incubated without further additions. Pyruvate kinase activity was assayed by following the decrease in absorbance at 340 nm in a coupled reaction with lactate dehydrogenase at 25 °C, according to Valentine and Tanaka [6]. The reaction mixture contained 0.2 M Tris–maleate buffer, pH 8.2, 2.0 mM ADP, 0.09 mM NADH, 65 mM KCl, 20 mM $MgSO_4$ and 0.1 mg lactate dehydrogenase in a final volume of 3.0 ml. The reported experiments were repeated after a three-month period, at which time a complete new population of erythrocytes is present, and these experiments were completely reproducible.

ADP, phosphoenolpyruvate, NADH and Fru-1,6-P_2 were obtained from Boehringer (Mannheim, Germany). Mercaptoethanol was obtained from Fluka, Basel. All other reagents were of an analytical grade purity.

RESULTS

Figs. 1A and 1B show the v vs [phosphoenolpyruvate] plot at [ADP] = 2 mM in the presence and absence of 0.5 mM Fru-1,6-P_2 for pyruvate kinase, obtained from normal red blood cells. Fig. 1B shows the activity curve of the enzyme after incubation with 10 mM mercaptoethanol, whereas for Fig. 1A this compound was omitted. In accordance with earlier reports [5, 6] a sigmoidal curve was obtained, in the absence of Fru-1,6-P_2, with an n value of 1.7 (see Hill plot insert Fig. 1A). Addition of 0.5 mM Fru-1,6-P_2 stimulates the enzymatic activity especially at low phosphoenolpyruvate concentrations and the sigmoidal curve was converted into a hyperbolic curve (n value of 1.0). Incubation of the enzyme with mercaptoethanol for 1 h does not alter the allosteric properties and exactly the same n values, 1.7 in the absence and 1.0 in the presence of Fru-1,6-P_2, were obtained.

Fig. 2A shows the v vs [phosphoenolpyruvate] plot of pyruvate kinase, obtained from a patient with increased hemolysis, which has been classified as pyruvate kinase deficient (cf. ref. 4 patient M.V.). In agreement with an earlier report [4] the enzyme from this patient has lost its allosteric properties towards phosphoenolpyruvate and an n value of 1.0 was obtained (insert Fig. 1A). Incubation of this enzyme

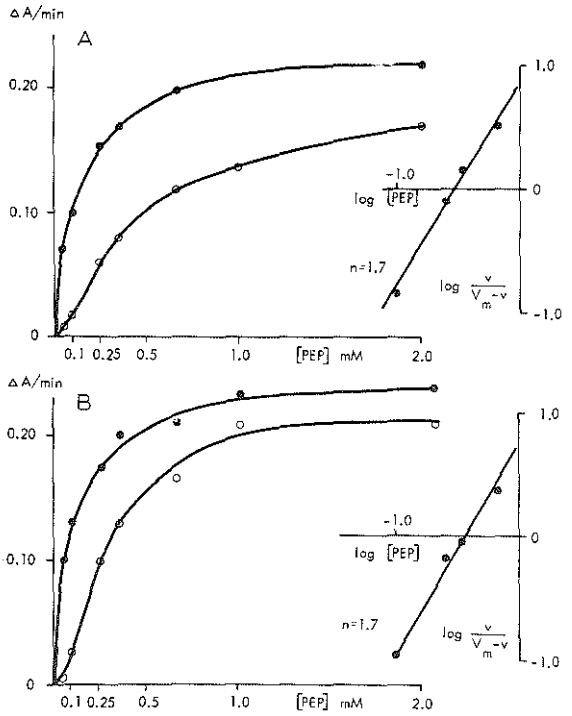

Fig. 1. The activity vs [phosphoenolpyruvate]plot of normal erythrocyte pyruvate kinase, measured in the presence and absence of Fru-1,6-P_2 (0.5 mM). The inserts are the Hill plots of the values obtained in the absence of Fru-1,6-P_2. The calculated Hill coefficients (n) are indicated. (A) The activity curve after incubation without mercaptoethanol, measured in the absence (○—○) and in the presence of Fru-1,6-P_2 (●—●). (B) Activity curve after a 1-h incubation with 10 mM mercaptoethanol at 10 °C, measured in the absence ○—○ and in the presence of Fru-1,6-P_2 ●—●.

in the presence of mercaptoethanol introduced an allosteric behavior towards phosphoenolpyruvate, as can be concluded from the obtained n value of 1.9 (insert Fig. 2B).

