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ENZYMATIC REGENERATION OF ATP FROM AMP AND ADP

PART II : ENZYME IMMOBILIZATION AND REACTOR DEVELOPMENT

G.M. Whitesides, A. Chmurny, P. Garrett,
A. Lamotte and C.K. Colton

Departments of Chemistry and Chemical Engineering
Massachusetts Institute of Technology
Cambridge, Massachusetts

The biosynthesis of many natural products consumes ATP (1). The involvement of ATP in biosynthetic reactions can be illustrated by two well established pathways for the activation of an alkyl carboxylate ion (Fig. 1). In one, transfer of the terminal phosphate

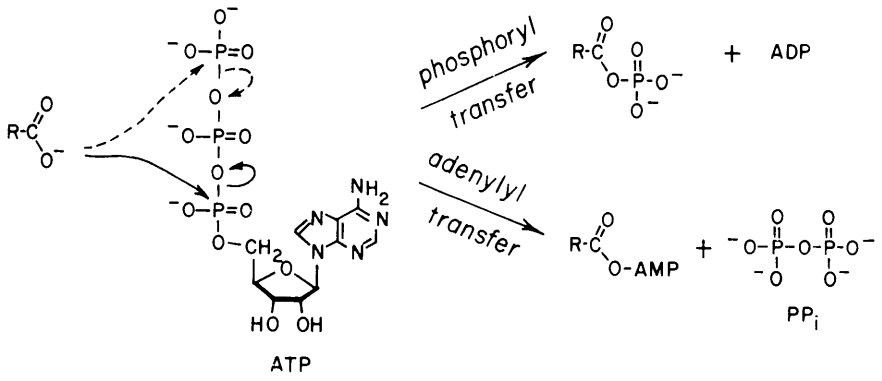


Fig. 1: Pathways for Activation of Alkyl Carboxylate

group of ATP to the carboxylate grouping (phosphoryl transfer) results in the production of an acyl phosphate and ADP. In a second pathway, nucleophilic attack of carboxylate ion on the α-phosphate grouping of ATP (adenylyl transfer) generates an acyl adenylate and inorganic pyrophosphate ion. These two types of acyl derivatives are both active esters and can take part in further reactions at the carbonyl group.

As part of a project to test the practicality of large scale

synthesis of the cyclic decapeptide antibiotic Gramicidin S using cell-free enzymes (2), we have explored methods for the economical regeneration of ATP from AMP or ADP. We have settled on a coupled enzyme system consisting of adenylate kinase and acetate kinase (Fig. 2). Acetyl phosphate is chosen as the ultimate phosphate

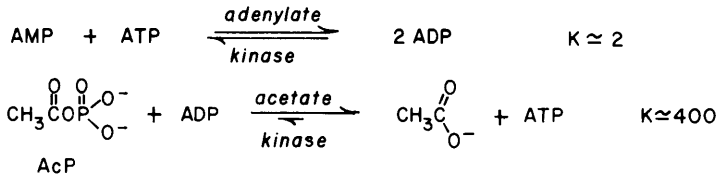


Fig. 2: Coupled System for ATP Regeneration

source. Reaction of acetyl phosphate with ADP, catalyzed by acetate kinase, generates acetate ion and ATP (3,4). ATP readily disproportionates with AMP in the presence of adenylate kinase to generate two molecules of ADP (5-7). Thus, reaction of AMP with acetyl phosphate in the presence of small amounts of ATP and adenylate kinase and acetate kinase results in consumption of acetyl phosphate and the generation of acetate ion and ATP.

