Large-Scale Enzymatic Synthesis with Cofactor Regeneration: Glucose 6-Phosphate

Sir:

Many important reactions in enzyme-catalyzed biosynthesis consume cofactors in stoichiometric quantities. The cost of the most commonly required cofactors has discouraged the use of these enzymatic reactions for the synthesis of organic compounds on any scale greater than a fraction of a mole. We have previously proposed a scheme for the enzymatic regeneration of ATP from ADP or AMP, and outlined its possible use in large-scale cofactor-requiring synthesis. Here we demonstrate the practicality of this scheme by the preparation of glucose 6-phosphate (G-6-P) from glucose on a mole scale.

A representative reaction was carried out in a 5-L flask modified to accept a pH electrode. The flask was charged with 1200 mL of solution (pH 6.6) containing glucose (1.4 mol), ATP (10 mmol), MgCl₂ (98 mmol), EDTA (4.8 mmol), and dithiothreitol (18 mmol). Polyacrylamide gel particles (20–50 μm in diameter) containing covalently immobilized hexokinase (ATP: D-hexose-6-phosphotransferase, E. C. 2.7.1.1, 1200 U.) and acetate kinase (ATP: acetate phosphotransferase, E. C. 2.7.2.1, 1100 U.) were suspended in this solution. Diammonium acetyl phosphate (AcP, 0.7 M) was added continuously over 48 h at 40 mL/h to the magnetically stirred reaction mixture. The solution was maintained between pH 6.6 and 6.9 by addition of 4 M potassium carbonate solution using an automatic pH controller. The reaction was conducted at 25 °C, and the reaction mixture and reagent solutions were deoxygenated before use and maintained under argon. After 50 h of operation (1.36 mol of AcP added), enzymatic assay indicated that 1.09 mol of G-6-P had been formed: its final concentration was 0.31 M. The polyacrylamide gel particles were allowed to settle, and the solution was decanted. Inorganic phosphate (0.27 mol, estimated by the difference between the AcP added and the G-6-P formed) was precipitated by addition of a stoichiometric quantity of Ba(OH)₂ and removed by filtration. G-6-P was then precipitated by addition of 1.2 mol of Ba(OH)₂: the resulting solid (502 g) contained 92% Ba G-6-P.H₂O (0.89 mol) by enzymatic assay. This quantity corresponds to a 65% yield based on AcP added. The activities of hexokinase and acetate kinase were recovered in the gel in 93 and 75% yield, respectively. The turnover number for ATP during the reaction was >100; no effort was made to recover it.

Three points concerning experimental details deserve mention. First, the initial quantities of ATP and Mg(II) were chosen such that the concentration of MgATP and MgADP would be well above the Michaelis constants for the soluble enzymes, even after dilution by the AcP solution. Second, the reaction proceeded satisfactorily with AcP having >80% purity. If the purity fell below 80%, complexation and precipitation of Mg(II) by the phosphate impurities made it difficult to maintain adequate concentrations of MgADP and MgATP in solution, and troublesome to isolate Ba G-6-P in high purity. Third, it was useful to carry out the reaction so that addition of AcP to the solution was overall rate-limiting and AcP was never present in the reaction mixture in high concentrations, to minimize spontaneous hydrolysis of AcP with concomitant release of phosphate.

Comparison of this preparation of G-6-P with existing chemical or enzymatic methods illustrates the potential of ATP-requiring enzymatic synthesis for the regioselective modification of unprotected, water-soluble, polyfunctional substrates. Since the hexokinases have broad substrate specificity, this sequence should be directly applicable to the preparation of phosphates of a number of other sugars (e.g., fructose, mannose, deoxy-D-glucose, glucosamine). In broader terms, this conversion establishes that it is practical to couple enzymatic ATP regeneration with ATP-requiring enzymatic synthesis to achieve large-scale organic transformations. Reactions which require regeneration of ATP from AMP are also accessible using this reaction sequence, by adding adenylate kinase (AMP:ATP phosphotransferase, E. C. 2.7.4.3) to catalyze the conversion of AMP and ATP to ADP; we will provide examples of this type of reaction sequence in the imme-
The good stability of the immobilized enzymes, and the ease of their recovery, suggests that these synthesis and regeneration schemes should have broad applicability in preparative organic chemistry.1 2

References and Notes

(1) Supported by the National Science Foundation (RANN), Grant No. GI 34284.
(2) Estimated costs, $/mole: ATP, 2,000; NAD+, 2,500; NADH, 18,000; NADP+, 60,000; NADPH, 18,000.
(6) Enzymes were immobilized by addition to a solution containing a polymerizing mixture of acrylamide, N,N'-methylenebisacrylamide, and the N-hydroxysuccinimide active ester of methacrylic acid, 10 s before gel formation. The procedure used is a modification of that described (G. M. Whitesides et al., Methods Enzymol., in press). Immobilization yields were 35% for hexokinase, and 40% for acetate kinase. The enzymes were commercial preparations (Sigma), and were used without purification: their specific activities (nmol min⁻¹ mg⁻¹) were: hexokinase (from yeast), 420; acetate kinase (from E. coli) following activation with dithiothreitol, 300.
(7) Diammonium acetyl phosphate was prepared in a separate step by reaction of ketene with anhydrous phosphoric acid, followed by neutralization and precipitation with anhydrous ammonia. G. M. Whitesides, M. Siegel, and P. Garrett, J. Org. Chem., 40, 2516 (1975). The material used was 80-85% pure, with ammonium acetate and acetamide as the principal impurities. The acetyl phosphate solution was maintained at 0 °C before addition to minimize hydrolysis.
(8) Preliminary experiments indicated that, under simulated reactor conditions, the rate of G-6-P formation was faster at pH 6.7 than at higher pH where the soluble enzymes would be expected to be more active (A. Sols, G. delaFuent, C. D. Villar-Palasi, and C. Ascensio, Biochem. Biophys. Acta, 30, 92 (1958); I. A. Rose, M. Grunberg-Manago, S. T. Korey, and S. Ochoa, J. Biol. Chem., 211, 737 (1954)).
(14) G. M. Whitesides, in ref 3, part 2, Chapter VII.

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