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NUCLEOTIDE SPECIFICITY OF PYRUVATE KINASE AND PHOSPHO*ENOL*PYRUVATE CARBOXYKINASE

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Summary

Various analogues of adenosine 5′-diphosphate with modifications in the heterocyclic base residue were tested as substrates of rabbit muscle pyruvate kinase (ATP:pyruvate 2-*O*-phosphotransferase, EC. 2.7.1.40) and guinea pig liver mitochondrial phospho*enol*pyruvate carboxykinase (GTP:oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32).

The significance of different structural elements for the enzyme-substrate interaction is discussed. While pyruvate kinase shows a rather broad specificity for these analogues, phospho*enol*pyruvate carboxykinase has a more stringent requirement for nucleotides, the intact keto and NH groups at C_6 and N_1 of the pyrimidine ring representing essential sites for the phospho*enol*pyruvate carboxykinase substrate interaction.

The biological significance of the different substrate specificities of pyruvate kinase and phospho*enol*pyruvate carboxykinase is discussed as a possible metabolic control factor.

Introduction

Pyruvate kinase (ATP:pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40) is known to have a rather broad specificity for nucleoside diphosphates which can act as substrates [1—3], while the only reported substrates for mammalian phospho*enol*pyruvate carboxykinase (GTP:oxaloacetate carboxyl-lyase (tranphosphorylating) EC 4.1.1.32) are IDP (or ITP) and GDP (or GTP) [4—6].

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* Abbreviations: o^1ADP: adenosine-1-oxide 5′-diphosphate; o^1IDP: inosine-1-oxide 5′-diphosphate; 8-BrADP: 8-bromoadenosine 5′-diphosphate; iGDP: isoguanosine 5′-diphosphate; XDP: xanthosine 5′-diphosphate; εADP: 1,N^6-ethenoadenosine 5′-diphosphate.

In the present investigation we have tested a number of purine nucleotides with specific modifications in the base moiety as substrates for both pyruvate kinase and for phospho*enol*pyruvate carboxykinase, with the aim of identifying possible correlations between certain structural elements and their catalytic properties. The selection of these particular two phosphotransferases for our investigation lies in their opposite metabolic role. While the first enzyme participates in glycolysis where it transfers the phosphoryl group from phospho*enol*pyruvate to ADP, the second one yields phospho*enol*pyruvate in gluconeogenesis.

Materials and Methods

Chemicals

The following commercially available chemicals were used: ADP, GDP, NADH, phospho*enol*pyruvate, lactate dehydrogenase, malate dehydrogenase (Boehringer, Mannheim), rabbit muscle pyruvate kinase (Sigma Chem. Co. St. Louis), crystalline bovine serum albumin (BDH Chemicals Ltd.). o^1ADP was synthesized from ADP by oxidation with permaleic acid in aqueous solutions [7]. iGDP was obtained from o^1ADP by a photoisomerization reaction [8]. IDP, o^1IDP and XDP were prepared by oxidative deamination of ADP, o^1ADP and GDP, respectively. 8-BrADP was obtained by bromination of ADP in aqueous solution [9]. The purity of each of the nucleotides was checked by their spectroscopic properties (Fig. 1) and by thin layer chromatography [10].

Assay of pyruvate kinase activity

The reaction medium contained per 1 ml solution 50 mM Tris · HCl pH 7.4, 80 mM KCl, 6 mM $MgCl_2$, 0.5 mM phospho*enol*pyruvate, 0.05 mM NADH, 3 units lactate dehydrogenase and different concentrations of nucleoside diphosphates. For the study of the pH dependence of the activity, the Tris · HCl buffer was replaced by Tris/maleate (pH between 5.5 and 8.5). The reaction was triggered by addition of pure or crude samples of pyruvate kinase, sufficient to yield a ΔA value between 0.02 and 0.1 per min at 366 nm.

