ENZYMATIC ATP FORMATION FROM ADP AND PHOSPHORAMIDATE

H. Leigh Auleb, Michael J. Dowler
and Henry I. Nakada

Department of Biological Sciences
University of California at Santa Barbara

Received March 29, 1966

Phosphoramidate:ADP Phosphotransferase has been partially purified from dried Baker's yeast. This enzyme catalyzes the phosphate transfer from phosphoramidates to ADP to form ATP. A similar non-enzymatic reaction has been studied by Moffett and Khorana (1). Previously, Smith and co-workers (2,3) reported on phosphoramidases and phosphoramidic:hexose transphosphorylase, and Auleb and Nakada (4) extended these findings to a number of different species. Furthermore, a phosphorylated histidine residue has been found to be an important part of the phosphate transfer mechanism of succinic thiokinase (5). Of immediate and great interest is the recent report of Perlgut and Wainio (6) who have presented evidence for phosphoiodohistidine being an intermediate in the oxidative phosphorylation process.

Materials and Methods

All materials were purchased from commercial sources with the following exceptions. Phosphoramidate (NP) was synthesized by the method of Stokes (7) using a reflux time of 2 hours for the synthesis of diphenylphosphorylchloride to obtain higher

yields. $^{32}POCl_3$ was synthesized by an exchange reaction using $H_3{}^{32}PO_4$ (8). N-phosphorylhistidine was prepared by the Rathlev and Rosenberg procedure (9) and N-phosphorylglycine by the method of Winnick and Scott (10).

The Phosphoramidate:ADP Phosphotransferase (PAP) was assayed by coupling ATP formation to hexokinase and glucose-6-P dehydrogenase reactions and following the rate of formation of NADPH using the Beckman DK-2A spectrophotometer. An alternative procedure involved the enzymatic formation of ^{32}P labeled ADP and ATP from $N^{32}P$ and counting the organic phosphate, which were separated by the method of Nielson and Lehninger (11). Typical reaction mixtures are shown in Table 1 and counting of radioactive samples was accomplished using liquid scintillation techniques.

Isolation and Partial Purification of Yeast Phosphoramidate:ADP Phosphotransferase

The enzyme was partially purified by the following typical procedure. Thirty-five gm. of Fleischmann's air-dried Baker's yeast was autolyzed overnight in 250 ml. 0.2M ammonium sulfate adjusted to pH 9.3. The extract was centrifuged to remove cellular debris and the supernatant liquid was made 0.01M in 2-mercapto-ethanol to stabilize the enzyme. (Thiol concentration was maintained at this value throughout the purification procedure). The extract was chilled to 0-5°, and adjusted to pH 4.5 with 1M HCl. After 15 minutes, precipitated protein was removed by centrifugation and the supernatant fluid was fractionated with solid ammonium sulfate. The fraction precipitating between 50 and 70% of ammonium sulfate saturation was dissolved in 0.01M 2-mercaptoethanol and further purified by the cautious addition of small amounts of $Ca_3(PO_4)_2$ gel or Alumina Cγ gel.

Table I. Some conditions for ATP synthesis by Phosphoramidate:ADP Phosphotransferase

Conditions	μ moles ^{32}P incorporated into organic phosphates			
Complete system	1.94;	1.76;	2.11;	1.65
^{32}Pi replaces $N^{32}P$	0;	0;	0;	0
Minus ADP	0;	0;	0;	0
Minus Enzyme	0;	0;	0;	0
AMP replaces ADP	0;	0;	0;	0
Minus Enzyme and ADP*	0;	0;	0;	0

The complete system contained: 7.5 μmoles $N^{32}P$, 7.5 μmoles ADP, .25 ml. enzyme, .75 ml. .2M citrate buffer pH 6.0 (total volume, 1.5 ml.). The final reaction mixture was incubated at room temperature for 20 minutes.

*This value was taken as the background level of radioactivity.

