increase is compared with the increase obtained when a sample of the influent is treated in the same way. When the former is negligible and A_{340} is maximal, both indicating complete conversion, the effluent is acidified to pH 5 with 2 M HCl. The solution is evaporated in vacuo to a small volume, cooled (0°C), and filtered and the residue extensively washed with water and dried. Yields over 60% are possible.

Conclusion

The procedures described in this paper are simple and yield immobilized xanthine oxidase preparations which meet the desired requirements (Table II) to a large extent. The rather low operational stability is more than compensated for by the very low price when milk is used as starting material. All the procedures can in practice be conveniently used for oxidation of many compounds on a preparative scale and, as a result of the reaction specificity, products of high purity can be collected. Figure 5 illustrates the reaction specificity of enzymes. The occurrence of several sharp isosbestic points strongly indicates the absence of side reactions. Tramper\(^{11}\) provides an extensive overview of the work on xanthine oxidase and on the application of immobilized enzymes to syntheses in general.

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[25] Enzymatic Regeneration of Adenosine 5'-Triphosphate: Acetyl Phosphate, Phosphoenolpyruvate, Methoxy carbonyl Phosphate, Dihydroxyacetone Phosphate, 5-Phospho-α-d-ribo-syl Pyrophosphate, Uridine-5'-diphosphoglucose

By Debbie C. Crans, Romas J. Kazlauskas, Bernard L. Hirschbein, Chi-Huey Wong, Obsidiana Abril, and George M. Whitesides

ATP is a key cofactor in enzyme-catalyzed synthesis. It must be used in catalytic amounts and regenerated in situ in enzyme-catalyzed reactions in order to minimize expense and simplify isolation of products.\(^2\) Here we describe the best methods currently available for regeneration of ATP from ADP and AMP and detail several applications of these methods in enzyme-catalyzed syntheses. In particular, we give procedures for synthesis of three phosphate donors—acetyl phosphate,\(^3\) phosphoenolpyruvate,\(^4\) methoxy carbonyl phosphate\(^5\)and apply these reagents to syntheses of sn-glycerol 3-phosphate,\(^6\) dihydroxyacetone phosphate,\(^7\) glucose 6-phosphate, 5-phospho-α-d-ribo-syl pyrophosphate,\(^8\) and uridine-5'-diphosphoglucose.\(^9\)

Three procedures for the enzymatic regeneration of ATP are presently available which are useful in practical-scale organic synthesis (Fig. 1). One is based on acetyl phosphate (AcP) as the phosphorylating agent and acetate kinase as the catalyst; the second uses phosphoenolpyruvate (PEP) and pyruvate kinase; the third uses methoxy carbonyl phosphate [CH\(_3\)OC(O)OP\(_2\)O\(^-\)] and acetate kinase. The advantages and disadvantages of each method are summarized in Table I. Acetyl phosphate is

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1 Research was supported by National Institutes of Health (NIH) Grants GM 26543 and GM 30367. R. J. K. gratefully acknowledges support as a NIH postdoctoral fellow 1983-1984.


very easily prepared; it is a phosphoryl donor of intermediate strength; it is moderately stable in solution. Acetate kinase is subject to modest inhibition by acetate ion but this product inhibition is practically important only for reactions carried out in solutions containing acetate concentrations greater than 1 M. Phosphoenolpyruvate has excellent stability in solution, and is a very strong phosphoryl donor. Its synthesis is, however, more complex than that of AcP. Moreover, pyruvate kinase is subject to inhibition by pyruvate. To minimize the effects of this inhibition, the reaction must be carried out in dilute solution to keep the pyruvate concentration low, pyruvate must be removed from the reaction mixture as it is formed, or high concentrations of enzymes and PEP must be used. Methoxycarbonyl phosphate is comparable to PEP in its high phosphoryl donor strength, but resembles acetyl phosphate in its ease of synthesis. The product remaining after phosphoryl transfer from MCP to ADP—methyl carbonate—hydrolyzes rapidly in solution to methanol and carbon dioxide. This decomposition minimizes problems arising during isolation of products and resulting from product inhibition (bicarbonate, which can also inhibit acetate kinase, is easily removed by purging). The principal disadvantage of methoxycarbonyl phosphate is that it decomposes in solution inconveniently rapidly ($t_{1/2} = 20$ min, pH 7, 25°C) under conditions used for enzymatic synthesis.

