ENZYME KINETIC PARAMETERS OF THE FLUORESCENT ATP ANALOGUE, 2-AMINOPURINE TRIPHOSPHATE

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Received 12 February 1973
Revised version received 21 March 1973

1. Introduction

The importance of ATP as phosphoryl donor, energy source, and enzyme modifier in numerous reactions of cellular metabolism has been repeatedly demonstrated. As a means of examining the individual enzymes in these metabolic pathways, various analogues of ATP have been profitably employed [1–8]. Spectroscopic work has been done using derivatives that induce an NMR signal [9] or that have an intrinsic fluorescence [6, 8]. The fluorescence properties of the 2-aminopurine nucleotides have been particularly well characterized by Ward et al. [6].

We have been using the 2-aminopurine nucleotides to good advantage for certain reactions in polynucleotide metabolism and surmised that they should be equally useful for studying enzymes in intermediary metabolism. We have therefore compared the substrate properties of the 2-aminopurine nucleotides to the natural adenine nucleotides using a representative sampling of these enzymes.

2. Materials and methods

Acetate kinase (EC 2.7.2.1) from *E. coli*, creatine kinase (EC 2.7.3.2) from rabbit muscle, glycerokinase (EC 2.7.1.30) from *Candida mycoderma*, hexokinase (EC 2.7.1.1) from yeast, myokinase (EC 2.7.4.3) from rabbit muscle, 3-phosphoglycerate kinase (EC 2.7.2.3) from yeast, pyruvate kinase (EC 2.7.1.40) from rabbit muscle, and lactate dehydrogenase (EC 1.1.1.27) from porcine muscle were purchased from Boehringer-Mannheim, as were the following substrates: phosphoenolpyruvate, NADH, AMP, ADP, ATP, fructose-6-phosphate, 3-phosphoglycerate. Creatine was purchased from Schuchardt, and glucose from Merck. Crystalline phosphofructokinase was a gift of Dr. H.A. Lardy. The preparation of 2-aminopurine nucleotides followed standard procedures [10–12]. Other reagents were of analytical quality or better.

Adenine and 2-aminopurine nucleotide concentrations were determined spectrophotometrically in neutral buffer using extinction coefficients of 15.4 mM^{-1} cm^{-1} at 259 nm and 6.3 mM^{-1} cm^{-1} at 303 nm, respectively.

The standard reaction solution contained in 1.0 ml, triethanolamine-HCl, pH 8.0, (50 mM); $MgCl_2$ (10 mM); KCl (100 mM); phosphoenolpyruvate (1 mM); NADH (0.18 mM); lactate dehydrogenase (10 U). When pyruvate kinase was used as a coupling enzyme, it was added at 10 U/ml. Using the kinetic constants determined for 2-APDP in the pyruvate kinase reaction and equations derived for the analysis of coupled assays [13], this assay system resulted in linear initial rates in less than 12 sec. Other substrate concentrations were at least ten times their respective Michaelis constants. Following temperature equilibration at 22°, the reactions were initiated with enzyme; rate measurements were made with a Zeiss spectrophotometer by recording the decrease

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Abbreviations:
2-APMP, 2-aminopurinenucleoside-5'-phosphate;
2-APDP, 2-aminopurinenucleoside-5'-diphosphate;
2-APTP, 2-aminopurinenucleoside-5'-triphosphate.

in absorption at 340 nm on an X—Y recorder. Kinetic constants were evaluated using the method of Wilkinson [14] which at least six to eight initial velocities for each nucleotide examined.

3. Results and discussion

The apparent kinetic constants for each of the enzymes tested are shown in table 1. The Michaelis constants may be directly compared for each reaction. The maximal velocities have been normalized for more ready comparison. The error in this treatment is not large because the assays containing either the adenine or 2-aminopurine nucleotides were carried out using the same reaction solution and enzyme dilution within a few hours of one another. In addition to the maximal velocity, the ratios of V/K should be compared for an enzyme in which two different substrates are reactive. In fact this ratio is a more valid indication of the "apparent affinity" of the enzyme for the two substrates than is a simple comparison of the two Michaelis constants. This arises from the fact that in the simple Michaelis equation, $v=VA/(K+A)$, $v=V$ at saturating A concentration; and as A becomes very small $v=V/K(A)$. Thus, in the first order region of A concentration, V/K is the corresponding first order rate constant, As such, V/K is a measure of the steady-state selectively of the enzyme for the substrate. To facilitate comparison, we have normalized V/K for the 2-aminopurine nucleotides by dividing each value by the corresponding V/K determined when the adenine nucleotide was employed. The values thus obtained have been entered in table 1 as relative V/K for each enzyme tested.

