BBA Report

BBA 61255

Inhibition of rabbit skeletal muscle adenylate kinase by the transition state analogue, P^1,P^4-di(adenosine-5′)tetraphosphate*

DANIEL L. PURICH and HERBERT J. FROMM

Department of Biochemistry and Biophysics, Iowa State University, Ames, Iowa 50010 (U.S.A.)

(Received June 12th, 1972)

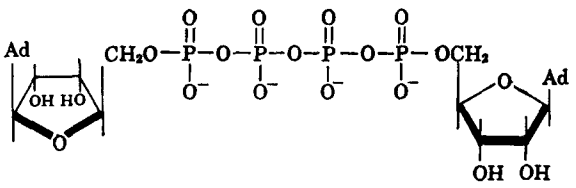
SUMMARY

Inhibition studies of rabbit muscle adenylate kinase (ATP:AMP phosphotransferase, EC 2.7.4.3) indicate that the transition state analogue, P^1,P^4-di(adenosine-5′)-tetraphosphate, is a competitive inhibitor relative to ATP and AMP. These studies suggest that adenylate kinase most probably has a kinetic mechanism involving the random addition of $MgATP^{2-}$ and AMP^{2-} to the enzyme to form a $E \cdot MgATP^{2-} \cdot AMP^{2-}$ Michaelis complex.

Rhoads and Lowenstein[1] demonstrated that the kinetic reaction mechanism of rabbit skeletal muscle adenylate kinase (ATP:AMP phosphotransferase, EC 2.7.4.3) is most probably of the random type and suggested that the productive ternary complex, enzyme·$MgATP^{2-}$·AMP^{2-}, is formed in the course of the reaction. Their conclusions were based upon initial rate studies, competitive inhibition experiments with adenosine-5′-monosulfate, and kinetic measurements of the equilibrium exchange reactions catalyzed by this phosphotransferase. These studies are in accord with earlier studies of rabbit muscle adenylate kinase[2,3]. Su and Russell[4] also found that a similar kinetic mechanism is suggested from rate studies of the yeast enzyme. This kinetic mechanism is in contrast to that exhibited by nucleoside diphosphokinases (EC 2.7.4.6)[5,6] or acetate kinase (EC 2.7.2.1)[7,8], but is in harmony with mechanisms associated with a wide variety of "kinase"-type phosphotransferases[9–13]. Our general interest in the kinetic mode of action of phosphotransferase-catalyzed reactions led to a study of the mode of inhibition of muscle

*Journal Paper J-7309 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa, 50010 (U.S.A.), Project 1666.

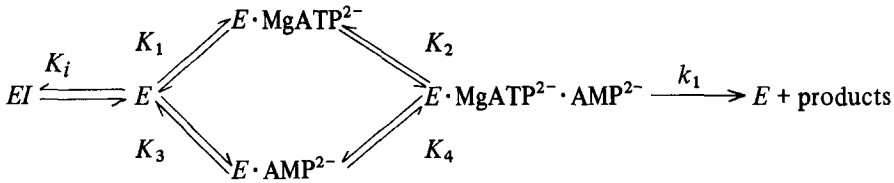
adenylate kinase by P^1,P^4-di(adenosine-5′)tetraphosphate (Ap_4A), which appeared to incorporate the features of both adenylate kinase substrates into a single molecule.



We wished to employ this compound to survey the geometrical features of the entire adenylate kinase active site, rather than the separate substrate binding sites. In addition, it appeared that this compound may resemble the proposed transition state of the adenylate kinase reaction[14], and it was of interest to test this possibility by measuring the inhibition constant for Ap_4A in the adenylate kinase reaction, as has been done with other transition state analogues[15–17]. Use of this symmetrical dinucleotide for such studies could serve as a model for subsequent studies of other enzymes such as hexokinase (EC 2.7.1.1), which should be similarly inhibited by the unsymmetrical polyphosphate, P^1-(adenosine-5′)-P^3-(glucose-6)triphosphate. Ap_4A is especially suited for this purpose as it can be prepared as a major product of the reaction of ATP with adenosine-5′-phosphoromorpholidate[18]. Finally, the mode of Ap_4A inhibition relative to each of the substrates can be used to differentiate sequential kinetic mechanisms[19], and should be of value in checking the kinetic mechanism proposed for this reaction.