It was essential that activity be retained in the supernatant liquid, since elution was difficult and recovery was low if adsorption occurred. An apparent purification of 50-fold resulted; however, this value is misleading because of the presence of certain contaminating enzymes and deterioration of enzymatic activity during the purification. Although the preparation was usually free of hexokinase, phosphoramidic:hexose transphosphorylase and glucose-6-P dehydrogenase, some preparations contained an ATP-ase, myokinase and phosphoramidase.

Formation of ATP

The coupled enzyme assay procedure used for enzyme purification and in some of the early experiments suggested the formation of ATP. This indication was unreliable because of the

presence of myokinase. Parallel results were obtained by using ^{32}P labeled phosphoramidate and isolating the organic phosphates by Nielson and Lehninger procedure (11). Results of four separate runs using the radioactive assay procedure are shown in Table I.

As shown, inorganic phosphate could not replace $N^{32}P$, ADP was a required phosphate acceptor and AMP was not phosphorylated in this system. In order to show that the labeled organic phosphates were adenosine phosphate, reaction mixtures were separated by chromatography on Dowex-1 by the method of Bartlett (12). Results of such a separation appear in Figure 1. Note that the AMP peak contained no radioactivity while both the ADP and ATP contained ^{32}P. Label in ADP can be accounted for by the action of myokinase. That myokinase was not responsible for the

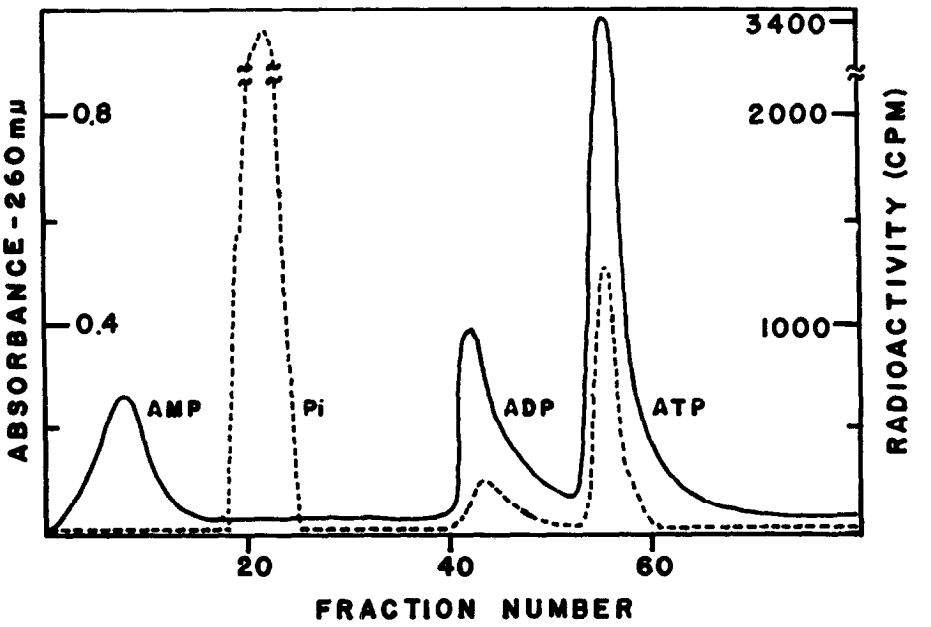

Figure 1: Chromatographic Separation of Reaction Mixture. The dotted line represents radioactivity in counts per minute. The solid line indicates 260 m adsorbing material. The reaction mixture was identical to the one shown in Table 1.

observed PAP activity was shown by the inability of myokinase preparations to catalyze the synthesis of ATP from NP, by differential activities observed during enzyme purification, and by the inhibition of myokinase by ammonium sulfate concentrations that activated PAP.

Other Properties of PAP

Phosphoramidate, N-phosphorylglycine, and N-phosphorylhistidine were effective donors but creatine phosphate was inactive (Table II).