Figs 3A and 3B show the v vs [phosphoenolpyruvate] plot of the second, unrelated, patient P.R. Pyruvate kinase from this patient also shows a loss of its allosteric behavior towards phosphoenolpyruvate (n value of 1.0, insert Fig. 3A). By incubating the altered enzyme from this patient with mercaptoethanol, the normal kinetic behavior towards phosphoenolpyruvate was obtained (n value 1.7, insert Fig. 3B).

From Figs 1, 2 and 3 we can conclude that the abnormal kinetics of these mutant enzymes can be converted into a kinetic behavior towards phosphoenolpyruvate, which is identical with the normal enzyme. In order to investigate the nature of this conversion more closely, we studied the effect of ATP on the reduced and untreated enzyme of patient M.V. It can be seen (Fig. 4) that the untreated enzyme of this patient possesses a low affinity for the inhibitor ATP, whereas incubation with mercaptoethanol causes a shift in the inhibition curve; the affinity for ATP is increased, which is accompanied by a lowering of the n value (calculated by the method of Jensen and Nester [7]) from 2.5 to 1.6. With both enzymes Fru-1,6-P_2 (0.5 mM)

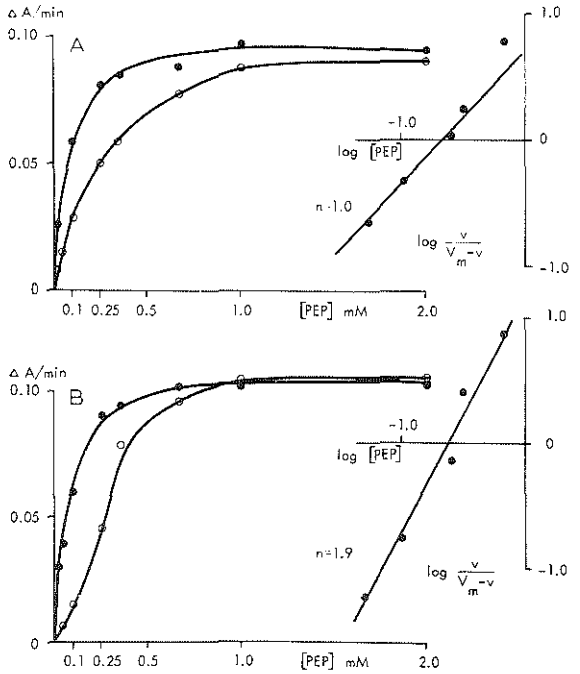

Fig. 2. The activity vs [phosphoenolpyruvate] plot of erythrocyte pyruvate kinase from a patient M.V., measured in the presence and absence of Fru-1,6-P_2 (0.5 mM). The inserts are the Hill plots of the values obtained in the absence of Fru-1,6-P_2. The calculated Hill coefficients (n) are indicated. (A) The activity curve after incubation without mercaptoethanol, measured in the absence (○—○) and in the presence of Fru-1,6-P_2 (●—●). (B) Activity curve after a 1 h incubation with 10 mM mercaptoethanol at 10 °C, measured in the absence (○—○) and in the presence of Fru-1,6-P_2 (●—●).

is able to abolish the inhibition, suggesting that this effector site is intact. With pyruvate kinase from patient P.R. the results obtained are qualitatively similar (Fig. 5), although the enzyme from this patient seemed less sensitive to ATP. Comparison of the ATP inhibition plots of patients and controls reveals the conclusion that the ATP inhibition, obtained with the patients' enzymes after incubation with mercaptoethanol, is similar to the inhibition plot of the controls (cf. ref. 4). This allows the conclusion that incubation with mercaptoethanol is able to convert the abnormal enzyme, isolated from the two patients into an enzyme which cannot be distinguished from pyruvate kinase from control persons.

