ENZYMES

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ORGANIC SYNTHESIS USING COFACTOR-REQUIRING ENZYMES

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Many of the present large-volume applications of enzymes in chemical and food processing are based on enzyme-catalyzed hydrolysis. Synthetic processes -- i.e., processes involving the preparation of more complex products by transformations on simpler starting materials -- are seldom used, for several reasons. First, these processes usually require cofactors, especially ATP and the nicotinamide-derived cofactors NAD, NADH, NADP, and NADPH, and these materials are expensive (from \$2,500/mole to \$250,000/mole). Second, the enzymes required are usually relatively expensive. Third, these synthetic enzymes may be relatively unstable. Our research has been concerned, in part, with finding practical solutions to these problems.

Our work with cofactors has centered on the development of practical methods for regenerating ATP. The regeneration procedure developed is outlined in Scheme I, which follows.

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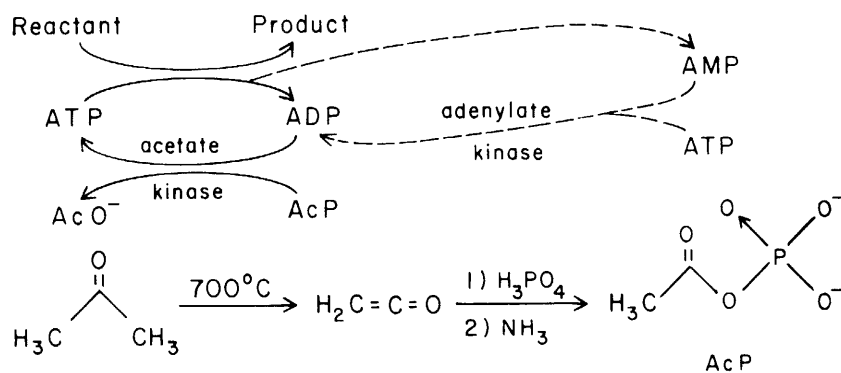
Scheme I   ATP regeneration coupled with ATP-requiring organic synthesis.

ATP can be generated from ADP by phosphorylation by acetyl phosphate (ACP) catalyzed by acetate kinase. If the synthetic route produces AMP rather than ADP, the latter can be generated from the former by a reaction with ATP catalyzed by adenylate kinase. The acetyl phosphate required as the ultimate phosphorylating agent in either event is easily prepared by acylation of phosphoric acid with ketene (itself prepared by thermal cracking of acetone).

Acetyl phosphate was chosen as the phosphate donor in this scheme for several reasons: it is easily prepared; it is thermodynamically a good phosphate donor; it is acceptably stable toward hydrolysis in solution; and it is utilized by acetate kinase, and readily available and reasonably stable enzyme. The only practical alternatives to acetyl phosphate as phosphate donors are carbamyl phosphate and pyrophosphate. The former has very poor solution stability; the latter is a poor phosphate donor and requires enzymes that are not readily available.

The major problem encountered in stabilizing adenylate kinase and acetate kinase has been that of protecting the enzyme against autoxidation (see Scheme II).
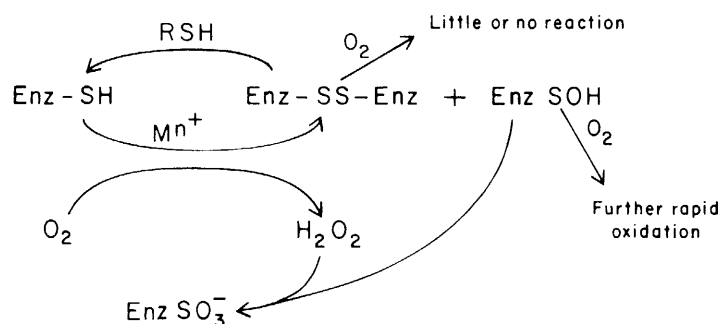
Scheme II   Important oxidation pathways for cysteine-containing enzyme oxidation.