Both pyruvate kinase and acetate kinase have high specific activity and show excellent stability in immobilized form. Pyruvate kinase is currently the less expensive enzyme. Further, it is effective for regeneration of ATP from ADP at lower concentrations of ADP than is acetate kinase since the Michaelis constant for pyruvate kinase $K_m$ (MgADP) = 0.1 mM is lower than that for acetate kinase $K_m$ (MgADP) = 0.4 mM. In practice, for most synthetic applications, either acetyl phosphate/acetate kinase or phosphoenolpyruvate/pyruvate kinase is used for regeneration of ATP. The former is preferable for large-scale work in which economy is important; the latter is used in instances in which the requirement for a strong phosphorylating reagent outweighs the relative inconvenience of the preparation of phosphoenolpyruvate, or in which a slow rate of enzyme-catalyzed reaction dictates the use of a hydrolytically stable phosphorylating agent. The syntheses of each phosphoryl donor

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**TABLE 1**

<table>
<thead>
<tr>
<th>Property</th>
<th>AcP</th>
<th>PEP</th>
<th>MCP</th>
</tr>
</thead>
<tbody>
<tr>
<td>O</td>
<td>CH$_3$COPO$_4$$^2$</td>
<td>CO$_2$$^\text{2-}$</td>
<td>CH$_3$COPO$_4$$^2$</td>
</tr>
<tr>
<td>Ease of preparation</td>
<td>+++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>$\Delta G_{\text{m}}^\circ$ (kcal/mol)</td>
<td>$-10.1$</td>
<td>$-12.8^*$</td>
<td>$-12.4$</td>
</tr>
<tr>
<td>Half-life for hydrolysis (hr)</td>
<td>21$^*$</td>
<td>$10^*$</td>
<td>0.3</td>
</tr>
<tr>
<td>pH 7.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0$^\circ$</td>
<td>960</td>
<td>$10^*$</td>
<td>15</td>
</tr>
<tr>
<td>pH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Product inhibition</td>
<td>Acetate</td>
<td>Pyruvate</td>
<td>HCO$_3$$^-$</td>
</tr>
<tr>
<td>$K_m$ (mM)</td>
<td>400, NC</td>
<td>10, C</td>
<td>500, NC</td>
</tr>
</tbody>
</table>

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$^*$ Other authors have used $-14.8$ kcal/mol [H. G. Wood, J. J. Davis, and J. Lochmuller, J. Biol. Chem. 241, 5692 (1966)]. This value, however, is based on a $\Delta G_{\text{m}}^\circ$ for ATP of $-9.1$ kcal/mol instead of $-7.3$ kcal/mol which was used to calculate the $\Delta G_{\text{m}}^\circ$ for acetyl phosphate and methoxycarbonyl phosphate. Measurement of the equilibrium constant for the ATP-mediated equilibrium:

acetate + PEP $\rightleftharpoons$ acetyl phosphate + pyruvate

showed that the phosphonolpyruvate donor potential of PEP is $-2.5$ kcal/mol greater than that for acetyl phosphate at pH 7.6. This value is consistent only with $-12.8$ kcal/mol as the value of $\Delta G_{\text{m}}^\circ$ of PEP.


$^*$ Other authors have used this value determined using activation parameters. Half-life for diaminophosphate calculated using activation parameters determined at 75°F from S. J. Benkovic and K. J. Schray, Biochemistry 7, 4090 (1968).

$^*$ Neutral: competitive; C: competitive.

$^*$ A. S. Mildvan and M. Cohn, J. Biol. Chem. 241, 1178 (1965).