From the phosphoenolpyruvate dependence and ATP inhibition of the mutant enzyme of the patients, it can be concluded that the mutant enzyme is characterized by a shift of the R ⇌ T equilibrium to the R state. Recently we showed that M-type pyruvate kinase from leucocytes [8] and liver [9] possess allosteric properties, similar to the L-type. To obtain more information about the molecular basis of pyruvate kinase deficiency we isolated leucocytes from the patients, described in this report, and characterized pyruvate kinase. No significant differences in the activity curve towards phosphoenolpyruvate could be detected. Also the alanine inhibition and the

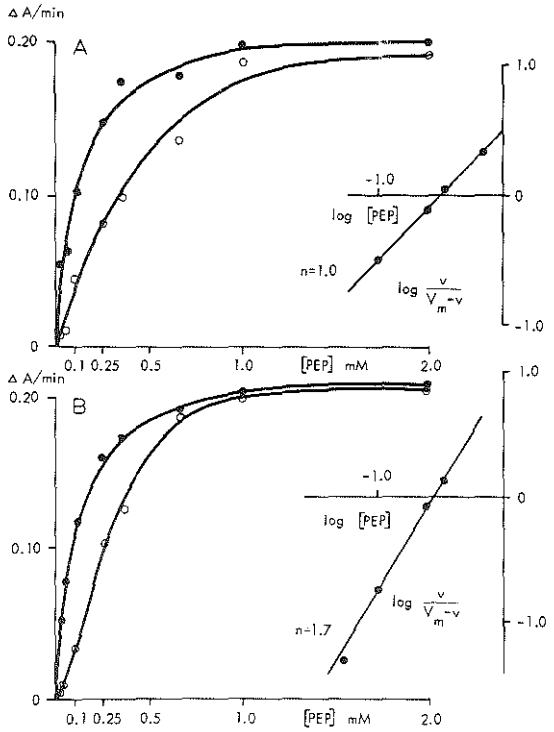

Fig. 3. The activity vs [phosphoenolpyruvate]plot of erythrocyte pyruvate kinase from a patient P.R., measured in the presence and absence of Fru-1,6-P_2 (0.5 mM). The inserts are the Hill plots of the values obtained in the absence of Fru-1,6-P_2. The calculated Hill coefficients (n) are indicated. (A) The activity curve after incubation without mercaptoethanol, measured in the absence (○—○) and in the presence of Fru-1,6-P_2 (●—●). (B) Activity curve after a 1-h incubation with 10 mM mercaptoethanol at 10 °C, measured in the absence ○—○ and in the presence of Fru-1,6-P_2 (●—●).

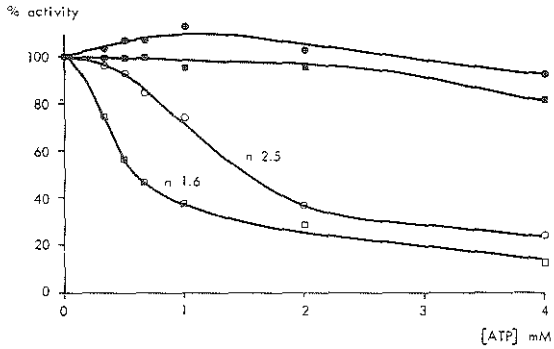

Fig. 4. The influence of ATP on the activity of erythrocyte pyruvate kinase of patient M.V. at 0.25 mM phosphoenolpyruvate, 0.5 mM ADP and pH 8.2. ○—○, the activity curve after incubation without mercaptoethanol. ●—●, the activity curve after incubation without mercaptoethanol measured in the presence of Fru-1,6-P_2 (0.5 mM). □—□, the activity curve after incubation with 10 mM mercaptoethanol for 1 h at 10 °C. ■—■, the activity curve after incubation with 10 mM mercaptoethanol for 1 h at 10 °C, measured in the presence of Fru-1,6-P_2 (0.5 mM). The calculated Hill coefficients (n) are indicated. The 100% value is the activity measured in the presence of various additions at [ATP] = 0 mM.