Assay of guinea pig mitochondrial phosphoenolpyruvate carboxykinase activity

The formation of oxaloacetate from phospho*enol*pyruvate and nucleoside diphosphates was coupled with the oxidation of NADH by an excess of malate dehydrogenase [11]. The reaction medium contained per 1 ml solution 50 mM Tris · HCl, pH 7.4, 1 mM $MnCl_2$, 0.5 mM phospho*enol*pyruvate, 0.05 mM NADH, 17 mM $NaHCO_3$ (no bicarbonate in control experiments), 2 units malate dehydrogenase, 2 μg rotenone and mitochondrial phospho*enol*pyruvate carboxykinase. After stabilization of the absorbance, the reaction was triggered by addition of different concentrations of nucleoside diphosphates. Guinea pig liver mitochondria were isolated as previously described [12]. To 0.5 ml of mitochondrial suspension (about 25 mg of protein) 0.5 ml of 2.5% Lubrol XW and 2 ml of distilled water were added. After 20 min at 0°C the suspension was centrifuged at 15 000 $\times g$ for 15 min and the supernatant containing most of the mitochondrial phospho*enol*pyruvate carboxykinase was used for kinetic experiments. Proteins were estimated by the method of Gornall et al. [13].

All enzymatic reaction rates were determined at 366 nm and 25°C, using an Eppendorf 1101 type photometer equipped with a W+W type 4410 recorder (full scale deflection 0.25 absorbance units).

Apparent formation constants

The corresponding formation constants for ADP, o[1]ADP and 8-BrADP with Mg^{2+} were determined using the ion-exchange resin method of Schubert [14] as modified by Haley and Yount [15].

Results and Discussion

Pyruvate kinase

It has been shown that modifications in the phosphate chain structure or in the ribose moiety of ADP lead to a substantial inhibition of both the apparent binding and the catalysis with pyruvate kinase [1,3,16]. However, nucleoside diphosphates with the fairly extensive alterations in the base moiety as those shown in Fig. 1 are all substrates of rabbit muscle pyruvate kinase. Although the numerical values of the maximal velocity and the Michaelis constant vary over an order of magnitude as compared to ADP (Table I), it is difficult to single out specific groups in the base part which could constitute essential sites for the enzyme-substrate interaction. The integrity of the C_6 amino group, for instance, seems not to be essential since the etheno derivative ϵ-ADP as well as

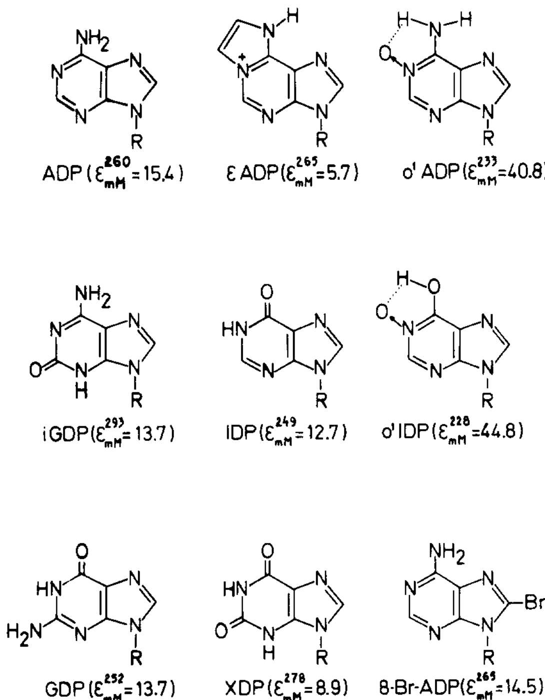


Fig. 1. Structure and extinction coefficients of nucleoside diphosphates tested in this work with R = ribosyl diphosphate.

TABLE I

KINETIC CONSTANTS OF RABBIT MUSCLE PYRUVATE KINASE FOR ADP AND ITS ANALOGUES

The V values are relative to the normal substrate ADP; the actual maximal velocity observed for ADP was 283 μmol/min per mg protein. k and k^* represent the relative V/K_m ratios for ADP and its analogues. The k^*/k ratio is a better measure than the corresponding K_m value for defining the efficiency of substrate analogues.