For a clear analysis of the Ap_4A inhibition of rabbit muscle adenylate kinase, it was necessary to demonstrate that this inhibitor is not degraded by the enzyme to any appreciable extent. Since the Ap_4A used in this report was nonradioactive, it was not possible to quantitate the rate or extent of degradation. Use of diethylaminoethyl-cellulose ion-exchange paper did, however, permit the separation of the adenine nucleotides, and inspection of the chromatograms under ultraviolet light permitted a qualitative assessment of the degradation. Preliminary experiments suggest that very little nonenzymatic degradation occurs after 12 h at 20°C, and gave no evidence that the rate of degradation was accelerated by the adenylate kinase. It was therefore assumed that little or no degradation of the inhibitor occurred during the period elapsing in initial rate measurements (usually less than 5 min) in subsequent experiments.

Reversible inhibitors can often provide significant information on the kinetic mechanism and the nature of the substrate binding sites. This is generally accomplished by use of product inhibition[6,8,9,12] and competitive inhibition[8,9,13,19] studies of multisubstrate enzymatic reactions. Inhibitors that simultaneously span both substrate binding sites can also provide information on the kinetic reaction mechanism[15]. The following scheme outlines the kinetic reaction mechanism proposed by Rhoads and Lowenstein[1] including the interaction of the free enzyme, E, with the geometrical inhibitor, Ap_4A, designated as I.



Scheme I

EI, $E \cdot MgATP^{2-}$, $E \cdot AMP^{2-}$, and $E \cdot MgATP^{2-} \cdot AMP^{2-}$ represent the various enzyme complexes with ligands, I, $MgATP^{2-}$, and AMP^{2-}, and the K's represent dissociation constants for these enzyme ligand complexes. The following rate expression accounts for the interactions described in Scheme I:

$$\frac{V}{v} = 1 + \frac{K_4}{[MgATP^{2-}]} + \frac{K_2}{[AMP^{2-}]} + \frac{K_1 K_2}{[MgATP^{2-}]\,[AMP^{2-}]}\left(1 + \frac{[I]}{K_i}\right) \quad (1)$$

where v and V represent the initial reaction velocity and the maximal initial reaction rate, respectively. It is clear from Eqn 1 that the inhibition by I relative to either substrate is competitive. This is in contrast with the inhibition patterns expected for an ordered ternary complex mechanism, where inhibition relative to the first and second substrate should be competitive and noncompetitive, respectively[15]. For example, Collins and Stark[15] found that the transition state analogue, *N*-(phosphonacetyl)-L-aspartate, acts as a competitive inhibitor relative to carbamyl-*P* and as a noncompetitive inhibitor relative to aspartate in the reaction catalyzed by the *Escherichia coli* aspartate transcarbamoylase (EC 2.1.3.2). These findings were in accord with the experiments of Changeux *et al.*[20] indicating that succinate, an unreactive analogue of aspartate, binds tightly to this enzyme only in the presence of carbamyl-*P*, and with initial rate measurements in the absence and presence of product inhibitors[21]. It is important to note that for random mechanisms of substrate addition, the mode of inhibition by transition state analogues is unique. On the other hand, for enzymes displaying ordered kinetic mechanisms, the inhibition by transition state (or geometrical) analogues resembles the action of a normal competitive inhibitor on an enzyme having a random kinetic reaction mechanism. For this reason, it appears that one cannot unambiguously differentiate between these kinetic mechanisms solely on the basis of inhibition studies with transition state (or geometrical) analogues; additional evidence using product or competitive inhibitors is essential.

