Enzymatic Synthesis of Arginine Phosphate with Coupled ATP Cofactor Regeneration[1]

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Arginine phosphate, a compound having high phosphate transfer potential which occurs in invertebrates, has been prepared in a 2-mol scale by enzymatic synthesis from arginine and ATP using arginine kinase as catalyst. ATP was regenerated *in situ*, using phosphoenolpyruvate or acetylphosphate as the ultimate phosphate donors.

Arginine phosphate (ArgP) is a compound with high phosphate transfer potential found in the muscle of invertebrates (*1, 2*). Although various chemical syntheses of the compound have been described (*3–5*), none are convenient, and extraction of ArgP from lobster or crayfish muscle still remains a competitive preparative procedure (*6*). We describe here a large-scale enzymatic synthesis of ArgP from arginine and ATP using arginine kinase (EC 2.7.3.3) as catalyst (*7, 8*). ATP was regenerated *in situ* using either phosphoenol pyruvate (PEP) or acetyl phosphate (AcP) as the ultimate phosphate donor (Eq. [1]).

$$[1]$$
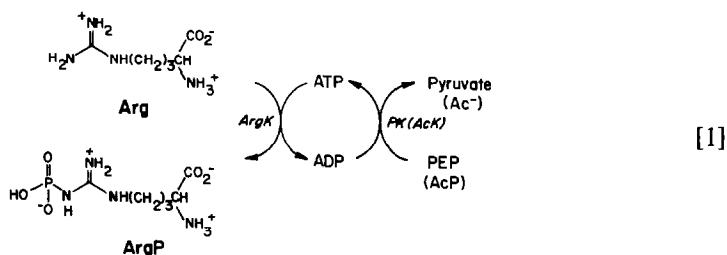
The free energy of hydrolysis of ArgP at pH 7 ($\Delta G'^0$) is -7.7 kcal/mol; for AcP, -10.3 kcal/mol; and for PEP, -14.8 kcal/mol (*9*). Thus, either PEP or AcP could, in principle, drive the reaction to completion. In practice, ArgK is subject to pronounced inhibition by the product ArgP.[3] Thus, although rates can be increased by increasing the concentration of Arg, it is difficult to achieve high conversions of Arg to ArgP and high concentrations of ArgP simultaneously. Under these circumstances, the ATP-regeneration system based on PEP is much superior to that based on AcP, even though AcP is more easily prepared than PEP

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[3] Inhibition by arginine is competitive ($K_i = 0.8$ mM) and by MgATP is noncompetitive ($K_i = 1.5$ mM) (*8*).

(*10, 11*). The overall rate of reaction is necessarily slow as the concentration of ArgP increases and ArgK becomes subject to product inhibition. As the reaction slows, the spontaneous hydrolysis of AcP to acetate and phosphate becomes increasingly important. Acetate kinase is itself subject to inhibition by acetate.[4] Thus, the overall rates of both the conversions of Arg to ArgP and ADP to ATP slow markedly toward the end of the reaction. We include under Experimental Procedures a format for the preparation of ArgP using AcP; this procedure may be useful for small-scale preparations, for preparations in dilute solutions, or for preparations requiring radioactive phosphorus. For general synthetic work, however, the procedure based on PEP is clearly preferable.

The optimal pH for ArgK is 8.6 (*7*). At this pH, arginine phosphate is reasonably stable; only 3% decomposition occurs in 24 hr.[5] It is therefore practical to work with limited amounts (100–400 U) of the relatively expensive[6] enzyme and to let the reaction proceed over several days. Arginine kinase was immobilized in a polyacrylamide gel (PAN 1000) using a procedure described previously (*12*), and using ADP, Mg^{2+}, Arg, and NO_3^-[7] to protect the active site. Immobilization is used both to facilitate recovery and reuse of the enzyme, and to improve its stability under operating conditions.[8]

Three successive reactions were carried out using the same preparation of enzyme-containing gel. The results were comparable for the three. In the last, it was possible to reach a concentration of 0.2 M in ArgP with a yield of 75% based on PEP and an ATP turnover number of 64 (Table 1). We did not observe loss of enzymatic activity during the 6 weeks required to conduct these experiments. The pyruvate formed is not stable; only 40% of the theoretical amount is found by enzymatic assay.

When extracted from biological material, arginine phosphate is isolated as a barium salt (*6*); the procedure requires selectively precipitating inorganic phosphates from an aqueous solution of ArgP, and then isolating the arginine phosphate as its barium salt by addition of ethanol. We have found it easier to isolate ArgP directly as a free acid; after removal of the enzyme-containing gel, the solution is adjusted to pH 4 by addition of Dowex 50 W (H^+ form). Arginine phosphate precipitates in ~60% purity as a dense, crystalline, easily filtered white solid on addition of 1.5 vol of 95% ethanol. This material can be easily brought to 95% purity by a further purification.