The initial reaction in this scheme conversion of a cysteine SH group to a disulfide or sulfinic acid, is strongly transition metal catalyzed. This reaction is easily reversed by reduction of the initially formed product with excess thiol (2-mercaptoethanol, dithiothreitol). The initial oxidation is, however, often accompanied by the generation of hydrogen peroxide. Hydrogen peroxide is, itself, reactive toward disulfide groups, and the products of this reaction in general cannot be reversed to thiols. Thus, it is essential in maintaining high enzymatic activity insofar as possible, to prevent the initial oxidation of the enzyme by excluding O_2, by adding transition metal chelating agents to the solution, and by running the reactions in the presence of added thiols. So long as they are protected against autoxidation, both acetate kinase and adenylate kinase show excellent stability in solution and in immobilized form.

The procedure we use for enzyme immobilization is outlined in Scheme III.

The essential reactant in this scheme is a water-soluble copolymer of acrylamide and N-acryloxysuccinimide, poly(acrylamide-co-N-acryloxysuccinimide), PAN. Reaction of this polymer with a mixture of an alpha, omega-diamine (typically triethylenetetramine) and enzyme results in simul-
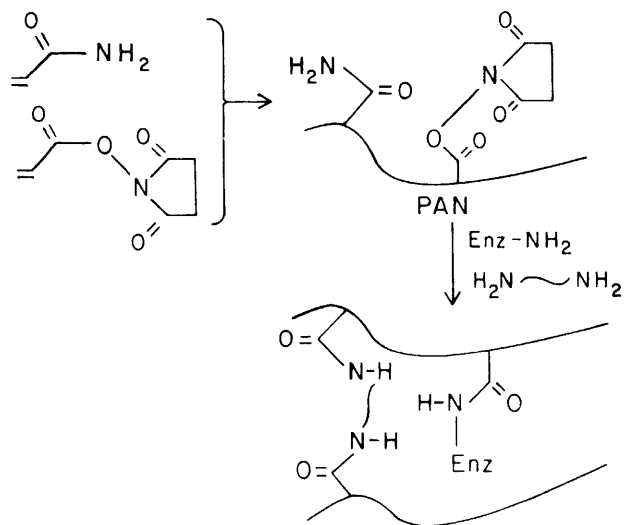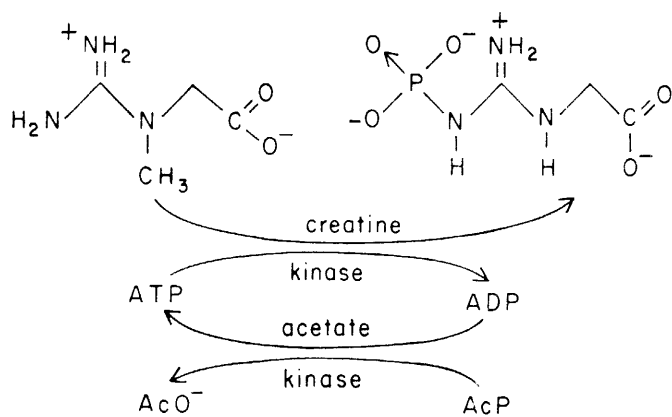
Scheme III   Enzyme immobilization by copolymerization
with reactive, soluble polymers.

taneous cross-linking of the polymer as an insoluble gel and
covalent incorporation of the enzyme into this gel. The
reaction is easily and rapidly carried out (10 minutes at room
temperature) and results in excellent yields of immobilized
enzyme. Although the physical characteristics of the enzyme-
containing gel are not ideal (the gel is mechanically soft), the
gel can be formed, if desired, on a number of support struc-
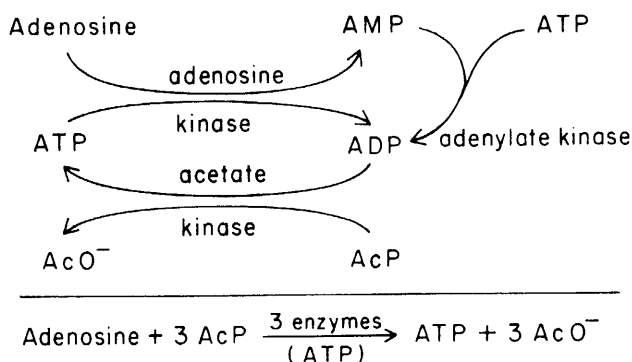tures, or mixed with diatomaceous earths after formation.

