CARBOXYLIC-PHOSPHORIC MIXED ANHYDRIDES ISOSTERIC WITH AMP AND ATP AS REAGENTS FOR ENZYMIC AMP AND ATP SITES

Alexander Hampton, Peter J. Harper, Takuma Sasaki,

Paul Howgate, and Robert K. Preston

The Institute for Cancer Research
The Fox Chase Cancer Center
Philadelphia, Pa. 19111

Received May 16,1975

SUMMARY: A carboxylic-phosphoric mixed anhydride was synthesized which differs from adenosine 5'-triphosphate (ATP) by replacement of the 5' methylene group of ATP by a carbonyl group. This compound did not inhibit rabbit adenylate kinase, but it rapidly inactivated rabbit pyruvate kinase; the effect was prevented by ATP, ADP and phosphoenolpyruvate and therefore appears to be ATP-site-directed. The analogous anhydride isosteric with adenosine 5'-phosphate (AMP) rapidly inactivated rabbit AMP aminohydrolase; inactivation was prevented by an equimolar level of AMP. These findings, together with previous studies with this AMP analog, suggest that these anhydrides are powerful and potentially useful reagents for adenine nucleotide binding sites of enzymes.

A previous communication (1) reported that a carboxylic-phosphoric mixed anhydride (I) (Figure 1) isosteric with AMP inactivates the AMP-utilizing enzyme adenylosuccinate lyase (EC 4.3.2.2), probably by acylating or phosphorylating an amino acid at the AMP binding site. We now present evidence that I inactivates AMP aminohydrolase (EC 3.5.4.6) in a similar manner. In addition, we describe the synthesis of an analogous anhydride (II) which is isosteric with ATP, and we report studies which indicate that II is an ATP-site-directed inactivator of pyruvate kinase (EC 2.7.1.40).

MATERIALS AND METHODS

Preparation of II. A solution of sodium tripolyphosphate in aqueous 50% methanol was passed through a column containing a 15-fold excess of tri-n-butylammonium Dowex-50 ion-exchange resin. The solvent was removed in vacuo and the residue thrice coevaporated in vacuo with anhydrous pyridine, then dissolved in anhydrous pyridine to give a 0.5 M solution of tri-n-butylammonium tripolyphosphate.

The following operations were carried out in a glove box in a dry nitrogen atmosphere with freshly prepared anhydrous reagents and solvents. To the sodium salt of 9-(β-D-ribofuranosyluronic acid)adenine (0.5 mmol) (2) was added N,N-dimethylformamide (2 ml). Diphenyl phosphorochloridate (0.48 mmol)

was added and the suspension was stirred for 5 hr. The mixture was centrifuged, when unreacted sodium salt was removed quantitatively together with sodium chloride. The supernatant was treated with diethyl ether (50 ml) and the precipitated diphenyl ester of I (1) was collected by centrifugation, dissolved in dioxane (2 ml), the solution clarified by centrifugation and the supernatant treated with diethyl ether (50 ml). The precipitate was collected, freed of diethyl ether *in vacuo*, and dissolved in 2 ml of the above 0.5 M tri-n-butylammonium tripolyphosphate solution. After 5 hr the solution was clarified by centrifugation and treated with diethyl ether (50 ml). The precipitate was collected, dissolved in pyridine - N,N-dimethylformamide (4 ml of 1:1) and reprecipitated with ether (50 ml). That the product contained no diphenyl ester of I was shown by the absence of ir peaks at 690 and 782 cm^{-1} due to monosubstituted phenyl groups; furthermore, the amination described below gave no diphenyl phosphate as judged by chromatography in previously described systems (1). The white precipitate, which contained a 90% yield (calculated from uv absorption) of the tri-n-butylammonium salt of II in admixture with *ca.* an equimolar amount of tri-n-butylammonium tripolyphosphate, was freed of ether *in vacuo* and dissolved in the minimum of anhydrous N,N-dimethylformamide and the solution stored at -25° under nitrogen. The spectral properties were the same as those reported for I (1). The identity and homogeneity of the product was established by treatment of a portion of the solution with dry ammonia whereafter paper chromatography showed a single ultraviolet-absorbing component of R_f 0.45 in ethanol-1 M ammonium acetate (7:3) and R_f 0.70 in n-butanol-acetic acid-water (5:2:3) which corresponded to the authentic carboxamide (1) of the starting material. The reaction mixture was subjected to tlc on PEI-cellulose using two developments with 4 M sodium formate of pH 3.4 and the plates were exposed to the vapor of concd HCl for 3 min and then sprayed with a molybdate-perchloric acid solution (3), when phosphate-containing components were seen as white spots on a yellow background; this showed that the reaction mixture contained much tripolyphosphate (a streak at R_f 0.1-0.3) but no pyrophosphate (R_f 0.5) and a trace of phosphate (R_f 0.9); the latter was found to be formed in the same proportion by the action of ammonia on the stock solution of tri-n-butylammonium tripolyphosphate.