(Fig. 2) are detailed below. Syntheses which produce AMP can also be coupled to these systems for ATP regeneration using adenylic kinase, a phosphotransferase which catalyzes formation of 2 ADP from ATP and AMP.\(^1\) The principles of ATP regeneration are also applicable to the regeneration of other nucleoside triphosphates since acetate kinase and pyruvate kinase accept other nucleoside phosphates as substrates.\(^4\)

Dihydroxyacetone phosphate and sn-glycerol 3-phosphate are synthesized on 1 M scales via phosphorylation catalyzed by glycerol kinase using acetyl phosphate as the ultimate phosphoryl donor. These represent the best currently available syntheses for these synths. Dihydroxyacetone phosphate is useful for sugar phosphate syntheses via aldolase-catalyzed reactions\(^5\); sn-glycerol 3-phosphate can be used for synthesis of enantiomerically pure phospholipids.\(^6\) Synthesis of 5-phospho-\(\alpha\)-D-ribose 5-phosphopentose (PRPP), an important intermediate in nucleoside and nucleotide biosynthesis,\(^7\) can be effected starting from either ribose or ribose 5-phosphate and using phosphoenolpyruvate as the ultimate phosphoryl donor.\(^8\) Ribose 5-phosphate is readily accessible from AMP via acid-catalyzed hydrolysis (Fig. 3). Both acetyl phosphate and phosphoenolpyruvate are used in the preparation of a mixture of nucleoside triphosphates from a hydrolysis of yeast RNA and the further conversion of the UTP in this mixture to uridine-5'-diphosphoglucone, an intermediate in oligosaccharide biosynthesis\(^9\) (Fig. 4).

Phosphoryl Group Donors

**Disodium Acetyl Phosphate.**\(^3\) Reaction of acetic anhydride with phosphoric acid in ethyl acetate, followed by extraction of the acetyl phosphate into water, extraction of acetic acid from the aqueous solution, and neutralization of the reaction mixture yields acetyl phosphate as an aqueous solution. This solution may be used for ATP regeneration without further manipulation. This procedure was designed to avoid the isolation of acetyl phosphate as the diammonium salt\(^1\) since ammonium ion causes the precipitation from solution of the magnesium ion required for activity of the kinase as \(\text{NH}_4\text{MgPO}_4\) during ATP regeneration. Several other procedures which yield aqueous solutions of acetyl phosphate have been described,\(^2,3\) but the procedure described here is generally the most convenient. Phosphoric acid (85%, 2.0 mol, 135 ml) is dissolved in 1.2 liters of ethyl acetate in a 2-liter flask. The solution is cooled to 0°C, and precooled


\(^{12}\) R. Radakrishnan, R. J. Robson, Y. Takagaki, and H. G. Khorana, this series, Vol. 72, p. 408.

(0) acetic anhydride (4.0 mol, 376 ml) is added over 40 min. The mixture is stirred for 6 hr at 0° and added to a suspension of 1 liter of water, 500 g of ice, and 168 g of sodium bicarbonate in a 5-liter flask. The resulting mixture is stirred at 0° until no more carbon dioxide evolves (approximately 30 min). The organic layer is separated and discarded. The resulting solution (pH ~3.0) is washed with one 1.8-liter portion and one 1.0-liter portion of ethyl acetate to remove most of the acetic acid. After neutralization of the aqueous solution of acetyl phosphate to pH 7 by addition of 10 M sodium hydroxide (~200 ml), approximately 40 ml of ethyl acetate separates as a second phase. The ethyl acetate layer is separated and discarded. Using this procedure, the concentration of acetyl phosphate in the final solution (1.68 liters) was 1.10 M as determined by enzymatic assay. The yield was 1.86 mol (93%). The acetyl concentration was 0.4 M as determined by 1H NMR spectroscopy.

