The Reaction of 1-Acetylimidazole with Orthophosphate*

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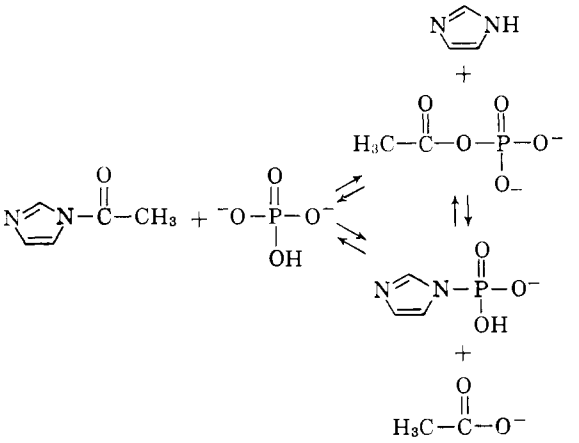
ABSTRACT: The reaction of 1-acetylimidazole, orthophosphate, and adenosine-5′-phosphate (AMP) to produce adenosine triphosphate (ATP) in *N,N*-dimethylacetamide solution was found to produce no measurable ATP when morpholine was added to the equilibrium mixture prior to the addition of AMP. This result eliminates 1-phosphoimidazole as the phosphorylating intermediate. Acetyl phosphate was identified as the only phosphorylated product in the equilibrium mixture resulting from the reaction of 1-acetylimidazole and orthophosphate both in aqueous and in *N,N*-dimethylacetamide solutions.

The isolation of phosphorylated histidine residues from mitochondrial protein (Boyer, 1963) has created considerable interest in elucidating the pathways involved in their formation, since these phosphoproteins are probably involved in oxidative phosphorylation (Boyer, 1963), substrate level phosphorylation (Kreil and Boyer, 1964; Mitchell *et al.*, 1964), and/or transphosphorylation (Kundig *et al.*, 1964) reactions. Information concerning nonenzymatic chemical reactions of 1-phosphoimidazole (1-imidazolylphosphonate) should be helpful in defining the biological pathways of protein phosphohistidine, and it is the purpose of this paper to report some of these reactions which seem particularly pertinent to biological systems.

A possible mechanism for the formation of protein phosphohistidine was proposed by Boyer (1963), involving the reaction of protein *N*-acylimidazole with orthophosphate. It has been observed in our laboratory that a mixture of 1-acetylimidazole and P_i readily phosphorylates AMP and ADP in polar organic solvents (Brinigar and Wang, 1964a). In connection with possible pathways for the formation of protein phosphohistidine, it is of interest to identify the intermediate(s) in the model reaction. Since both acetyl phosphate and 1-phosphoimidazole are excellent phosphorylating agents under the same conditions, the identity of the intermediate was not evident from the observation that phosphorylation of AMP and ADP did occur.

Previous studies of the reaction of Ac-Im[1] and P_i indicate that in aqueous solution acetyl phosphate represents at least the major product (Stadtman, 1954; Jencks and Carriuolo, 1959). Therefore, if P-Im is also a product of this reaction it will probably be present in relatively small amounts, and be formed either by a S_N2' type mechanism, or by reaction of imidazole with



acetyl phosphate. The hydrolysis of acetyl phosphate catalyzed by a variety of nucleophilic agents including imidazole was studied by Di Sabato and Jencks (1961) who demonstrated that although P—O bond cleavage occurred in the presence of pyridine and triethylenediamine, only C—O cleavage was detected in the presence of imidazole and *N*-methylimidazole.

Experimental

Materials. 1-Acetylimidazole was prepared from imidazole and isopropenyl acetate as described by Boyer (1952), dried over P_2O_5, and transferred to small vials in a drybox. The vials were stored in a desiccator below 0° until immediately before use. Transfers of solid material were carried out in a drybox.