AMP Aminohydrolase. Initial reaction rates were measured with a Cary Model 15 spectrophotometer as the decrease in absorbance at 265 nm and calculated as mμmol/min from $\Delta\varepsilon$ = 6,600. In all experiments the final volume of 0.90 ml contained 0.01 M potassium citrate (pH 6.5), 0.016 μg of enzyme (Sigma Chemical Co., Grade IV, from rabbit muscle), 0.005 M KCl, 60 μM I and 50 μM AMP. The order of addition for inactivation experiments was (1) buffer, (2) enzyme, (3) I, followed 1 min later by AMP. In the control assays the order of addition was (1) buffer, (2) I, followed 1 min later by (3) the enzyme and (4) AMP. Several experiments in Table I were performed by adding the same amount of I to the enzyme in 0.1 ml of buffer and then diluting the mixture to 0.90 ml after 1 min.

Rabbit Muscle Pyruvate Kinase. Initial reaction rates were measured at 340 nm. For all experiments (except where noted) the final volume of 1.00 ml of 0.1 M Tris-Cl (pH 7.6) contained 13 μg of lactic dehydrogenase (Sigma, rabbit muscle, type 1), 0.05 μg of pyruvate kinase (Sigma, type II), 0.1 M KCl, 0.025 M $MgSO_4$, 1.5 mM PEP (sodium salt), 0.25 mM ADP (sodium salt) and 0.25 mM NADH. The order of addition of the components was the same as with AMP aminohydrolase.

Rabbit muscle adenylate kinase was studied in a coupled assay with the pyruvate kinase system above in which ADP was replaced by the same level of ATP and more pyruvate kinase (4.8 μg) was used; 0.1 μg of Boehringer adenylate kinase was present per assay (final volume 1 ml).

RESULTS AND DISCUSSION

The present work revealed that the method used previously (1) to convert adenosine-5'-carboxylic acid to the AMP anhydride I is satisfactory also for conversion of the same acid to the ATP anhydride II. A number of the operations were simplified, thus reducing the time required to synthesize I and II; the modifications are detailed in the preceding section. The method did not convert uridine- or thymidine-5'-carboxylic acids (4) to the analogous UMP or TMP anhydrides.

I rapidly inactivated AMP aminohydrolase (Table I); the degree of inactivation increased slightly with a 9-fold higher level (540 μM) of I and with portionwise addition of I. The results of Table I were obtained with a sample of I which had been prepared the same day. After the solution of I in N,N-dimethylformamide had been stored at -25° for 24 hr, its ability to inactivate this enzyme decreased markedly, possibly as a consequence of acylation of the 6-amino group of I which, as noted previously (1), appears to occur upon storage of this solution. As in the case of adenylosuccinate lyase (1), the inactivation of AMP aminohydrolase was abolished by as little as 15-second

TABLE I: Inactivation of AMP Aminohydrolase by I.

Initial concn. of I (μM)	Additions prior to I	Rate, mμmol/min[a]		Inactivation (%)
		Enzyme plus hydrolyzed I	Enzyme plus I	
60	None	1.11	0.77	32
60	50 μM AMP	1.11	1.13	0
540[b]	None	1.13	0.68	40
540[c]	None	1.13	0.60	47

[a] Hydrolyzed I was not inhibitory at the levels used. [b] Enzyme and I mixed in 0.1 ml of buffer; see Methods. [c] I added to the enzyme as for [b], but in 3 portions at 20-second intervals.

hydrolysis of I in the assay buffer prior to contact with the enzyme, thus indicating that inactivation is the result of acylation or phosphorylation of the enzyme by the mixed anhydride, and that the reaction between I and the enzyme is extremely rapid. AMP at a level (50 μM) which was 12% that of its K_m value prevented inactivation of the enzyme by a 60 μM initial level of I, thus suggesting that the inactivation is brought about by covalent reaction of I at an AMP binding site.

AMP kinase of rabbit muscle was not inactivated by 1 mM nominal initial levels of I (a 2-day old sample) or of II (freshly prepared).