Monopotassium Phosphoenolpyruvate. This procedure is an improvement over an earlier procedure of Clark and Kirby in that the hydrolysis of the dimethyl ester of PEP occurs more rapidly using the conditions described here and the isolation of K⁺PEP⁻ is faster and more convenient. Pyruvic acid (480 g of 95% pure material, 5.17 mol), 20 drops of concentrated H₂SO₄, and 450 ml of CH₂Cl₂ are added to a 3-liter, three-necked flask equipped with an overhead stirrer, an addition funnel, and a reflux condenser connected to a bubbler. Bromine (265 ml, 5.17 mol) is added dropwise over a 3.5-hr period to the stirred solution. A white precipitate forms when the addition of bromine is nearly complete. The suspension is stirred for one additional hour and diluted with 40 ml of cyclohexene and 200 ml of ligroin (bp 35–60°). The reaction mixture was cooled in an ice bath. The bromopyruvic acid is collected by filtration, washed with 300 ml of ligroin, and dried at 0.1 torr for 12 hr. Yield: 804 g [mp 64–67° (lit. 67°, 97% pure, 46.5 mol, 90% yield based on pyruvic acid)] . Bromopyruvic acid is converted to the dimethyl ester of PEP (2-hydroxyacryl acid dimethyl phosphate) in a 12-liter, three-necked flask equipped with a reflux condenser connected to a bubbler, an additional funnel, and a magnetic stirrer. A solution of 752 g (4.37 mol) of bromopyruvic acid (97% pure, used without further purification) in 1.25 liters of dry ether is added dropwise at a rate sufficient to maintain the ether at reflux (3.5 hr) to a stirred solution of 357 g (4.72 mol) of trimethyl phosphate in 3.85 liters of dry ether. The reaction mixture is stirred for 1 hr at ambient temperature, and the ether removed by rotary evaporation. Crude dimethyl PEP (1000 g) is obtained as a brown viscous oil. This oil is dissolved in 1.67 liters of water, and the solution is stirred at 20° for 15 hr. The spontaneous hydrolysis reaction proceeds to completion in this time and produces 2.64 mol (60% of PEP) and 0.2 mol (5%) of pyruvate (by enzymatic assay). The solution is cooled in an ice bath, and 267 g of solid KOH (85% pure, 4.0 mol) is added (to produce a solution with pH 2.8) followed by 2.7 liters of absolute ethanol. The white precipitate which forms is collected by filtration, washed with 800 ml of cold absolute ethanol, and dried at 0.1 torr, yielding 531 g of K⁺PEP⁻ (95% pure by enzymatic assay, 2.45 mol, 50% yield based on crude pyruvic acid).

The 31P NMR spectrum of K⁺PEP⁻ (0.5 M, D₂O) consisted of a single peak at -4.51 ppm (85% H₂PO₄, external reference; resonances downfield of H₂PO₄ are reported as positive). The 1H NMR spectrum of K⁺PEP⁻ in D₂O consisted of a multiplet at 5.88 ppm (1 H) and a multiplet at 5.54 ppm (1 H) downfield from an internal 2,2-dimethyl-2-silapentane-5-sulfonic acid reference.

The purity of bromopyruvic acid was determined by enzymatic assay using an assay for pyruvic acid and from its 1H NMR spectrum.

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**Enzymatic regeneration of ATP**

Typhimurium supplied by Switzer who has previously purified the enzyme to homogeneity.\(^{23}\)

Acetate kinase activity is measured in the direction of ATP synthesis by the reaction sequence:\(^{18}\)

\[
\text{Acetyl phosphate + ADP} \xrightarrow{\text{AcK}} \text{acetate + ATP} \\
\text{ATP + glucose} \xrightarrow{\text{HK}} \text{glucose-6-phosphate + ADP} \\
\text{Glucose 6-phosphate + NAD(P)}_0 \xrightarrow{\text{G-6-PDH}} \text{6-phosphogluconate acid + NAD(P)H}
\]

The assay solution (total volume 3.0 ml) contains: Tris buffer (0.15 M, pH 7.6), glucose (5 mM), ADP (10 mM), acetyl phosphate (5 mM), NAD(P)\(_0\) (0.6 mM), hexokinase (HK, 9 U), glucose-6-phosphate dehydrogenase (G-6-PDH, 9 U) and acetate kinase (AcK, -0.03 U). The formation of NAD(P)H is monitored spectrophotometrically at 340 nm \((e = 6220 \text{ M}^{-1} \text{ cm}^{-1})\) as a function of time at 25°. Acetyl phosphate and methoxycarboxylic phosphatase concentrations are measured using the same assay system by using acetate kinase (9 U), omitting acetyl phosphate but instead adding an aliquot of the solution containing acetyl phosphate. Glucose 6-phosphate (G-6-P) is measured in the same assay system using NAD(P)\(_0\) (0.6 mM) and glucose-6-phosphate dehydrogenase (9 U) and then adding an aliquot of the solution containing glucose 6-phosphate.

