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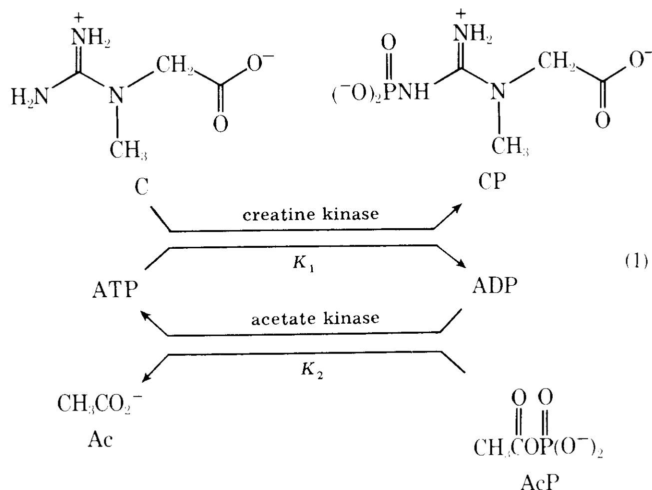
Large-Scale ATP-Requiring Enzymatic Phosphorylation of Creatine Can be Driven by Enzymatic ATP Regeneration[1]

Summary: Phosphorylation of creatine to creatine phosphate has been accomplished on a synthetically useful scale (0.16 mol) using creatine kinase (E.C.2.7.3.2), a catalytic quantity of ATP, and an ATP regeneration system based on acetate kinase (E.C.2.7.2.1) and acetyl phosphate.

Sir: We have previously used the hexokinase-catalyzed conversion of glucose to glucose 6-phosphate to illustrate the practicality of ATP regeneration in enzyme-catalyzed organic synthesis.[2] The equilibrium constant for phosphate transfer from ATP to glucose is large ($K \simeq 1.5 \times 10^2$ at pH 6.0),[3] and this reaction goes to completion. ATP is, however, only a moderately strong biological phosphorylating agent,[4,5] and many ATP-requiring enzymatic transformations of potential interest in organic synthesis have unfavorable equilibrium constants. Acetyl phosphate, the ultimate phosphorylating reagent in our ATP regeneration scheme, has a significantly greater thermodynamic potential for phosphorylation than ATP, and an important advantage of an ATP regeneration scheme based on acetyl phosphate is its ability to drive to useful conversion a reaction whose equilibrium constant is unfavorable based on the phosphate-donor potential of ATP alone.[4,6] Here we provide an example of a reaction of this type by the phosphorylation of creatine (C) to creatine phosphate (CP) on a practical scale (eq 1). The maximum value reported



for the equilibrium constant for phosphorylation of C to CP by ATP is $K_1 = 2.5 \times 10^{-1}$ (pH 9);[7] that for phosphorylation of ADP to ATP by AcP at this pH is $K_2 \simeq 1.5 \times 10^2$.[8] The equilibrium constant (eq 2) for the coupled equilibrium reactions (eq 1) was maximized empirically under conditions appropriate for large-scale synthesis by varying the pH, ionic strength, and composition of the solvent: $K = 140$ (pH 9, 10% v/v aqueous ethylene glycol solution).[9]

$$K = \frac{(\mathrm{CP})(\mathrm{Ac})}{(\mathrm{C})(\mathrm{AcP})} = \frac{(\mathrm{CP})(\mathrm{ADP})}{(\mathrm{C})(\mathrm{ATP})}\frac{(\mathrm{ATP})(\mathrm{Ac})}{(\mathrm{ADP})(\mathrm{AcP})} = K_1K_2 \quad (2)$$

Synthesis of CP was carried out in a 5-L round-bottomed flask equipped with a pH electrode, a magnetic stirring bar, and 6 g of glass beads to facilitate stirring the heterogeneous reaction mixture. The flask was charged with 3000 mL of 10% aqueous ethylene glycol solution (pH 9, no buffer)[9] containing creatine hydrate (100 g, 667 mmol, only partially soluble), ATP (5.0 mmol), $MgSO_4 \cdot 7H_2O$ (20 mmol), and dithiothreitol (5.0 mmol).[10] Polyacrylamide gel particles containing immobilized acetate kinase (AcK, E.C.2.7.2.1, 980 U, 4 mL of gel) and creatine kinase (CK, E.C.2.7.3.2, 312 U, 160 mL of gel) were suspended in the mixture.[11] Diammonium acetyl phosphate in 10% aqueous ethylene glycol solution (1 M, pH 9.0) was added continuously over 36 h at 25 mL h^{-1} to the stirred solution.[12] The solution was maintained between pH 8.8 and 9.2 by the addition of 5.0 N NaOH solution (10% aqueous ethylene glycol) using an automatic pH controller. The reaction was conducted at ambient temperature, and the reaction mixture and reagent solutions were deoxygenated before use and maintained under argon. After 36 h of operation (675 mmol of AcP added), enzymatic assay[13] indicated that the reaction was close to equilibrium. The concentration of CP was 56 mM. This quantity (234 mmol in 4200 mL) corresponds to a 63% yield based on dissolved C (56 g) and a 35% yield based on AcP.