1-Phosphoimidazole barium salt was prepared from imidazole and potassium phosphoramidate by a slight modification of the method reported by Rathlev and Rosenberg (1956). The molar quantity of imidazole used by the above-mentioned authors was doubled in order to reduce the amount of 1,3-diphosphoimidazole formed in the reaction. The barium salt was obtained by adding $BaCl_2$ to the reaction mixture and precipitating the product with alcohol. Purification was accomplished by redissolving in water and precipitating with alcohol. Paper chromatography, as described further on, gave

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[1] Abbreviations used in this work: Ac-Im, 1-acetylimidazole; P-Im, 1-phosphoimidazole; DMAC, *N,N*-dimethylacetamide.

a single spot with both reagents, R_F = 0.4. DMAC solutions of the diimidazolium salt were prepared by the addition of an equimolar quantity of diimidazolium sulfate to the barium salt in water, removal of the $BaSO_4$, evaporation of the water under high vacuum, and dissolving the residue in DMAC. Solutions were filtered before use. The concentration of 1-phosphoimidazole was determined by subjecting aliquots of the DMAC solution to acid hydrolysis and Fisk-Subbarrow phosphate determination. Total phosphate was taken as the P-Im concentration.

Diimidazolium hydrogen phosphate was obtained by mixing 2 moles of imidazole with 1 mole of 85% orthophosphoric acid. The resulting mixture was dried *in vacuo* over P_2O_5, giving a white crystalline solid.

AMP, ADP, ATP, acetyl phosphate (dilithium salt), and firefly lantern extract were obtained from Sigma Chemical Co. The *N,N*-dimethylacetamide was Eastman White Label which had been passed through alumina and distilled under vacuum. Morpholine was Fisher, reagent grade.

Chromatography. Whatman No. 2 paper was used with a descending solvent composed of ethanol–0.1 M aqueous K_2CO_3 (65:35). Chromatograms were sprayed with Hanes-Isherwood molybdate reagent followed by diazotized sulfanilic acid, as described by Rathlev and Rosenberg (1956). Hydroxylamine followed by $FeCl_3$ was also employed for acetyl phosphate. The following R_F values were observed: orthophosphate, 0.15; 1-phosphoimidazole, 0.4; acetyl phosphate, 0.3; imidazole, 0.8. Acetylimidazole failed to survive chromatography in this solvent system.

ATP Analysis. The quantitative determination of ATP was carried out by mixing diluted aliquots of the reaction mixtures with firefly lantern extract as previously described (Brinigar and Wang, 1964b).

Results

Position of Equilibrium. The rate of appearance of ATP from ADP with Ac-Im and P_i in *N,N*-dimethylacetamide (DMAC) was found to proceed much more slowly than reaction with comparable concentrations of either acetyl phosphate or P-Im. Therefore it was necessary to establish whether equilibrium concentration of the phosphorylated intermediate was sufficient to permit detection. To answer this question, identical solutions of Ac-Im and diimidazolium hydrogen phosphate in DMAC were equilibrated for various lengths of time before the addition of a DMAC solution of ADP. The results given in Figure 1 show that the reason for the slower rate of phosphorylation is the relatively slow production of the phosphorylated intermediate. The equilibrium in aqueous solution is to the right (Stadtman, 1954) and, although this experiment gives no information concerning the magnitude of the equilibrium constant in DMAC, it does indicate that the equilibrium is at least in the same direction in this solvent as in water.

Chromatographic Analysis. Mixtures of Ac-Im and diimidazolium hydrogen phosphate in approximately epuimolar amounts in DMAC were allowed to stand