**PRPP Synthetase.** A modification of the Ferrari method\(^{24}\) is employed:

\[
\text{ATP + ribose 5-phosphate} \xrightarrow{\text{PRPP synthetase}} \text{PRPP + AMP} \\
\text{AMP + ATP} \xrightarrow{\text{AdK}} 2 \text{ADP} \\
2 \text{ADP + 2 PEP} \xrightarrow{\text{2 ATP + 2 pyruvate}} \text{2 pyruvate + 2 NADH} \xrightarrow{\text{LDH}} 2 \text{ lactate + 2 NAD}
\]

The assay mixture (total volume 1.0 ml) contains triethanolamine buffer (100 mM, pH 7.6), K\(_2\)HPO\(_4\) (100 mM), ribose 5-phosphate (5-P, 5 mM), ATP (3 mM), PEP (0.8 mM), MgSO\(_4\) (10 mM), KCl (142 mM), NADH (0.2 mM), lactate dehydrogenase (LDH, 2 U), pyruvate kinase (PK, 2 U) and adenylate kinase (AdK, 2 U).

**PRPP.** The determination of PRPP concentrations is based on the decrease in optical density at 295 nm upon the conversion of 5-fluororibosyl-5-fluorouracil.\(^{25}\)

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\(^{23}\) R. L. Switzer and K. J. Gibson, this series, Vol. 51, p. 3.


\(^{25}\) J. G. Flaks, this series, Vol. 6, p. 473.
The assay solution (final volume 1 ml) contains Tris–HCl buffer, pH 8.5 (20 mM), MgCl\(_2\) (2 mM), 5-fluoroorotic acid (0.2 mM), orotidine-5-P-pprophosphorylase (0.5 U), and orotidine-5-P-decarboxylase (0.5 U).

Nuclease \(P_1\): The phosphodiesterase activity of nuclease \(P_1\) using yeast RNA (Boehringer-Mannheim) as substrate is used to assay activity\(^{26}\) (one unit of activity liberates 1 μmol of acid soluble nucleotide from RNA in 1 min at 37°C). The procedure involves incubation of nuclease \(P_1\) with yeast RNA, separation of the mononucleotide products from polynucleotides by precipitation of these polynucleotides with uranyl acetate, and measurement of the mononucleotide concentration in the supernatant spectrophotometrically at 260 nm.

A 1.10-ml aliquot of a dilute solution of nuclease \(P_1\) (containing ~5 U) initiates the reaction in a mixture containing 0.18 ml of 30 mM sodium acetate buffer, pH 5.33; 0.20 ml of a solution of yeast RNA (5 mg/ml) in the acetate buffer; and 20 μl of 10 mM ZnSO\(_4\). Distilled water (0.10 ml) instead of enzyme is added to the blank reaction sample. The test tubes are incubated for 15 min at 37°C. The reaction is quenched by the addition of 1.0 ml of a solution containing 0.25% (w/v) uranyl acetate in 10% (v/v) perchloric acid. The tube is rapidly vortexed, then placed in an ice-water bath for 20 min before sedimenting (5000 g) the undigested RNA. The supernatant is diluted by a factor of 10 and the absorbance of the sample is measured against the blank at 260 nm. An average extinction coefficient for nucleoside monophosphates at 260 nm of 10,600 M\(^{-1}\) cm\(^{-1}\) is used for calculations.

Polynucleotide Phosphorylase (PNPase). Activity is measured by monitoring the disappearance of NADH at 340 nm due to the following reactions:\(^{27}\)

\[
\text{Poly(A)} + \text{P, PNPase} \rightarrow \text{ADP} \\
\text{ADP} + \text{PEP} \rightarrow \text{ATP} + \text{pyruvate} \\
\text{Pyruvate} + \text{NADH} \rightarrow \text{1-lactate} + \text{NADH}
\]

To assay the PAN-immobilized PNPase, poly(A) is treated with nuclease \(P_1\) before the addition of PNPase. To a 3-ml plastic cuvette containing 1.5

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\(^{27}\) S. Ochoa and S. Mi, J. Biol. Chem. 236, 3303 (1961).
trodre. The pH of the reaction mixtures was controlled with a Weston Model 7561 pH controller which regulated the addition of acid or base using an I.K.B 10200 peristaltic pump. Prior to the addition of the immobilized enzymes, all solutions were deoxygenated by purging with argon using a gas dispersion tube. The enzymatic reactions were conducted at room temperature under an argon atmosphere unless otherwise specified.

