THE EFFECT OF STRUCTURAL ALTERATIONS ON THE REACTIVITY OF THE NUCLEOTIDE SUBSTRATE OF RABBIT MUSCLE PYRUVATE KINASE

David C. HOHNADEL and Cecil COOPER
Department of Biochemistry, School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106, USA

Received 1 January 1973

1. Introduction

Although the enzyme pyruvic kinase (EC 2.7.1.40) has been studied extensively and the specificity for monovalent and divalent cations and phosphoenolpyruvate have received considerable attention, there has been no detailed analysis of the specificity for the nucleotide substrate. Early work on this enzyme indicated that the natural analogs of ADP such as GDP, IDP, UDP and CDP [1–3] could all serve as substrates but these findings were believed to be ambiguous because of the contamination of the enzyme with nucleoside diphosphokinase [4]. The possible contribution of such a contamination to the apparent lack of specificity of pyruvic kinase was re-examined by Plowman and Krall [5] with a more highly purified preparation and they concluded that this was not the explanation. They also measured the kinetic constants for ADP, IDP, GDP, CDP, UDP and dADP and found relatively small variation in the apparent V_{max} for the purine nucleotides over the pH range of 6.5 to 8.0 suggesting that either there is little structural specificity or that key alterations in the structure had not yet been examined. An indication that specificity for the pyrophosphate group exists was provided by Setondji et al. [6] who reported that adenosine-5′-hypophosphate is a very poor substrate or inhibitor for pyruvic kinase. The structural variations existing in the compounds studied to date are relatively small and we felt a more detailed examination may provide important clues regarding the requirements for binding and catalysis. We have used a series of synthetic and natural analogs of ADP to evaluate the relative importance

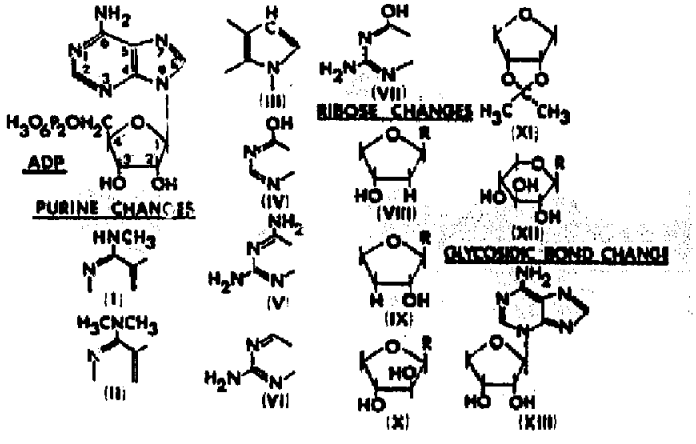

Fig. 1.

of the adenine ring, ribose and glycosidic bond. Our conclusion is that 2′- and 3′-*cis* hydroxyls of the ribose and the N^9-glycosidic bond are of major importance in determining the reactivity of the nucleotide substrate of rabbit muscle pyruvic kinase.

2. Materials and methods

Rabbit muscle pyruvic kinase (A grade) and rabbit muscle lactic dehydrogenase (A grade) were obtained from Calbiochem and aliquots were prepared for use daily by dilution with 50 mM Tris-HCl pH 7.5. Desalting these enzymes with Sephadex G-25 prior to use had no significant effect of the K_m and V_{max} for ADP and this treatment was therefore not carried out for the experiments described below. Solutions of NADH (P-L Biochem-

North-Holland Publishing Company – Amsterdam

Table 1

Variation of apparent K_m and V_{max} for the nucleotide substrate of rabbit muscle pyruvic kinase with alterations of the adenine ring, ribose and position of the glycosidic bond.

Expt. No.	Compound	K_m ± SD (mM)	V_{max} ± SD (μmoles/min/mg)	$\frac{K_m\ (XDP)}{K_m\ (ADP)}$	$\frac{V_{max}\ (XDP)}{V_{max}\ (ADP)}$
1	ADP	0.35 ± .13	121 ± 3	– –	– –
	I	0.53 ± .06	64 ± 1	1.51	0.53
	II	0.58 ± .28	69 ± 5	1.66	0.57
	III	0.49 ± .12	135 ± 6	1.40	1.11
	IV	2.32 ± .18	101 ± 2	6.57	0.84
	V	0.17 ± .13	108 ± 6	0.49	0.89
	VI	0.19 ± .19	49 ± 8	0.54	0.41
	VII	1.22 ± .04	84 ± 1	3.43	0.69
2	ADP	0.29 ± .03	130 ± 4	– –	– –
	VIII	1.67 ± .16	29 ± 0.5	5.53	0.22
	IX	2.11 ± .20	11 ± 0.2	7.24	0.08
	X	1.43 ± .20	4 ± 0.2	4.93	0.03
	XI	Not a substrate			
	XII	Not a substrate			
	XIII	0.72 ± .22	2.8 ± 0.1	2.49	0.02

The assay conditions were as follows: 50 mM Tris-HCl, 7.0 mM $MgCl_2$, 75 mM KCl, 1.0 mM PEP, 0.2 mM NADH, 3 units lactic dehydrogenase, varying amounts of nucleoside diphosphate and water to a final volume of 305 μl and a final pH of 7.5. After preincubation for 5 min at 25° the reaction was started by the addition of a 5 μl aliquot containing 0.05 units of pyruvate kinase.

icals) were prepared daily. The coupling enzyme, lactic dehydrogenase, was present in 60-fold excess as recommended by Pon and Bondar [7]. The minimum concentration range tested with all nucleotides was 0.5 to 5 times the K_m value and in most cases the range was larger. Seven to nine different concentrations were tested in duplicate. All volume measurements were made with Hamilton syringes and the rates determined with an IBM 1620 computer and a modification of the program described by Cleland [8]. The synthetic nucleotides were prepared as previously described [9] and were converted to the potassium salts prior to use. The structures of the compounds used are shown in fig. 1.