The kinetic data presented in Figs 1 and 2 suggest that Ap_4A is a competitive inhibitor relative to both $MgATP^{2-}$ and AMP^{2-}. These data are therefore in harmony with the earlier kinetic studies of rabbit muscle adenylate kinase[1-3], and appear to exclude an ordered binding of substrates to this phosphotransferase. It is also unlikely that the mechanism involves the formation of a covalent phosphoryl-enzyme intermediary in view of the observation that inhibition relative to either substrate is competitive. Moreover, one would not expect that Ap_4A binding would be very strong for an enzyme forming such an intermediate. The dissociation constant, however, for the $E \cdot Ap_4A$ complex was

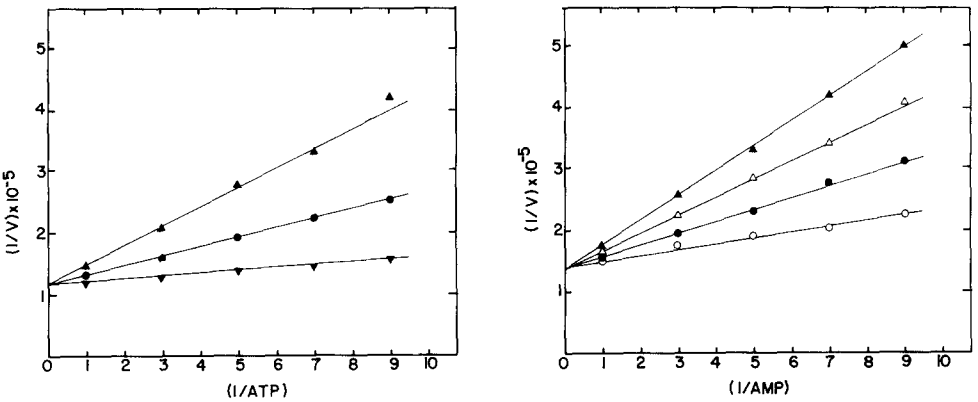


Fig. 1. Plot of the reciprocal of the initial reaction velocity (v) *versus* the reciprocal of the millimolar concentration of ATP in the absence and presence of Ap_4A. The concentration of AMP was maintained at 0.2 mM, and the ATP was varied in the concentration range of 0.11 to 1.0 mM. The concentrations of Ap_4A were none (▼), 0.09 mM (●), and 0.18 mM (▲). The velocity, expressed as the molar concentration of ADP formed in the reaction mixture over a 1-min period after the addition of enzyme at 28°C, in a Cary Model 15 recording spectrophotometer (0–0.1 slide wire) in 1.0 ml reaction mixtures using rabbit muscle adenylate kinase (Calbiochem). Each sample contained, in addition to the above components, 50 mM Tris–HCl (pH 8.0), 10 mM cysteine, 75 mM KCl, 0.1 mM NADH, 1.0 mM *P*-enolpyruvate, excess lactate dehydrogenase (EC 1.1.1.27) and pyruvate kinase (EC 2.7.6.40) (Calbiochem), and 1.0 mM free uncomplexed Mg^{2+}. The amount of total Mg^{2+} (as $MgSO_4$) added to each mixture was computed as described earlier[13], and under these conditions less than 5% of the total ATP was uncomplexed. The ATP, AMP, and Ap_4A concentrations were assayed spectrophotometrically; a value of $30.8 \cdot 10^3$ was assumed for the molar absorbance of Ap_4A. Other experimental details are described in the text.

Fig. 2. Plot of the reciprocal of the initial reaction velocity (v) *versus* the reciprocal of the millimolar concentration of AMP in the absence and presence of Ap_4A. The concentration of ATP was maintained at 0.15 mM, and the AMP was varied in the concentration range from 0.11 to 1.0 mM. The Ap_4A concentrations were none (0), 0.05 mM (0), 0.1 mM (Δ), and 0.15 mM (▲). The other experimental conditions were as described in the legend to Fig. 1, and less than 10% of the AMP was in the form of the MgAMP complex.

calculated to be approximately 24 μM by assuming K_m values of 0.3 mM for $MgATP^{2-}$ and AMP^{2-} from previous studies[2,3].