Immobilization of Enzymes

Enzymes. Enzymes obtained as suspensions in ammonium sulfate solutions were dialyzed twice against 150 volumes of 50 mM HEPES buffer (pH 7.5) at 4°C before use; small quantities of ammonium ion left in solution compete with the α,ω-diamine (TET) for active ester groups on the PAN.

N-Acryloyoxyxuccinimide. N-Hydroxysuccinimide (115 g, 1.0 mol) and triethylamine (110 g) were dissolved in 1500 ml of chloroform at 0°C. Acryloyl chloride (Aldrich, 100 g, 1.1 mol) was added dropwise over a 20-min period to the stirred reaction mixture. After being stirred an additional 20 min at 0°C, the solution was washed with 800-ml portions of ice-cold water and saturated brine, dried with MgSO4, and filtered; 50 mg of 2,6-di-tert-butyl-4-methylphenol (polymerization inhibitor) was added to the chloroform solution which was concentrated to a volume of 300 ml in vacuo using a rotary evaporator and filtered. Ethyl acetate (30 ml) and 200 ml of n-hexane were added slowly with stirring to the chloroform solution which was left to stand at 0°C for several hours. The precipitated, colorless crystals were separated by filtration and washed with an ice-cold 100-ml portion of a mixture of n-hexane and ethyl acetate (4:1), then with another 100-ml portion of n-hexane and ethyl acetate (9:1), and finally with two 100-ml portions of n-hexane. The crystals were dried in vacuo at ambient temperature to constant weight; 118 g (70%) was obtained at this stage. mp 69.5–71.0°C (lit. mp 67°C). This material is pure enough for the PAN preparation. A slightly purer product (mp 70.5–71.5°C) could be contained by the recrystallization from a mixture of n-hexane and ethyl acetate. The product has the expected spectral characteristics: NMR (CDCl3) 2.85 (s, 4 H), 6.0–7.0 (mult, 3 H); IR (Nujol mull) 1800, 1775, 1735, 1260, 995, 870 cm⁻¹.

Poly(acrylamide-co-N-acryloyoxyxuccinimide)(PAN). A 5000-ml, round-bottomed flask, equipped with a Teflon-coated magnetic stirring bar (0.5 × 2.0 in.) and a reflux condenser, was charged with acrylamide (275 g, 3.85 mol), N-acryloxyxuccinimide (30 g, 178 mmol), AIBN (1.75 g, 11 mmol), and 2500 ml of THF (AR grade, distilled from CaH2). The reflux condenser was capped with a serum stopper and the flask degassed with nitrogen for 30 min with vigorous stirring to remove dioxygen. The flask was maintained in a constant-temperature water bath at 50°C under slight positive pressure of nitrogen for 24 hr. After 24 hr, 1000 ml of THF was added to the flask and the contents were stirred for 10 min. The precipitated white polymer was washed on the funnel four times with 1000-ml aliquots of dry THF transferred to a vacuum desiccator, and dried under vacuum (0.02 torr) for 24 hr at room temperature; 325 g (106%) of a white, very fluffy produce was obtained. An assay of this polymer showed that it contained 215 μmol of active ester groups per gram: IR (Nujol mull) 3340, 3200, 1730, 1660, 1210, 1070 cm⁻¹.

Assay for the Active Ester Content of PAN. PAN (~50 mg, ~50 mol of active ester groups, dried under vacuum at 0.01 torr and 45°C for 24 hr) was dissolved and made up to volume in distilled water in a 5-ml volumetric flask. A 50-μl aliquot of this solution was added into a 5-ml quartz cuvette containing 3000 μl of HEPES buffer (0.1 M, pH 7.5), 50 liters of 1 M ethylamine solution, and 10 μl of a 1 M solution of mercaptoethanol; the rate of the appearance of N-hydroxysuccinimide was followed spectrophotometrically at 259 nm at 25°C; after the reaction was completed (~90 min) and the increase of the absorbance leveled off, the active ester concentration was calculated. When required, the total concentration of neutral (NHSH) and deprotonated N-hydroxysuccinimide (NHS⁻) was estimated from the observed concentration of the anion using Eq. (1). At pH 7.5, for pKw[HHS⁻] = 6.0, this correction is approximately 3% ([NHS⁻]/[NHSH] + [NHS⁻]) = 0.969, and was ignored.