Nucleotide	K_m (mM)	V	k^*/k
ADP	0.28	1.00	1.000
εADP	0.30	0.80	0.747
iGDP	0.41	0.42	0.287
GDP	1.18	0.79	0.187
IDP	0.90	0.48	0.149
XDP	1.07	0.27	0.071
o^1ADP	1.76	0.28	0.045
o^1IDP	3.55	0.13	0.010
8-BrADP	1.18	0.044	0.010

N-methyl (or dimethyl-) adenosine 5′-diphosphate are both good substrates for pyruvate kinase [3,17]. Even if the amino group is replaced by oxygen such as in IDP, GDP or XDP, with or without substituents at C_2, there is not a complete loss of substrate properties although the binding is poorer. A certain significance could be attached to the modifications in o^1ADP, o^1IDP and 8-BrADP, which are by far the poorest substrates of pyruvate kinase. The efficiency of these three nucleotides expressed as the k^*/k ratio is only 4.5, 1.0 and 1.0% respectively of that of ADP. In order to explain the poor substrate property of 8-BrADP one could invoke the steric factor since it has been shown that the bulky bromine in position 8 forces this nucleotide into an unusual syn conformation with regard to the glycosidic C-N bond [9,18]. If this supposition is correct then generally all 8-bromoadenine nucleotides should be poor substrates (or competitive inhibitors) of phosphotransferases. Indeed, with phosphofructokinase which also has a relatively broad specificity for nucleotides, 8-BrATP has an efficiency of only 1.9% as compared to that of ATP (Bârzu, O. et al., unpublished). With o^1IDP and o^1ADP there are no evident structural modifications which could account for their low catalytic activity with pyruvate kinase, except perhaps the N_1 oxygen, which is acting not only as a simple substituent, but also has a far reaching contribution to the whole heterocyclic π-electron system [8]. Such a redistribution of the whole electron density pattern is expected to affect the stacking properties of the purine moiety and its binding to the enzyme by non-covalent forces.

In order to rule out a difference in the stability of the complex with divalent metals as a factor contributing to their low substrate activity, although generally the nature of the base does not strongly affect the formation constants [19], we also examined the interaction of o^1ADP and 8-BrADP with Mg^{2+}. As shown in Table II o^1ADP behaves much like ADP in binding Mg^{2+}, whereas 8-BrADP is able to yield an even stronger complex with Mg^{2+}. The ratio of the reaction rate with ADP and o^1ADP (or o^1IDP) remains constant at different pH values. These facts suggest that neither the ionisation degree of the nucleotides nor the com-

TABLE II

FORMATION CONSTANTS (K_f) *OF ADP*, o^1ADP AND 8-BrADP WITH Mg^{2+}

Measurements were made at pH 8 and 24°C at a final volume of 10 ml in a medium containing 50 mM KCl, 50 mM Tris · HCl, 0.05—0.30 mM nucleotide, 0—2 mM $MgCl_2$ and 50 mg Dowex 1X4. The number of experiments is given in parentheses.

Nucleotide	K_f (M^{-1})
ADP (3)	1600 ± 100
o^1ADP (4)	1650 ± 40
8-BrADP (3)	4700 ± 120

plex formation with Mg^{2+} can be invoked to explain the observed differences between the kinetic parameters of pyruvate kinase acting on ADP or on its analogues o^1ADP, o^1IDP and 8-BrADP.

In order to see if there are differences in the nucleotide specificity for pyruvate kinase from different tissues, we investigated the effect of ADP and o^1ADP on pyruvate kinase obtained from various rat tissues; the possible differences could serve for the identification of isoenzymes based on a substrate specificity. The data in Table III however show that there are no significant differences with regard to the effect of ADP or of its o^1ADP analogue on pyruvate kinase from the various examined tissues.

Phosphoenolpyruvate carboxykinase

The substrate specificity of this enzyme shows a completely different picture than that for pyruvate kinase. From all investigated nucleotide diphosphates only IDP, GDP and XDP are substrates for guinea pig liver mitochondrial phospho*enol*pyruvate carboxykinase as shown in Table IV. Consequently there must be severe structural restrictions for the interaction of the base part of the nucleotide and the catalytic center in phospho*enol*pyruvate carboxykinase. Inspection of the structures displayed in Fig. 1 reveals that the common features of the nucleotides which are able to act as substrates are a keto group at C_6 and an NH group at N_1 of the pyrimidine ring, suggesting that these represent essential sites for the enzyme-substrate interaction. We were particularly

TABLE III

DIFFERENCES IN REACTIVITY OF LIVER, BRAIN, HEART AND KIDNEY RAT PYRUVATE KINASE TO o^1ADP AND ADP

The corresponding tissue was homogenized in KCl (0.15 M) centrifuged at 20 000 × *g* for 30 min and the supernatant used in the optical test without any further treatment. The K_m values for ADP are given in parenthese; the *V* values for o^1ADP are relative to ADP; in the parentheses are given the values for ADP expressed as μmol/min per mg protein.