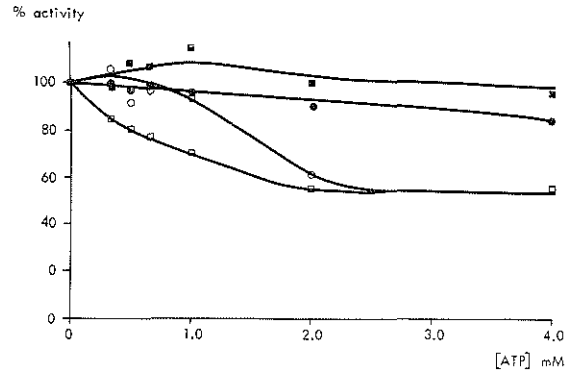

Fig. 5. The influence of ATP on the activity of erythrocyte pyruvate kinase of patient P.R. at 0.25 mM phosphoenolpyruvate, 0.5 mM ADP and pH 8.2. ○—○ the activity curve after incubation without mercaptoethanol. ●—●, the activity curve after incubation without mercaptoethanol, measured in the presence of Fru-1,6-P_2 (0.5 mM). □—□, the activity curve after incubation with 10 mM mercaptoethanol for 1 h at 10 °C. ■—■, the activity curve after incubation with 10 mM mercaptoethanol for 1 h at 10 °C, measured in the presence of Fru-1,6-P_2 (0.5 mM). The 100% value is the activity measured in the presence of various additions at [ATP] = 0 mM.

effect of Fru-1,6-P_2 was similar to the control values, which suggest that the R ⇌ T equilibrium of leucocyte M-type pyruvate kinase is not changed in the patients.

DISCUSSION

Since Valentine et al. [10] described in 1961 the first cases of non-spherocytic anemia with affected pyruvate kinase, more than 160 reports [1] about erythrocyte pyruvate kinase deficiency have been published. No quantitative correlation between the pyruvate kinase activity and the clinical severity, or between the pyruvate kinase activity and the degree of hemolysis could be demonstrated. Most of the cases showed a normal or even increased glycolytic rate, as can be concluded from the glucose consumption and lactate production [1]. Considering those facts, we suggested that the alteration in pyruvate kinase might be a secondary defect [2]. The heterogeneity, observed in the kinetics of pyruvate kinase from pyruvate kinase-deficient patients, suggest that this alteration can be the consequence of different primary defects. The most important condition for the primary defect must be that there exists a relation between this defect and the increased hemolysis, found in the patients.

Recently we showed [2] for the pyruvate kinase-deficient patients, characterized by a decreased affinity for phosphoenolpyruvate and Fru-1,6-P_2, that the alteration in the enzyme kinetics can be the consequence of oxidation of the thiol groups in the enzyme. This report shows that, also with patients characterized by an increased affinity for phosphoenolpyruvate, the thiol groups are involved in the enzyme modification. From the kinetic data obtained with the untreated mutant enzyme (a hyperbolic response to phosphoenolpyruvate and decreased sensitivity towards ATP), we can conclude that in the R ⇌ T model of Monod et al. [12], which is valid for erythrocyte pyruvate kinase [5, 12], this R ⇌ T equilibrium is shifted to the R state. It was speculated earlier [13] (based on the kinetics towards phosphoenolpyruvate) that the mutant enzyme, characterized by a hyperbolic activity curve

towards phosphoenolpyruvate in the absence as well in the presence of Fru-1,6-P_2, looks very similar to the conformation which can be obtained artificially with the normal enzyme, by lowering the pH and the temperature. The ATP inhibition curve, obtained with the patients, proves that this speculation was correct and that the molecular basis of the altered enzyme is a decrease of the allosteric constant L. Incubation of the mutant enzymes with mercaptoethanol converts the abnormal kinetics to normal kinetics. Since this compound affects the –SH groups of proteins, it seems likely that thiol groups are involved in this conversion. By restoring the thiol group(s) of the mutant enzyme to the "normal" reduction grade, the R $\rightleftharpoons$ T equilibrium is shifted to the equilibrium, found with the normal enzyme and the allosteric constant L reaches its normal value. At the moment the primary defect, responsible for the mutant enzymes described here, remains uncertain. However, it seems likely that the primary defect is related to the reduction state of the –SH groups in the red blood cell. This reduction state can form the link between alterations in pyruvate kinase and the increased hemolysis, found in these patients.

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