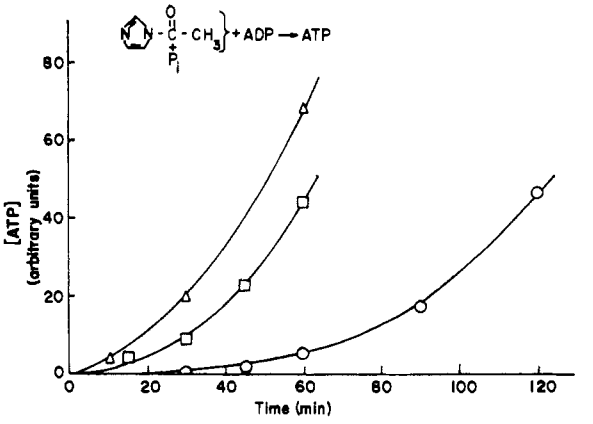

FIGURE 1: Phosphorylation of ADP by a mixture of Ac-Im and P_i in DMAC. ADP solution added: ○, simultaneously with 1-acetylimidazole; □, 1 hour later; △, 2 hours later. Final concentrations: 1-acetylimidazole, 10^{-4} M; diimidazolium hydrogen phosphate, 10^{-4} M; diimidazolium salt of ADP, 10^{-5} M. Reactions were conducted at room temperature.

at room temperature for several hours, and the solvent was removed under high vacuum at room temperature. The residue was dissolved in water and adjusted to *p*H 9 with K_2CO_3, and aliquots were chromatographed on paper as described in the experimental section. The only phosphorylated product detected was acetyl phosphate. Substitution of water for DMAC did not change the product composition. In neither case was the faintest color observed with either Hanes-Isherwood reagent or diazotized sulfanilic acid at the R_F where P-Im would have appeared. Although these results verify previous observations in aqueous solution that acetyl phosphate is the major product of the reaction, they do not exclude the possibility that small amounts of P-Im are present but remain undetected.

Reactions in the Presence of Morpholine. As a result of an attempt in a related phosphorylation experiment to substitute the dimorpholinium salt of phosphoric acid for the diimidazolium salt, it was observed that ATP was not produced from the dimorpholinium salt whereas significant amounts of ATP were formed under the same conditions from the diimidazolium salt. Subsequent experiments showed that the addition of morpholine to DMAC solutions of acetyl phosphate prior to the addition of AMP completely blocked ATP formation, whereas the addition of morpholine to solutions of P-Im did not affect the yield of ATP. This result is reasonable since *N*-acetylmorpholine has been shown to be the only product from the reaction of acetyl phosphate and morpholine (Di Sabato and Jencks, 1961), whereas, if morpholine and P-Im reacted under these conditions, the product anticipated would be *N*-phosphomorpholidate. In contrast to *N*-acetylmorpholine, which is incapable of "activating" phosphate, phosphomorpholidates are excellent phosphorylating agents.

TABLE I: ATP Synthesis in the Presence and Absence of Morpholine.[a]

Reactants	Reaction Mixtures						
	1	2	3	4	5	6	7
1-Acetylimidazole	5 mM	5 mM					5 mM
Diimidazolium-P_i	1.7 mM	1.7 mM			1.7 mM		1.7 mM
1-Phosphoimidazole			5 μM	5 μM	5 μM	5 μM	5 μM
Morpholine		0.04 M		0.04 M		0.04 M	0.04 M
Imidazolium acetate						3.3 mM	
ATP produced	21 μM	<0.008 μM	0.13 μM	0.16 μM	0.09 μM	0.14 μM	0.16 μM

[a] In addition to the above, each reaction mixture contained 0.85 mM AMP. The concentrations are expressed as final values per liter after all additions had been made. Reactions mixtures were initially prepared containing all reactants except morpholine and AMP, and allowed to stand at room temperature for 48 hours. Morpholine was then added as required and solutions allowed to stand an additional 12 hours after which time the AMP was added. ATP analysis was carried out 48 hours after the AMP addition.

Since the firefly method of ATP analysis is extremely sensitive, a means was hereby available for the detection of small amounts of P-Im which might be produced in the reaction between Ac-Im and P_i. Table I shows the results of a complete series of experiments carried out simultaneously using the same stock solutions for the preparation of each reaction mixture.

Two additional reaction mixtures were analyzed, one containing 5 mM *N*-acetylmorpholine and 1.7 mM P_i, the other approximately 4 mM acetyl phosphate and 0.04 M morpholine, both with 0.85 mM AMP. No detectable ATP (<0.008 μM) was produced in either solution.