3. Results and discussion

The kinetic constants obtained with the compounds tested as substrates for pyruvic kinase are shown in table 1. The average of about 0.3 mM for the K_m of ADP is in reasonable agreement with the previously reported values of 0.2 mM [5, 6, 10], 0.3 mM [11] and 0.36 mM [7]. Multiple experiments were carried out with ADP and dADP to evaluate the reproducibility of the K_m and V_{max} ratios. In four separate experiments the K_m ratios [K_m (XDP)/K_m (ADP)] were 5.53, 6.14, 5.90 and 5.41 while the V_{max} ratios [V_{max} (XDP)/V_{max} (ADP)] were 0.22, 0.18, 0.22 and 0.23.

Experiment 1 in table 1 summarizes the effects produced by alterations in the adenine ring of ADP. The most striking feature is that fairly extensive alterations cause little change in V_{max}. Even the least active member of the group, compound VI, has a V_{max} that is about 40% that of ADP. Similarly, the effects on the apparent K_m are not very large except in the case of IDP (compound IV) and GDP (compound VII).

In contrast to the results in experiment 1, all of the changes in the ribose that were tested (experiment 2) produced substantial inhibition of both the apparent binding and catalysis. Removal of the hydroxyl group at the 2'-position (compound VIII) or at the 3'-position (compound IX) or changing the adjacent *cis* hydroxyls to *trans* (compound X) all produce large deleterious effects. Compound XIII

has the glycosidic linkage at position 3 of the adenine ring rather than at position 9 as in ADP. It is interesting that this change causes an almost complete loss of catalytic activity. Leonard and Laursen [12] reported that this type of analog can function as a substrate for adenylate kinase, luciferase, polynucleotide phosphorylase and hexokinase although they did not measure the kinetic constants. Hohnadel and Cooper [13] found that the triphosphate analog of compound XIII gave a K_m ratio of 2.7 and a V_{max} ratio of 0.48 with yeast hexokinase. Hohnadel and Cooper [9] also found that compound XIII can serve as a phosphate acceptor for oxidative phosphorylation catalyzed by mitochondrial inner membrane particles. Converting the *cis* hydroxyls to the bulky isopropylidine derivative (compound XI) or replacing the ribose with glucose (compound XII) leads to a complete loss of activity. Compound XI, which is unable to serve as a substrate, was tested for its ability to inhibit the reaction when ADP was the substrate. There was no detectable effect at concentrations up to 6.8 mM indicating that it probably does not bind to the enzyme in the presence or absence of ADP. This is in contrast to the finding made with yeast hexokinase. In the latter case the triphosphate analog of compound XI behaved like a competitive inhibitor with a K_i of about 2.5 mM [13]. We did not have sufficient amounts of compound XII to test its ability to act as an inhibitor of pyruvate kinase. We have previously found that the 2'- and 3'-*cis* hydroxyls are also of major importance for yeast hexokinase [13].

Klenow and Anderson [14] were probably the first to note that dADP was a relatively poor substrate for pyruvic kinase but they tested only a single concentration. Although Plowman and Krall [5] carried out a kinetic analysis with dADP and found it had a very high K_m, it was the only compound of those they tested that could have provided information concerning the importance of the ribose in this reaction. Our results therefore provide the first clear demonstration that the ribose moiety of ADP and its linkage to the adenine ring are of considerable importance with regard to the specificity and catalysis of the pyruvic kinase reaction.

Acknowledgements

Supported by a research grant from the National Science Foundation (GB 12747) and a training grant from the National Institutes of Health (GM 00035).

References

[1] J.L. Strominger, Biochim. Biophys. Acta 16 (1955) 616.
[2] A. Tietz and S. Ochoa, Arch. Biochem. Biophys. 78 (1958) 477.
[3] H. Adam, Biochem. Z. 335 (1969) 25.
[4] E.Z. Davidson, Biochim. Biophys. Acta 33 (1959) 238.
[5] K.M. Plowman and A.R. Krall, Biochemistry 4 (1965) 2809.
[6] K. Setondji, P. Remy, J.P. Ebel and G. Dirheimer, Biochim. Biophys. Acta 232 (1971) 585.
[7] N.G. Pon and R.J.L. Bondar, Anal. Biochem. 19 (1967) 272.
[8] W.W. Cleland, Adv. Enzymol. 29 (1967) 1.
[9] D.C. Hohnadel and C. Cooper, Biochemistry 11 (1972) 1138.
[10] L. Kerson, D. Garfinkel and A.S. Mildvan, J. Biol. Chem. 242 (1967) 2124.
[11] J.T. McQuate and M.F. Utter, J. Biol. Chem. 234 (1959) 2151.
[12] J.J. Leonard and R.A. Laursen, Biochemistry 4 (1965) 365.
[13] D.C. Hohnadel and C. Cooper, European J. Biochem. 31 (1972) 180.
[14] H. Klenow and B. Andersen, Biochim. Biophys. Acta 23 (1957) 92.