Using enzymes immobilized by this procedure, we have
carried out several enzyme-catalyzed syntheses designed to
illustrate the practicality of our ATP regeneration scheme.
(Schemes IV-VI).
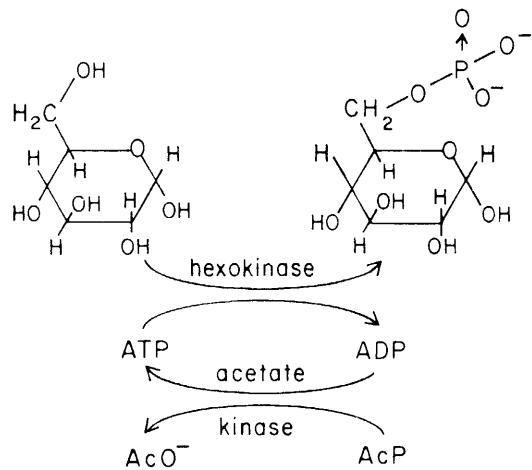
Scheme IV   Conversion of D-glucose to D-glucose-6-phosphate.

Scheme V   Conversion of creatine to creatine phosphate.

Adenosine   AMP   ATP
        adenosine
        kinase
ATP   ADP — adenylate kinase
        acetate
        kinase
AcO⁻   AcP

$$\text{Adenosine} + 3\,\text{AcP} \xrightarrow[\text{(ATP)}]{\text{3 enzymes}} \text{ATP} + 3\,\text{AcO}^-$$

Scheme VI   Conversion of adenosine to ATP.

The conversion of glucose to glucose-6-phosphate
illustrates the selective functionalization of a polyfunctional
water-soluble molecule, and is a representative of a type of
transformation which cannot be easily accomplished by conven-
tional chemical methods. The production of creatine phosphate
by reaction with ATP is an example of an endothermic reaction
which can be driven to useful conversion by coupling with the
exothermic phosphorylation of ADP by acetyl phosphate. The
phosphorylation of adenosine to ATP provides a demonstration
of a coupled, three-enzyme, reaction sequence.

The success of these reactions (all have been run on
scales greater than 100 g of product per reaction cycle) estab-
lishes that the problem of ATP regeneration is solved from a
practical point of view, at least so far as fine chemicals
synthesis is concerned.

Among the problems in extending this start to more
complex types of enzyme-catalyzed synthesis are those of
developing recovery systems which must be used in homoge-
neous solution, and broadening the selectivity and types of
reactions catalyzed by enzymes.

We have developed the use of ''generalized affinity
chromatography'' as a possible solution to the first of these
problems. (Scheme VII). Covalent attachment of arylsulfo-
namide moieties to an enzyme of interest results in an enzyme-
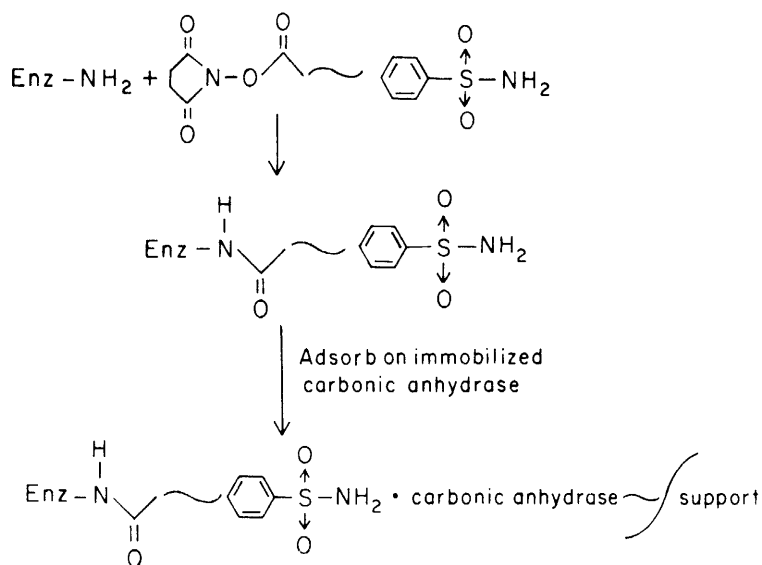
Scheme VII   Modification of enzymes for use in generalized affinity chromatography.