The polyacrylamide gel particles and a white precipitate composed primarily of magnesium phosphate were allowed to settle, and the solution was decanted and centrifuged. Inorganic phosphate (666 mmol, estimated by the difference between the AcP and phosphate-containing impurities added and the CP produced) was partly precipitated by the addition of a stoichiometric amount of $MgSO_4 \cdot 7H_2O$ (666 mmol) at pH 9.2–9.3 and removed by centrifugation. The supernatant was adjusted to pH 7.6 with 5.0 N HCl solution and was treated with $BaBr_2$ (370 mL of 1.8 M solution) to precipitate the remaining inorganic phosphate. The mixture was allowed to stand for 20 min, the precipitate was separated by centrifugation, and the supernatant was treated with 234 mmol of $BaBr_2$ (130 mL of 1.8 M solution) and four volumes of absolute ethanol precooled to 0 °C. The mixture was stirred for 20 min and allowed to stand for 5 h at 4 °C. The supernatant was discarded and the white precipitate was washed twice with 800-mL portions of absolute ethanol (0 °C) and with 1000 mL of anhydrous ether (0 °C). The precipitate (74.4 g) was dried over Drierite for 12 h under vacuum: it contained 79% BaCP (159 mmol) by enzymatic assay.[13] This quantity corresponds to a 24% yield based on AcP. The activities of creatine kinase and acetate kinase were recovered in the gel in 79 and 71% yield, respectively.

The conversion of C to CP using ATP provides a severe test for enzymatic synthesis: it is endothermic; neither the product (CP) nor AcP has high hydrolytic stability;[14] the enzymatic reaction is inhibited by CP at low concentrations;[10] the specific activity of CK is only moderate. Nonetheless, these results establish that by careful adjustment of reaction conditions it is possible to use the high phosphate donor potential of AcP to drive the coupled enzymatic reactions (eq 1) to synthetically useful conversions. This coupled pair of reactions defines the least thermodynamically favorable scheme that can be used in practical synthesis with the AcP-based ATP regeneration sequence: if the net equilibrium constant for the CP synthesis and ATP regeneration reactions were smaller by a factor of 10, problems with recovery of low concentrations of products from large volumes of phosphate-containing solution would begin to be troublesome.

References and Notes

(1) Supported by the National Science Foundation (RANN), Grant GI 34284.
(2) A. Pollak, R. L. Baughn, and G. M. Whitesides, *J. Am. Chem. Soc.*, **99**, 2366 (1977).

(3) E. A. Robbins and P. D. Boyer, *J. Biol. Chem.*, **244,** 121 (1957).
(4) The free energy of hydrolysis of phosphate esters to phosphate is taken as a measure of their phosphorylating ability. Pertinent values are ($-\Delta G°$, pH 7, kcal/mol): phosphoenol pyruvate, 14.8; carbamylphosphate, 12.3; AcP, 10.3; CP, 10.3; pyrophosphate, 8.0; ATP, 7.3; glucose 6-phosphate, 3.3 (W. P. Jencks, p J181 in ref 5).
(5) G. D. Fasman, Ed., "Handbook of Biochemistry and Molecular Biology", Chemical Rubber Publishing Co., Cleveland, Ohio, 1976.
(6) The potential of several of the systems proposed for ATP regeneration in driving thermodynamically unfavorable equilibria is discussed by R. S. Langer, B. K. Hamilton, C. R. Gardner, M. C. Archer, and C. K. Colton, *AIChE J.*, **22,** 1079 (1976).
(7) S. A. Kurdy and E. A. Noltman in "The Enzymes", 3rd ed, Vol. VIII, P. Boyer, Ed., Academic Press, New York, N.Y., 1970, pp 412–431.
(8) R. S. Langer, C. R. Gardner, B. K. Hamilton, and C. K. Colton, *AIChE J.*, **23,** 1 (1977) and references cited therein.
(9) No correction was made for the influence of ethylene glycol on the measured pH: cf, P. Maurel, G. Hui Bon Hoa, and P. Douzou, *J. Biol. Chem.*, **250,** 1376 (1975).
(10) The limiting solubility of $C \cdot H_2O$ in water is ~13 g L^{-1} = 110 mM: R. M. C. Dawson et al., Ed., "Data for Biochemical Research", Oxford University Press, London 1969, p 16. The presence of an excess of suspended creatine in the mixture assured that the solution was saturated, and had no apparent ill effects on the reaction. The Michaelis constants for CK are (mM) = 0.4 (MgATP), 0.14 (MgADP), 110 (C), and 3.3 (CP).[7]
(11) Enzymes, obtained from Sigma and used without purification, had specific activities (μmol min^{-1} mg^{-1}): AcK 300 U (following treatment with DTT); CK 2.5 U (defined for C → CP, pH 9.0, 25 °C). Immobilization yields were 48% for CK, and 55% for AcK. Enzyme immobilization was carried out as described by A. Pollak, R. L. Baughn, O. Adalsteinsson, and G. M. Whitesides, *J. Am. Chem. Soc.*, in press.
(12) G. M. Whitesides, M. Siegel, and P. Garrett, *J. Org. Chem.*, **40,** 2516 (1975). The AcP used was 70–80% pure, with NH_4Ac, $(NH_4)_3PO_4$, and CH_3CONH_2 as the principal impurities. The solution was maintained at 0 °C before addition to minimize hydrolysis.
(13) H. U. Bergmeyer, Ed., "Methods of Enzymatic Analysis", 2nd ed, Academic Press, New York, N.Y., 1974, p 1777.
(14) Qualitative examination indicated hydrolysis rates of ~4% h^{-1} for AcP and 0.17% h^{-1} for CP under the conditions used for the enzymatic synthesis.

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