Tissue	K_m (mM)	*V*	$100 \cdot k^*/k$
Heart	1.95 (0.31)	0.18 (1.37)	2.9
Liver	2.94 (0.39)	0.21 (0.49)	2.8
Brain	5.56 (0.90)	0.17 (2.24)	2.7
Kidney	3.45 (0.42)	0.11 (0.66)	1.3

TABLE IV

KINETIC CONSTANTS OF GUINEA PIG LIVER MITOCHONDRIAL PHOSPHO*ENOL*PYRUVATE CARBOXYKINASE FOR NUCLEOSIDE DIPHOSPHATES

The maximal velocity is expressed as nmol NADH oxidised/min per mg of protein. The k^*/k ratio is described in Table I. The remaining nucleotides listed in Fig. 1 have no substrate properties for phospho*enol*pyruvate carboxykinase.

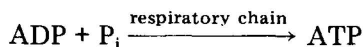
Nucleotide	K_m (mM)	V	k^*/k
IDP	0.034	29.0	1.000
GDP	0.026	20.8	0.938
XDP	0.057	10.6	0.218

interested in the behaviour of the newly synthesized iGDP which is a positional isomer of GDP. The lack of substrate properties shows that iGDP in this enzymatic system must be classified as an ADP analogue with an additional ketonic function at C_2. o^1IDP, which has the nitrogen in position 1 masked by an oxygen and exists mainly in the enolic C_6-OH isomer (Mantsch, H.H. et al., unpublished), is inert as substrate for phospho*enol*pyruvate carboxykinase and acts as a weak competitive inhibitor (K_i = 1.2 mM).

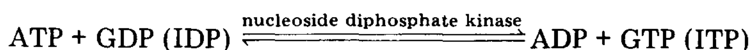
The biological significance of this high degree of specificity of phospho*enol*pyruvate carboxykinase can be correlated to the phospho*enol*pyruvate synthesis by coupling the succinic thiokinase reaction to phospho*enol*pyruvate carboxykinase [4]. Both enzymes exhibit a nearly identical nucleotide specificity pattern.

Biological implications

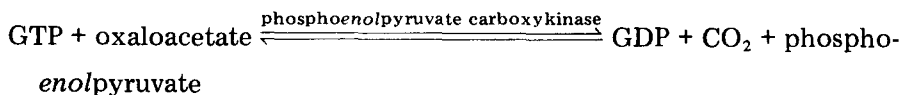
The question arises what functional significance can be attached to the different nucleotide specificities of the examined enzymes pyruvate kinase and phospho*enol*pyruvate carboxykinase as a possible metabolic control factor. Indeed, for mitochondrial phospho*enol*pyruvate carboxykinase, such as that of guinea pig [6], sheep or chicken liver [5], the coupling of energy release by the respiratory chain with formation of phospho*enol*pyruvate is possible either by the succinic thiokinase catalysed reaction or by a sequence involving the participation of nucleoside diphosphate kinase such as:



$$\text{ADP} + \text{P}_i \xrightarrow{\text{respiratory chain}} \text{ATP}$$



$$\text{ATP} + \text{GDP (IDP)} \xrightleftharpoons{\text{nucleoside diphosphate kinase}} \text{ADP} + \text{GTP (ITP)}$$



$$\text{GTP} + \text{oxaloacetate} \xrightleftharpoons{\text{phospho}\textit{enol}\text{pyruvate carboxykinase}} \text{GDP} + \text{CO}_2 + \text{phospho-}\textit{enol}\text{pyruvate}$$

A possible coupling of the pyruvate kinase reaction with phospho*enol*pyruvate carboxykinase could take place in the cytosol and lead to a 'futile' cycle similar to that between phosphofructokinase and fructose diphosphatase [20]. However such a coupling would require the participation of nucleoside diphosphate kinase in order to compensate for the different nucleotide specifi-

cities of the two enzymes. This is feasible since it is well known that the mediating enzyme nucleoside diphosphate kinase has a large specificity for nucleotides both as phosphate donors or as phosphate acceptors [21—24]. It also would follow that the course of the metabolic flux towards either glycolysis or gluconeogenesis does not depend only on the ATP/ADP ratio but also on the ΣNTP/ΣNDP ratio where the individual components of each sum may play an important regulatory role.

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