Table II. Assay of Potential Phosphoryl Donors

Phosphoryl Donor	spec. act. (mμmoles/mg.-min.)	
	ADP present	ADP absent
0.04M phosphoramidate	2.4	0.0
0.02M N-phosphorylhistidine	12.0	0.48
0.01M N-phosphorylglycine	12.0	0.64
0.01M creatine phosphate	0.0	0.0

Assayed by the coupled enzyme procedure. Each cuvette contained in 1 ml: 0.5 mM $NADP^+$, 5mM $MgCl_2$, 20 mM citrate buffer pH 6.0, 20 mM glucose, 3mM ADP, 1.5 units hexokinase, 0.29 units glc-6-P dehydrogenase and PAP. The reaction was started by the addition of the appropriate phosphoryl donor.

The pH optimum and the pH of least stability was 6.0. The reaction was found to have an apparent heat of activation of about 22 kcal/mole and an apparent Michaelis constant (phosphoramidate) of 2×10^{-2}M. Both of the latter values are only approximate because of the presence of contaminating enzymes. The presence of myokinase prevented the determination of a Michaelis constant for ADP.

Discussion

A number of different enzymes capable of utilizing phosphoramidate as substrates for hydrolytic or phosphate transfer reactions have been reported (1-4). To date, the absence of naturally occuring phosphoramidates has masked any possible important involvement of these compounds in biological systems. Recent studies are beginning to resolve this difficulty. Boyer and co-workers (5) have shown the presence of enzyme-bound phosphohistidines as intermediates in enzymatic phosphotransfer reactions. Perlgut and Wainio (6) have demonstrated the formation of phosphoiodohistidine in mitochondria. This compound is apparently connected to the oxidative phosphorylation via the electron transport system. They have also shown non-enzymatic and possible enzymatic transfer of phosphate from phosphoiodohistidine to ADP to yield ATP. The present work shows that an enzyme capable of phosphorylating ADP using various phosphoramidates can be isolated from the soluble portion of autolyzed yeast. Possible involvement of this enzyme in oxidative phosphorylation is not yet known.

The involvement of phosphoramidate in hexose phosphorylation, in enzymatic transphosphorylation and in ATP formation indicates that a group of important "high energy" compounds have been overlooked. In view of the widespread occurance of enzymes capable of utilizing phosphoramidates it is apparent that their involvement should be investigated more thoroughly.

Acknowledgements

This work was supported in part by a research grant from the Cancer Research Coordinating Committee of the University of California.

References

1. Moffett, J.G., and H.G. Khorana, J. Am. Chem. Soc., 80, 3756, (1958)

2. Fujimoto, A., and R.A. Smith, Biochem. Biophys. Acta, 56, 501, (1962)

3. Holzer, M.E., D.J. Burrow and R.A. Smith, Biochem. Biophys. Acta, 59, 491, (1962)

4. Auleb, H.L., and H.I. Nakada, Fed. Proc., 23, 2596, (1964)

5. DeLuca, M., K.E. Ebner, D.E. Hultquist, G. Kreil, J.B. Peter, R.W. Moyer, and P.D. Boyer, Biochem. Z., 338, 512, (1963)

6. Perlgut, L.E., and W.W.Wainio, Biochem., 5, 608, (1966)

7. Stokes, H.N., Am. Chem. J., 15, 19[illegible], (1893)

8. Vigne, J.P., and R.L. Tabau, Bull. Soc. Chem. France, 1194, (1958)

9. Rathlev, T., and T. Rosenberg, Arch. Biochem. Biophys., 65, 319, (1956)

10. Winnick, T., and E.M. Scott, Arch. Biochem. Biophys., 12, 201, (1947)

11. Nielson, S.O., and A.L. Lehninger, J. Biol. Chem., 215, 555, (1955)

12. Bartlett, G.R., J. Biol. Chem., 234, 459, (1959)