It is evident that no measurable amount of ATP is formed from Ac-Im and P_i in the presence of morpholine. Further, the addition of P-Im to a mixture of Ac-Im and P_i, to which morpholine is also subsequently added, produces the same amount of ATP as does P-Im alone, indicating that there is no active equilibrium involving P-Im. This conclusion finds further support from reaction mixture 6 where a relatively large concentration of acetate added to P-Im failed to decrease the amount of ATP produced.

This series of experiments was carried out several times at slightly different reactant concentrations. With the exception of number 5, containing P-Im and P_i, all mixtures gave a reproducible yield of ATP. However, solutions containing both P-Im and P_i yielded varying amounts ranging from 50 to 80% as much ATP as the corresponding solution without added P_i (number 3). In the presence of a relatively high concentration of P_i, pyrophosphate formation would be expected to reduce the P-Im concentration prior to the addition of AMP; and since pyrophosphate does not phosphorylate AMP at a perceptible rate under these conditions, the yield of ATP is thereby reduced. However this side reaction should not adversely affect the measurement of P-Im in mixtures from Ac-Im to P_i because an excess of Ac-Im relative to P_i was employed in order to leave little free P_i in the solution at equilibrium.

Since at least 42 μM of phosphorylating agent was produced in reaction mixture 1, and the limit of sensitivity is approximately 0.01 μM of ATP corresponding to 0.5 μM of P-Im, a reasonable estimate of the amount of P-Im which would remain undetected by this experiment is roughly 1% of the total concentration of phosphorylating intermediates.

When reaction mixtures analogous to those in Table I were prepared in aqueous solution and allowed to stand at room temperature for 1 hour, and water was removed *in vacuo* and replaced by DMAC, similar results were obtained, although the amounts of ATP produced were considerably lower owing to competitive hydrolysis.

Discussion

The phosphorylation of AMP and ADP by a mixture of Ac-Im and P_i in DMAC appears to be mediated solely by acetyl phosphate. The addition of P-Im to the equilibrium mixture from Ac-Im and P_i produced the same amount of ATP in the presence of morpholine as did P-Im alone. Likewise the addition of acetate and morpholine failed to alter the amount of ATP produced from P-Im. If P-Im were a product of the reaction of Ac-Im and P_i, one would expect the equilibrium to be shifted to the left in both cases by the formation of *N*-acetylmorpholine, resulting in little or no ATP formation. Therefore it may be concluded that reactions leading to and away from P-Im are exceedingly slow compared with those directly involving Ac-Im, P_i, acetyl phosphate, and OAc^-, both in aqueous and DMAC solutions. It is, of course, possible for these same functional groups associated with a protein to exhibit relative rates quite different from those observed with simple model compounds, and conditions may well exist which favor the rate of P-Im formation. Indeed, preliminary results in our laboratory indicate that the hydrolysis of acetyl phosphate at *p*H 9 in

the presence of 2- to 10-fold excess imidazole is accompanied by substantial amounts of P-Im.

An interesting feature of the reactions is the yield of ATP. From acetyl phosphate (Ac-Im + P_i) the yield of ATP, based on P_i, is only 2.5% of theoretical; from P-Im, approximately 5%. Although these figures are disappointingly low (and as such serve as dramatic testimony to the restrictions which enzymes are able to impose upon chemical reactions), they are in the same range as the yield obtained from chlorocruorohemin dimethyl ester, P_i, and AMP, a model system possibly related to the cytochrome oxidase site in oxidative phosphorylation (Brinigar and Wang, 1964b).

The reaction mixtures were not examined for other phosphorylated compounds, and doubtlessly contain considerable amounts of pyrophosphate, ADP, adenosine tetraphosphate, and the like. It has been shown that P-Im in neutral aqueous solution at 100° produces pyrophosphate (Baddiley *et al.*, 1956). Another reaction which may well be involved is the disproportionation of of P-Im into 1-pyrophosphoimidazole and imidazole (Cramer and Schaller, 1961).

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