One final point concerning the data in Table 1 deserves mention. The apparent (or effective) activity of the enzymes under the conditions encountered in the synthesis reaction is approximately 10% of that expected on the basis of activities

[4] Inhibition is appreciable for acetate concentrations above 0.6 M (*10*).

[5] Arginine phosphate barium salt is stable a room temperature. Although Morrison and Coll claimed the free acid to be unstable (*6*), we observed less than 0.1% decomposition at $-10°C$ over 4 months.

[6] Arginine kinase costs ~$30/1000 U.

[7] The addition of nitrate during immobilization increases the immobilization yield. Nitrate also increase the binding of arginine to arginine kinase (*13*).

[8] Arginine kinase in suspension in ammonium sulfate solution is stable. In solution, in Hepes buffer, a gradual precipitation and partial loss of activity occurs after a few minutes. It is oxygen sensitive and must be stored in the presence of antioxidant (usually 0.01 M mercaptoethanol). Arginine kinase immobilized in PAN gel retained 65% of its activity when stored at 5°C for 4 months.

TABLE 1

PREPARATION OF ARGININE PHOSPHATE FROM
ARGININE AND PHOSPHOENOL PYRUVATE
USING RECYCLED, GEL-IMMOBILIZED
ENZYMES[a]

Arginine (mol)	1.2
PEP (mol)	1.2
ArgP (mol,[b])	0.90, 75
Isolated ArgP (mol, %)[b,c]	0.80, 67
Reaction time (hr)	281
TN[d] (ATP)	64
TN[d,e] (ArgK)	3.3×10^6
TN[d,e] (PK)	0.8×10^6
Apparent activity (U)[f]	53

[a] The reaction mixture initially contained 550 U of ArgK and 500 U of PK immobilized in PAN gel. Before this reaction, these immobilized enzymes had been used in two other reactions conducted on the same scale.

[b] Yields are based on PEP as the limiting reagent.

[c] The isolated ArgP was 65% pure by enzymatic assay. Material of 95% purity can be obtained by further manipulation (see Experimental Procedures).

[d] TN = turnover number = moles of product formed/mole of cofactor or enzyme present.

[e] The *cumulative* TN for the enzymes for the three reactions was 7.3×10^6 for ArgK and 1.8×10^6 for PK.

[f] This term is the apparent average activity of the coupled enzyme system under the reaction conditions; that is, the average number of micromoles of ArgP produced/minute during that reaction.

measured under assay conditions. A difference of this magnitude is relatively common in synthesis using immobilized enzymes, and reflects both differences between the V_{max} conditions used in assays and the high-concentration conditions used in the reactors, and other types of nonidealities encountered in large-scale reactions (agglomeration of the gel particles or coating with precipitates with consequent mass transport limitations to rates, and loss of in magnesium ion from solution due to formation of insoluble magnesium phosphates).

EXPERIMENTAL PROCEDURES

General. All reagents and solvents were obtained commercially and were used without further purification. Biochemicals were obtained from Sigma. Phos-

phoenol pyruvate (*11*), acetyl phosphate (*10*), and PAN-1000 (*12*) were prepared using published procedures.

Analytical methods. Enzymes and substrates were assayed using procedures described in Bergmeyer (*14*); acetyl phosphate and acetate kinase (p. 425), pyruvate and phosphoenol pyruvate (p. 1446), and pyruvate kinase (p. 509). Arginine and arginine phosphate concentrations were determined using a colorimetric method (*15*). Arginine phosphate was determined using the coupled reactions of arginine kinase, hexokinase, and glucose phosphate dehydrogenase. Arginine kinase was assayed using the coupled reactions of pyruvate kinase and lactate dehydrogenase (*16*).

Immobilization of enzymes. Pyruvate kinase and acetate kinase were immobilized as described previously (*12*). Arginine kinase was immobilized in a similar way; a suspension of 25 mg (850 U) of commercial ArgK was separated from the ammonium sulfate-containing solution by centrifugation, and dissolved in 2 ml of deoxygenated Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] buffer (pH 7.5, containing 20 mM ADP, 20 mM MgCl$_2$, 30 mM Arg, and 100 mM NaNO$_3$). PAN-1000 (4 g) was rapidly (~1 min) dissolved in 16 ml of the same buffer solution. Dithiothreitol (DTT, 200 μl) was added to the PAN-containing solution, then the ArgK-containing solution, and finally, (after 5 s) 4 ml of a 0.5 M solution of triethylenetetramine (TET). The gel was allowed to form over 1 hr, then transferred to a Waring blender containing 200 ml of Hepes buffer (50 mM, pH 7.5, containing 50 mM ammonium sulfate, 10 mM MgCl$_2$, and 10 mM mercaptoethanol). After blending for 1 min at low speed and 20 sec at higher speed, the enzyme-containing gel suspension was separated by centrifugation, and the gel was washed several times, first with Hepes buffer (50 mM, pH 7.5, containing 10 mM MgCl$_2$ and 10 mM mercaptoethanol), and then with glycine buffer (100 mM, pH 8.6, containing 10 mM mercaptoethanol). The immobilization yield was 45%.