In searching for a synthesis of acetyl phosphate that is both inexpensive and amenable to process development on an industrial scale, we have settled on the acylation of phosphoric acid with ketene (8). Ketene is readily generated on a large scale by the

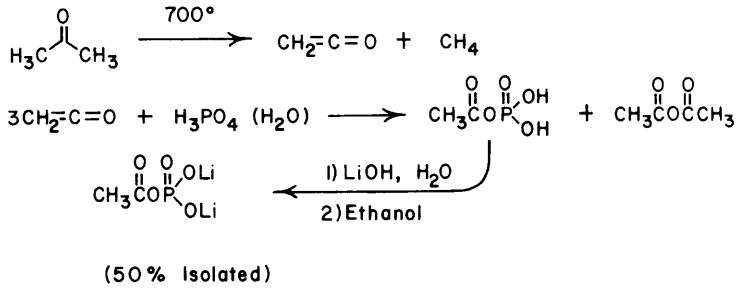


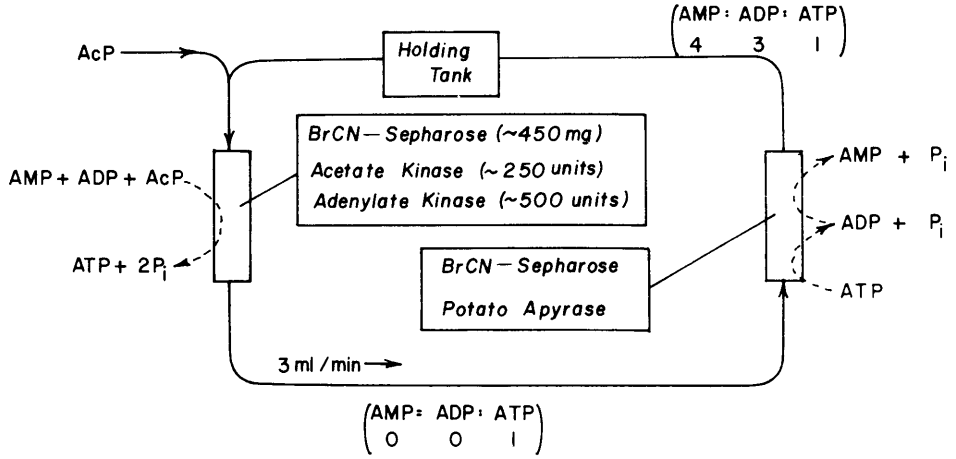
Fig. 3: Synthesis of Acetyl Phosphate

thermal cracking of acetic acid or acetone (Fig. 3). Reaction of ketene with phosphoric acid generates, initially, monoacetyl phosphate. Further reaction of ketene with the product mixture containing the monoacetyl phosphate also generates di- and triacetyl phosphates. In addition, water originally present in the phosphoric acid reacts with ketene to produce acetic anhydride. Procedures for the conversion of phosphoric acid to monoacetyl phosphate are

not yet optimized; nonetheless, using existing procedures, phosphoric acid can be readily converted into the dilithium salt of acetyl phosphate in approximately 50% yield, based on starting phosphoric acid. It should ultimately be possible to make large quantities of acetyl phosphate, very inexpensively, using this procedure.

With an assured supply of acetyl phosphate as starting material, attention has been focused on important problems dealing with the two enzymes required for the conversion of AMP to ATP. A matter of central concern in considering potential commercial applications of enzymes is the stability of the enzyme. An enzyme that can be used for extended periods of time is a practical subject for developmental work, even if its initial cost is high. An enzyme with a short lifetime under operating conditions is less attractive, even if appreciably less expensive. The principal contributor to the inactivation of rabbit muscle myokinase is autoxidation of mercaptan groupings in the protein. The conversion of the sulfydryl groups of cysteine to disulfide groupings, and subsequently to cysteine sulfonic acid, is well known (9). In the particular case of myokinase, this autoxidation can be partially reversed by addition of appropriate reducing agents: dithiothreitol, dithioerythrytol and β-mercaptoethanol. If the solution containing the enzyme is kept rigorously free of oxygen, and the oxidation potential of the solution is stabilized by addition of dithiothreitol, the enzyme retains its activity for weeks. Surprisingly, the stability of myokinase immobilized on Sepharose is much higher than that of myokinase in free solution. The immobilized enzyme has retained its activity for four to six weeks, even in suspensions from which oxygen has not been excluded. The origin of the increased stability of the immobilized enzyme relative to that of the same enzyme in free solution is not clear at present. If general, the latter phenomenon should prove to be of enormous practical importance in the commercial applications of immobilized enzymes.