The ATP isostere II effected ca. 50% inactivation of pyruvate kinase (Table II) when a nominal initial level of 100 μM was added in three increments. The relationship between the degree of inactivation and the initial concentration of II was not examined further at this stage of the study of the potential of

TABLE II: Inactivation of Rabbit Pyruvate Kinase by II[a]

Additions prior to II	Rate x 10^2 (ΔA_{340}/min)		Inactivation (%)
	Enzyme plus hydrolyzed II	Enzyme plus II	
None	3.10	1.80	43
None[b]	3.00	1.45	52
None[c]	3.06	1.81	41
0.1 mM ATP	3.00	2.95	2
1.5 mM PEP	3.10	2.95	5
2.5 mM ADP[d]	3.02	2.96	2

[a] The nominal initial concentration of II was 100 μM. [b] II added in 3 equal increments at 20-second intervals. [c] After treatment with II the enzyme solution was stored at 22° for 16 hr before the velocity determination. [d] The enzyme was exposed to 2.5 mM ADP, then 0.1 mM II, in 0.1 ml of buffer and assayed after dilution to the standard volume of 1 ml.

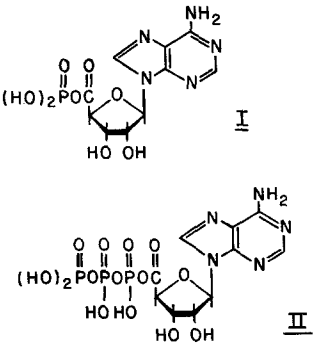

Figure 1: Structures of the carboxylic-phosphoric mixed anhydride isosteres of AMP (I) and of ATP (II).

I and II as reagents for enzymic AMP and ATP sites. As in the case of I, inactivation was prevented by 15-second hydrolysis of II in the buffer prior to addition of enzyme. Pyruvate kinase inactivated by II did not regain activity when stored in the assay medium for 16 hr at 22°. Protection of the enzyme from a 100 μM nominal level of II was afforded by 100 μM ATP, 2.5 mM ADP or 1.5 mM phosphoenolpyruvate, the levels of the two latter compounds being selected so as to be in excess of their enzyme-substrate dissociation constants [0.8 mM and 0.08 mM respectively (6)]. Protection by these three substrates implies that the action of II is probably ATP-site-directed because rabbit muscle pyruvate kinase exhibits random-order addition of substrates and kinetic (5,6) and magnetic resonance (7) evidence indicates that there is partial overlap between the phosphoenolpyruvate site and the ADP-ATP site and that ATP (and hence II) can bind neither to the enzyme-phosphoenolpyruvate complex nor to the enzyme-ADP complex.

We have studied the anhydrides I and II because their isosteric relationships to AMP and ATP, respectively, suggests that they might selectively adsorb to the AMP and ATP sites of many enzymes and thereafter acylate or phosphorylate those sites if a nucleophilic group of the enzyme is sufficiently near to the carbonyl carbon or to the phosphorus of the mixed anhydride. I and II inactivate three of the four enzymes which have been studied in this

laboratory, and the present evidence indicates that in each case they do so by reacting at AMP or ATP binding sites. These compounds therefore hold promise of reacting in the same manner with other enzymes for which AMP or ATP are substrates or effectors. Further work is required to determine the degree to which I and II react preferentially at the AMP or ATP sites of individual enzymes.

This work was supported by U.S.P.H.S. Research Grant CA-11196 from the National Cancer Institute, an award from the Pennsylvania Science and Engineering Fund, and by grants to The Institute for Cancer Research (U.S.P.H.S. grants CA-06927 and RR-05539 and an appropriation from the Commonwealth of Pennsylvania). T.S. undertook this work during the tenure of a Research Training Fellowship awarded by the International Agency for Research on Cancer.

REFERENCES

1. Hampton, A., and Harper, P. J. (1971), Arch. Biochem. Biophys., 143, 340-341.
2. Harper, P. J., and Hampton, A. (1970), J. Org. Chem., 35, 1688-1689.
3. Hanes, C. S., and Isherwood, F. A. (1949), Nature, 164, 1107-1112.
4. Moss, G. P., Reese, C. B., Schofield, K., Shapiro, R., and Todd, A. R. (1963), J. Chem. Soc., 1149-1154.
5. Reynard, A. M., Hass, L. F., Jacobsen, D. D., and Boyer, P. D. (1961), J. Biol. Chem., 236, 2277-2283.
6. Ainsworth, S., and Macfarlane, N. (1973), Biochem. J., 131, 223-236.
7. Mildvan, A. S., Fung, C. H., Melamud, E., Sloan, D. L., and Gupta, R. K. (1975, submitted) Abstracts, Division of Biological Chemistry, 170th National Meeting, American Chemical Society, August, 1975.