Wolfenden[22] has argued that the binding of the transition state, which incorporates both substrate molecules, should exceed that which would result if their individual affinities for the enzyme were merely combined into a single molecule. On the other hand, his treatment does not consider the possible effects of conformational transitions following the binding of the first substrate or, possibly more importantly, charge differences between the $E \cdot$analogue and $E \cdot \text{substrate}_1 \cdot \text{substrate}_2$ Michaelis complex. In addition, the effect of Mg^{2+} on the structure of the $E \cdot MgATP^{2-} \cdot AMP^{2-}$ Michaelis complex may differ from the effect on the structure of the $E \cdot MgAp_4A^{2-}$ complex. It is therefore difficult to evaluate whether Ap_4A is a true transition state analogue in the adenylate kinase reaction. Since the Ap_4A inhibition relative to either substrate is competitive, it does, however, appear

that the mechanism involves the random addition of $MgATP^{2-}$ and AMP^{2-} to form the productive ternary complex, and that geometrical analogues can be advantageously employed to exclude some kinetic reaction mechanisms.

The authors thank Dr J.G. Moffatt for his generous gift of the P^1,P^4-di(adenosine-5')-tetraphosphate used in these experiments.

This work was supported in part by Research Grant GB-33400 from the National Science Foundation. One of us (D.L.P.) was the recipient of a United States Public Health Service Predoctoral Fellowship.

REFERENCES

1 D.G. Rhoads and J.M. Lowenstein, *J. Biol. Chem.*, 243 (1968) 3963.
2 L. Noda, *J. Biol. Chem.*, 232 (1958) 237.
3 O.H. Callaghan and G. Weber, *Biochem. J.*, 73 (1959) 473.
4 S. Su and P.J. Russell, Jr., *J. Biol. Chem.*, 243 (1968) 3826.
5 N. Mourad and R.E. Parks, Jr., *J. Biol. Chem.*, 241 (1966) 271.
6 E. Garces and W.W. Cleland, *Biochemistry*, 8 (1968) 633.
7 R.S. Anthony and L.B. Spector, *J. Biol. Chem.*, 245 (1970) 6739.
8 D.L. Purich and H.J. Fromm, *Arch. Biochem. Biophys.*, 149 (1972) 307.
9 H.J. Fromm and V. Zewe, *J. Biol. Chem.*, 237 (1962) 3027.
10 A.M. Reynard, L.F. Hass, D.D. Jacobsen and P.D. Boyer, *J. Biol. Chem.*, 236 (1961) 2277.
11 A.S. Mildvan and M. Cohn, *J. Biol. Chem.*, 241 (1966) 1178.
12 J.F. Morrison and E. James, *Biochem. J.*, 97 (1965) 37.
13 J. Ning, D.L. Purich and H.J. Fromm, *J. Biol. Chem.*, 244 (1969) 3840.
14 A.S. Mildvan, in P.D. Boyer, *The Enzymes*, Vol.2, Academic Press, New York, 3rd edn, 1970, p.446.
15 K.D. Collins and G.R. Stark, *J. Biol. Chem.*, 246 (1971) 6599.
16 B. Evans and R. Wolfenden, *J. Am. Chem. Soc.*, 92 (1970) 4751.
17 R. Wolfenden, *Nature*, 223 (1969) 704.
18 J.R. Reiss and J.G. Moffatt, *J. Am. Chem. Soc.*, 87 (1965) 3381.
19 H.J. Fromm, *Biochim. Biophys. Acta*, 139 (1967) 221.
20 J.P. Changeux, J.C. Gerhart and H.K. Schachman, *Biochemistry*, 7 (1968) 531.
21 K.D. Collins and G.R. Stark, *J. Biol. Chem.*, 244 (1969) 1869.
22 R. Wolfenden, *Acc. Chem. Res.*, 5 (1972) 10.