sulfonamide conjugate. This conjugate retains much of the enzymatic activity of the native enzyme, but has the advantage that it can be rapidly recovered from homogeneous solution following use by biospecific adsorption on a column of immobilized carbonic anhydrase (sulfonamides are inhibitors for carbonic anhydrase, with values of $\underline{K}_i \sim 10^{-6}$ to 10^{-7} $\underline{M}$).

As an approach to changing the specificity of enzymes, we are exploring the use of "semi-synthetic enzymes", i.e., proteins which have been modified chemically so that they catalyze reactions which the native proteins will not effect. An example of this approach is outlined in Scheme VIII. Covalent attachment of biotin to a chelating diphosphinerhodium (I) moiety is easily accomplished by conventional chemistry. Exposure of this biotin ∿ rhodium adduct to the biotin-binding protein avidin results in site-specific incorporation of this diphosphinerhodium (I) group into the protein. The resulting protein-rhodium conjugate is a homogeneous hydrogenation catalyst, which shows activity (per rhodium atom) in water
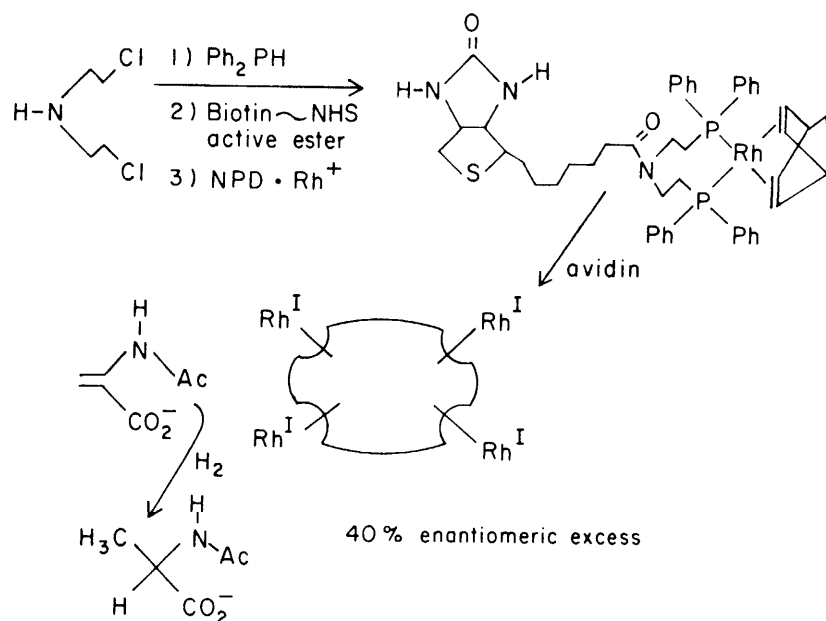
Scheme VIII   Preparation of a semi-synthetic enzyme with asymmetric hydrogenation activity.

comparable to that observed by homogeneous rhodium catalysts in organic solvents. More importantly, this hybrid protein-transition metal catalyst shows modest enantioselectivity in reduction of amino acid precursors. Control experiments have established that this enantioselectivity reflects chirality intrinsic to the protein biotin binding site. The combination of enzymatic selectivity with catalytic activity normally not associated with enzymes offers interesting prospects for new types of catalysts.