Several of the entries in table 1 deserve special mention. It is clear that pyruvate kinase is well suited as a coupling enzyme for the conversion of 2-APDP to the triphosphate. The maximal velocity using 2-APDP was 89% that found with ADP and, more importantly for a coupling enzyme, the relative V/K was about 60% that determined for ADP. On the other hand, the indetectable activity of 2-APTP in the hexokinase reaction did not allow us to use this as a coupling enzyme for determining formation of the triphosphate in other reactions. In addition, 2-APTP had only a small inhibitory effect (K_i = 4 mM) in this reaction when ATP concentration was varied.

The myokinase reaction was interesting in that 2-APTP functioned as a substrate when AMP was present, but the complementary experiment in which 2-APMP and ATP were tested as the substrate pair did not yield a detectable reaction. This is, however, not surprising because this enzyme is known to have a higher specificity for the monophosphate than for the triphosphate [15]. When 2-APMP was used as an inhibitor, the K_i was in excess of 10 mM.

The major effect of 2-APTP substitution in the phosphofructokinase reaction was a reduction in the maximal velocity by one-half. The ratio V/K was comparable to that observed with ATP as substrate. Under these assay conditions, we would not expect to see an inhibitory effect of 2-APTP at high concentrations as has been reported for ATP at pH 7 [16]. However, Bloxham and Lardy have investigated this aspect of the reaction and have found no inhibitory function by 2-APTP [17].

The other enzymes tested showed varying degrees of reactivity or selectivity when 2-APTP was substituted for ATP. In general, it appears that those enzymes with a very high specificity for the adenine ring do not accept the 2-aminopurine substitution. Furthermore, in the two cases for which no activity was observed, there was also only a negligible inhibitory effect indicating that if the nucleotide is bound at all it is used as substrate, i.e., there appeared to be no nonproductive addition to the enzyme tested.

It is to be expected that the 2-aminopurine series of adenine analogs, as well as other recently reported adenine derivatives [8], will be useful in elucidating the mechanisms of individual enzymes. The marked fluorescence of these compounds accompanied by an absorption maximum well removed from protein absorption recommend these analogues to many physical studies. In addition, the differences found in the utilization of the adenine and 2-aminopurine nucleotides will yield further information concerning the specificity of enzymes studied. We have not used the 2-aminopurine derivatives as guanidine analogues, but on the basis of their structural similarity they may be useful in reactions requiring GTP.

Beyond this, however, we would suggest that the 2-aminopurine derivatives should be helpful in metabolic studies where the function of only certain pathways or portions of pathways is to be examined. The fact that adenylate kinase does not phosphorylate 2-APMP

Table 1
Comparison of kinetic constants.

Enzyme	Nucleotide	K_A (mM)	$K_{2\text{-AP}}$ (mM)	rel. V	rel. V/K
1. Pyruvate kinase	ADP	0.29	0.41	0.89	0.63
2a. Myokinase	ATP	0.065	1.84	0.52	0.02
2b. Myokinase	AMP	0.17	*	0.01	–
3. Phosphofructokinase	ATP	0.043	0.023	0.48	0.87
4. Creatine kinase	ATP	0.52	4.2	0.22	0.04
5. Hexokinase	ATP	0.43	*	0.01	–
6. 3-Phosphoglyceratekinase	ATP	0.43	4.1	1.1	0.12
7. Acetate kinase	ATP	0.16	0.11	0.39	0.57
8. Glycerokinase	ATP	0.044	1.76	1.40	0.04

Apparent Michaelis constants were determined for the indicated adenine nucleotide (K_A) as well as for the corresponding 2-aminopurine nucleotide ($K_{2\text{-AP}}$). Relative maximal velocities (rel. V) are expressed as a ratio of that observed using the 2-aminopurine nucleotide divided by that determined for the respective adenine nucleotide. The ratio V/K has been similarly normalized (see text). The assay conditions and EC number for each enzyme is given in Materials and methods.

* For these two enzymes the rate of reaction with the 2-aminopurine analogue was less than 1% of the rate observed employing adenine nucleotides.

may help to resolve problems in intermediary metabolism in which the usually rapidly equilibrating adenylate kinase reaction leads to ambiguity of interpretation. Similarly, if only ATP and not 2-APTP can be converted to ADP using glucose-hexokinase, it may be possible to study differential transport of these nucleotides in mitochondria.

Acknowledgement

We thank Dr. Thomas M. Jovin, in whose laboratory this work was done, for helpful discussions.

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