Procedure for Immobilization of Enzymes. PAN (3.0 g, 450 μmol/g, 1350 μmol of active ester groups) was placed in a 50-ml beaker and 12.0 ml of 0.3 M HEPES buffer (pH 7.5, containing the appropriate substrates of this enzyme to be immobilized; see below) was added. The polymer was quickly (within 1 min) dissolved by mechanical grinding with a glass rod against the glass wall of the beaker and a 1-in. magnetic stirring bar was dropped into the polymer solution. The solution was stirred magnetically for 30 sec, and 150 μl of 0.5 M dithiothreitol solution and 1.275 ml of triethylene tetraine (1275 μmol of primary amino groups) were added with vigorous stirring; 30 sec later, 1000 μl of a solution of the enzyme was added. In less than 2 min, the solution set to a transparent, resilient gel. The gel was allowed to stand for 1 hr at ambient temperature and then was transferred to a blender containing 185 ml of HEPES buffer (50 mM).
pH 7.5, containing 50 mM ammonium sulfate). Blending at low speed for 3 min followed by 30 sec at high speed reduced the gel to a suspension of particles having ~100 μm diameter. The suspension was separated by centrifugation and the gel washed twice with buffer containing no ammonium sulfate. The final suspension was diluted to 150 ml using the same HEPEs buffer. Immobilizations were carried out in the presence of substrates or products intended to occupy the active site and protect it against modification during immobilization. The concentration of substrates used to protect the enzyme active site during immobilization and the immobilization yields were glycero! (3-mercaptoethanol (17 mmol) was adjusted to pH 7.0 and deoxygenated. A suspension of immobilized glycero! (600 U) and acetate kinase (800 U) was added and stirred magnetically at ambient temperatures under argon. Disodium acety! phosphate (1.1 mol in 1.2 liters of solution) kept at 0 °C was added via a peristaltic pump over 5 days. The pH was kept at 7.0 (±0.5) using a pH controller and the automatic addition of 4 M sodium hydroxide solution. The reactor was kept for 2 days after the end of acetyl phosphate addition at which time enzymatic assay showed 97% conversion of glycero! to sn-glycero!-3-phosphate. The solution was separated from the enzyme-containing gel by decantation followed by centrifugation. The enzymes were washed with 200 ml of deoxygenated water and centrifuged, and the supernatant added to the main reaction fraction. The solution was passed through charcoal (~50 g) to remove ATP, and concentrated in vacuo to 0.5-1 liters. A saturated solution of barium chloride (0.2 mol) was added, and the precipitate, consisting primarily of barium phosphate, was separated by filtration. Additional barium chloride (1 mol) and ethanol (3 liters) was added to precipitate the barium salt of sn-glycero!-3-phosphate. The salt was allowed to precipitate and settle for 2 days to facilitate filtration. After drying in vacuo, a total of 0.92 mol (92%) of sn-glycero!-3-phosphate was obtained (314 g of solid containing 90% barium sn-glycero!-3-phosphate as determined by enzymatic assay). The turnover number for ATP during the synthesis was 115, and the activities of enzymes recovered in the gel were GK 95%; AcK 83%.

Dihydroxyacetone Phosphate. The enzyme reaction was repeated for three consecutive runs each of 0.32 mol generating a total of 0.95 mol of DHAP. To a I-liter deoxygenated solution containing dihydroxyacetone (36.0 g, 0.4 mol), ATP (2.20 g, 4 mmol), MgCl₂·6H₂O (1.63 g, 8 mmol), and 2-mercaptoethanol (4 mmol) was added PAN-immobilized glycero! kinase (1500 U, determined with glycero! as substrate) and PAN-immobilized acetate kinase (1700 U). To this mixture a deoxygenated solution of acetyl phosphate (0.45 mol, 410 ml) was added using a peristaltic pump over 16 hr. The mixture was stirred at room temperature under argon, and the pH was automatically controlled at 6.7-7.0 by addition of aqueous NaOH (4 M) through a peristaltic pump. The reaction was stopped when enzymatic assay indicated 98% conversion (16 hr). After separation of the reaction solution from the enzyme-containing gel, the solution was filtered through charcoal (~25 g). The solution was adjusted to pH 4.0 and stored at 4°C; the concentration of DHAP retained 90% of its original value after 1 month.