We have successfully immobilized both acetate kinase and adenylate kinase on cyanogen bromide - Sepharose and operated a small demonstration reactor for the conversion of AMP and ADP to ATP (Fig. 4). The circulating solution flows through the column containing acetate kinase and adenylate kinase, and subsequently through a column containing potato apyrase immobilized on Sepharose. The potato apyrase column converts ATP to ADP and AMP. The product stream emerging from the apyrase column is mixed with a solution containing acetyl phosphate and fed into the acetate kinase and adenylate kinase containing column. This column has been operated for a number of hours, converting all of the input AMP to ATP. The total activity of the reactor was sufficient to generate about one gram of ATP per hour. Although the long term stability of this reactor has not been studied in continuous operation, the ATP regeneration column retained activity for greater than six weeks.



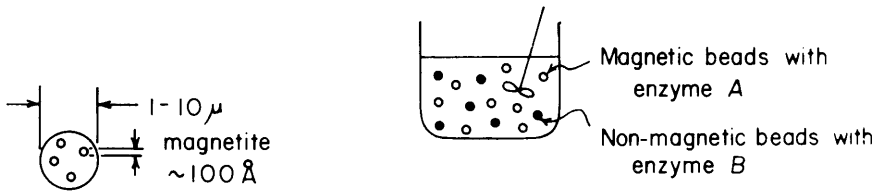
Residence Time in Regeneration Column < 1 min

$(ATP)_{out} / (ATP)_{in} = 6 - 9$

Productivity ~ 1 g ATP/hr

Fig. 4: AMP to ATP Regeneration Reactor

As part of an effort to design enzymatic reactors for ATP regeneration and other problems that provide alternatives to the commonly used fixed bed, we have begun work on a reactor based on a stirred bed of polyacrylamide beads containing both entrapped enzymes and small magnetic particles (Fig. 5). These beads, 1-10 microns in diameter, are sufficiently small that diffusional limitations on the rates of reaction of substrate solution with the enzymes are relatively unimportant. Conventional filtration of these particles is a



magnetic polyacrylamide beads containing entrapped enzyme

stirred or fixed bed reactor for coupled enzymes having different stabilities

Fig. 5: Magnetic Processing

slow and inefficient process. However, they are very readily separated from solution in the presence of high magnetic field gradients (10,11). More importantly, magnetic beads offer a method of dealing with enzyme systems in which two enzymes have drastically different stabilities under the operating conditions of the reactor. If these two enzymes were coimmobilized on a common support, it would be necessary to discard the activity of the longer lived enzyme when the shorter lived enzyme had become inactive. In the stirred magnetic bead reactor, this problem can be dealt with by enclosing the two enzymes in different beads, one magnetic and one nonmagnetic. When the activity of one of the two enzymes has decreased to a useless value, it is then easy to separate the active and inactive enzymes by magnetic filtration. The inactive enzyme can be discarded and the active enzyme mixed with a new batch of its reaction partner and added back to the reactor. The potential of magnetic separations for dealing with this and other separation problems in applied enzymology is high and is being actively exploited.

The central conclusion from this work is that the enzymatic regeneration of ATP from AMP and/or ADP using acetyl phosphate, acetate kinase and adenylate kinase is an entirely practical proposition. Acetyl phosphate appears to be readily available, the two enzymes have good stability under the operating conditions, and the thermodynamics of the coupled enzymatic reaction system are attractive for possible use in large scale reactors. Of the various schemes that have been proposed for enzymatic regeneration of ATP, this scheme seems to have most appeal, because it is capable of handling both ADP and AMP, and because the phosphate source on which it ultimately relies appears to be the most economical.

ACKNOWLEDGEMENT

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