Arginine phosphate using phosphoenol pyruvate. A 5-liter flask was charged with PAN-immobilized ArgK (550 U) and PK (900 U) in 3000 ml of solution containing ATP (15 mM), MgCl$_2$ (40 mM), and 2 g of mercaptoethanol. Arg and PEP were added in portions during the course of the reaction, so that their concentrations remained above 0.05 M. The pH of the reaction mixture remained stable, once adjusted to 8.6. The reaction was stopped after 281 hr. (The total amount each of Arg and PEP added was 1.2 mol.) The enzyme-containing gel was separated from the reaction mixture by centrifugation, and returned to the reac-flask. After reaction, the solution obtained after removal of the gel by centrifugation was adjusted to pH 4 with Dowex 50W-X H$^+$ form (ca. 200 g), filtered to remove the resin, and treated with 1.5 vol of ethanol. The resulting crystalline white solid was isolated by filtration, washed with ethanol and diethyl ether, and dried under vacuum giving the free acid form of ArgP. This experiment is summarized in Table 1.

Purification of arginine phosphate. Crude arginine phosphate (25 g) was dissolved in 200 ml of water and the pH was adjusted to 8.5 with 6 N NaOH. To the resulting suspension was added 9.5 g (0.04 mol) of BaBr$_2$ · 2H$_2$O in 50 ml of water. After centrifugation, the solution was treated with Dowex 50W Na$^+$ form (40 ml) to remove Ba^{+2}. After filtration of the resin, the pH was adjusted to 3.5 with

Dowex 50W H$^+$ form (about 50 ml). The resin was removed by filtration and arginine phosphate was precipitated with ethanol. The precipitate was washed successively with 10 ml of absolute ethanol and 100 ml of diethyl ether, and dried for 12 hr under vacuum at room temperature, giving 12 g of arginine phosphate, 95% pure by enzymatic assay.

Arginine phosphate using acetyl phosphate. A 5-liter flask was charged with 1 liter of a solution containing arginine (0.05 M), ATP (8.3 mM), MgCl$_2$ (40 mM), DTT (1 g), and a suspension of PAN-immobilized ArgK (400 U) and AcK (850 U). The pH of the mixture was adjusted to 8.0 and maintained at this value by addition of NaOH solution (6.0 M) using an automatic pH controller. Arg (as a 2.5 M solution) and AcP (as a 0.88 M solution) were added continuously during the course of the reaction to maintain concentrations of the latter above K_m while avoiding excesses of this reagent (and thus minimizing hydrolysis). The solution was diluted to a final volume of 3 liter over the course of the reaction to prevent product inhibition of the enzymes. After 107 hr and the addition of a total of 0.42 mol of arginine and 0.66 mol of AcP, the concentration of ArgP was 0.07 M. The enzyme-containing gel was separated from the reaction mixture by centrifugation and a concentrated solution of BaBr$_2$ (0.66 mol) was added. The very abundant precipitate which formed (Ba$_3$(PO$_4$)$_2$) was discarded after centrifugation. The resulting solution was adjusted to pH 8.5 and 4 vol of 95% ethanol was added. The resulting solid precipitate was isolated by centrifugation. This precipitate still contained inorganic phosphates; a part remained insoluble on treatment with 800 ml of water. The water-soluble portion was filtered and reprecipitated by the addition of 9 vol of ethanol. The final solid (after lyophilization) consisted of 49.3 g of a white solid (0.13 mol, a 30.3% yield of ArgP based on starting Arg by enzymatic assay). For further purification, 10 g of this solid was dissolved in 100 ml of water at pH 8.5 and the solution was treated with 100 ml (an excess) of Dowex 50W (Na$^+$ form). The solution was adjusted to pH 4 with concentrated HCl and diluted with 9 vol of ethanol. The resulting precipitate was filtered, washed with ethanol and diethyl ether, and dried for 20 hr under vacuum. This procedure gave 6 g (0.023 mol) of ArgP as the free acid, 95% pure by colorimetric and enzymatic assay.

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