A solution of 0.4 mol of dihydroxyacetone phosphate (1.6 liters, 0.25 M) was prepared as above using the recovered enzymes. Quantitative 31P NMR was used to determine inorganic phosphate content and a solution of barium chloride (14.6 g, 60 mmol) was added to precipitate it at pH 6. After the precipitate was removed by filtration, the dihydroxyacetone phosphate was precipitated by addition of barium chloride (0.4 mol, 97.6 g) and three volumes of ethanol. Drying in vacuo resulted in 116 g (0.33 mol, 87% pure calculated as Ba·DHAP by enzymatic assay, overall yield 82.5%).

A solution of 0.4 mol of dihydroxyacetone phosphate was prepared as above (1.6 liters, 0.25 M) using the recovered enzymes. After removal of the inorganic phosphate by precipitation as barium phosphate at pH 6.0, and reducing the pH to 3.0 with Dowex 50-X8 (hydrogen form), the solution was passed through charcoal (~25 g). Acetic acid was removed by extraction with two 1-liter portions of ethyl acetate, and the pH was adjusted to 4.8 by addition of NaOH (2 M). The solution was concentrated in vacuo to ~500 ml and lyophilized. The isolated monosodium dihydroxyacetone phosphate (119.0 g) was 82% pure by enzymatic assay (0.32 mol, 119 g, 79.7% overall yield). The turnover number for ATP was 83 and the recovered activities for the enzymes after three runs were GK 62%, AcK 30%.

enzymatic regeneration of ATP

The reaction was carried out at pH 8.0 in a mixture of 0.1 M NaCl and 0.1 M Tris-HCl buffer (pH 8.0). The reaction mixture contained ATP (1.0 mM), MgCl₂ (1.0 mM), and ATP synthase (2.0 mg/mL). The reaction was started by adding ATP to the mixture and incubating at 37°C for 20 minutes. The reaction was stopped by adding 1 M HCl to the mixture, and the reaction products were separated by thin-layer chromatography.
cally. The fractions with elution volumes 90-130 ml contained ATP, GTP and CTP, and were combined and lyophilized (0.6 g). This quantity is equivalent to a yield of dipotassium UDP-Glc of 92% based on glucose 6-phosphate.

Acknowledgments

Our work with PRPP synthetase depended on generous help and advice from Professor R. L. Switzer and co-workers, Drs. Akiva Gross, Jerome Lewis, and Alfred Pollak contributed significantly to the development of various of the procedures described.

[26] Equilibrium and Kinetically Controlled Synthesis with Enzymes: Semisynthesis of Penicillins and Peptides

By Volker Kasche, Ursula Hauffler, and Lutz Riechmann

The increased availability of hydrolases and their potential biotechnical application as catalysts in the synthesis of condensation products have led to an increased interest in studying these enzymes. Some products that have been synthesized using hydrolases as bio catalysts are given in Table 1.

The enzyme-catalyzed synthesis of the condensation product AN can be carried out either as an equilibrium-controlled process:

\[
\text{AOH} + \text{NH} \rightleftharpoons \text{AN} + \text{H}_2\text{O}
\]  

(1)

where the enzyme only accelerates the rate with which the equilibrium is obtained, or as a kinetically controlled process:

\[
\text{AB} + \text{NH} \rightleftharpoons \text{AN} + \text{HB} \rightleftharpoons \text{AOH} + \text{NH} + \text{HB}
\]  

(2)

where an activated substrate AB (ester or amide) is used. In the latter process the enzyme acts as a transerase transferring the group A from AB to a nucleophile NH. Generally the biosynthesis of condensation products is kinetically controlled [Eq. (2)].

Whether this kinetically controlled process can compete with the equilibrium-controlled process in the biotechnological synthesis of condensation products must be answered by studies on the mechanism and the yield-controlling factors. Some of these factors will be analyzed here in connection with the enzyme-catalyzed semisynthesis of β-lactam antibiotics and peptides.

Mechanism

Two different mechanisms have been proposed for hydrolase-catalyzed condensation reactions. One (Fig. 1A) involves no specific nucleophile binding to the reactive enzyme-substrate (acyl-enzyme or noncovalent complex) intermediate. This mechanism is most frequently used to explain condensation reactions catalyzed by proteases.

