Journal of Molecular Catalysis, 6 (1979) 177 - 198

POLYACRYLAMIDE GEL ENTRAPMENT OF ADENYLATE KINASE AND ACETATE KINASE

GEORGE M. WHITESIDES, ANDRE L. LAMOTTE, ORN ADALSTEINSSON, RAYMOND F. BADDOUR, ALAN C. CHMURNY, CLARK K. COLTON and ALFRED POLLAK

Departments of Chemistry and Chemical Engineering, Massachusetts Institute of Technology, Cambridge, Mass. 02139 (U.S.A.)

(Received in revised form July 14, 1978)

Summary

The factors that limit the stability of adenylate kinase and acetate kinase in solution have been examined and compared with those that determine stability under conditions encountered during photochemically initiated polymer gel formation in solutions of acrylamide and *N,N'*-methylenebisacrylamide. Both adenylate kinase (from rabbit and pig muscle) and acetate kinase (from *E. Coli*) contain cysteine residues close to their active sites. In solutions exposed to air, the rate of deactivation of these enzymes is determined by the rate of autoxidation (probably transition metal-catalyzed) of their cysteine sulfhydryl groups. Both enzymes are very stable if protected against autoxidation. At least four types of reactions contribute to deactivation during polyacrylamide gel formation: autoxidation of cysteine sulfhydryl groups by molecular oxygen; Michael addition of cysteine thiolate anion to acrylamide monomer and related electrophilic species; reaction of cysteine and of other amino acids with singlet oxygen (generated by energy transfer from excited riboflavin to ground-state molecular oxygen during irradiation); reaction of several amino acid residues with free radicals (presumably SO_4^- or buffer-derived radicals).

To avoid deactivation during acrylamide polymerization, it is helpful to exclude molecular oxygen, to work at low temperature and low pH, and to add thiols to the solution as radical scavengers. Both enzymes are less susceptible to deactivation in solutions having high concentrations of substrates. Additional protection against singlet oxygen is afforded by using a tertiary amine buffer, and by adding β-carotene to the solution; both are effective quenchers for singlet oxygen.

Adenylate kinase and acetate kinase have been modified by converting their cysteine –SH groups to $-SSCH_3$ moieties by reaction with *S*-methyl methanethiosulfonate; this blocking is completely reversed by treatment with DTT. These modified proteins show 70% and 30%, respectively, of the activity of the native enzymes. They are much more resistant to autoxidation and Michael addition than are the native proteins; their resistance to

singlet oxygen is slightly better than these proteins; their resistance to deactivation by $SO_4^{\cdot-}$ radical is indistinguishable from that of the fully reduced precursors. By taking advantage of a detailed accounting of the course of deactivation during polyacrylamide gel formation, it is possible to design experimental procedures that allow cross-linked polyacrylamide gels to be formed by free-radical polymerization in solutions containing adenylate kinase with preservation of 50 - 90% of the activity of the enzyme, and in solutions containing acetate kinase with preservation of 25 - 60% of the activity of the enzyme. If protected from atmospheric oxygen, the enzymes remain active in contact with these gels over periods of many months. Leakage of enzymes from the gels on washing is, however, rapid.

Introduction

Cross-linked polyacrylamide gels are widely used as insoluble matrices for the immobilization of biochemicals [1 - 4]. The simplest enzyme gel immobilization procedure involves free radical polymerization of acrylamide monomer and cross-linking agent in a solution containing protein, and generates a gel containing physically entrapped enzyme. Polyacrylamide gel entrapment has both advantages and disadvantages relative to other methods of immobilization. On the one hand, polyacrylamide is inexpensive, hydrophilic, and well-characterized [5]; gel formation is easily carried out; polyacrylamide is resistant to biodegradation; the gel network protects incorporated proteins against attack by microorganisms and proteases. On the other hand, acrylamide monomer is reactive toward proteins; the gel-forming polymerization often destroys enzymatic activity; leakage of protein from gel usually results in loss of activity, and polyacrylamide has poor mechanical properties.

As part of an effort to devise techniques for using cell-free enzymes as catalysts for large-scale organic synthesis utilizing cofactors, we have developed a coupled enzymatic process for the regeneration of ATP from AMP or ADP [6 - 11]. The ultimate phosphorylating agent in this scheme, acetyl phosphate (AcP*), can be synthesized readily [12]. The synthesis of complex organic chemicals by cell-free, enzyme-catalyzed reactions will compete

$$\text{AMP} + \text{ATP} \underset{\text{kinase}}{\overset{\text{adenylate}}{\rightleftharpoons}} 2\text{ADP} \qquad K = 1 - 9$$

$$\text{ADP} + \text{AcP} \underset{\text{kinase}}{\overset{\text{acetate}}{\rightleftharpoons}} \text{ATP} + \text{Acetate} \qquad K = 50 - 400$$

*Abbreviations used are: AdK, adenylate kinase; AcK, acetate kinase; AcP, acetyl phosphate; DTT, dithiothreitol; DTE, dithioerythritol; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; Hepes, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid; Mops, morpholinepropanesulfonic acid; Tea, triethanol amine; TMEDA, *N,N,N',N'*-tetraethylenediamine; NADP, nicotinamide adenine dinucleotide phosphate; Bis, *N,N'*-methylenebisacrylamide; G-6-PDH, glucose-6-phosphate dehydrogenase; MMTS, *S*-methyl methanethiosulfonate.

with conventional chemical and fermentation syntheses only if enzymes can be immobilized conveniently and in good yield, if the immobilized enzymes can be used under conditions which retain high activity for long periods of time, and if practical schemes for cofactor regeneration can be developed. The studies outlined here identify the reactions that result in loss of enzymatic activity during polyacrylamide gel entrapment of adenylate kinase and acetate kinase by free-radical polymerization of acrylamide monomer and cross-linking agent in solutions containing these enzymes. The reactions that deactivate the enzymes can be effectively suppressed by appropriate choices of reaction conditions, with the enzyme-containing gels being formed with good preservation of enzymatic activity. This study, by indicating the processes that result in loss of activity of these particular enzymes during gel formation, should be generally useful in the preparation of gels containing entrapped biochemicals. Relatively rapid leakage from the gels of the physically-entrapped enzymes limits their utility in synthesis: the accompanying paper outlines methods for modifying the gel-forming polymerization to include active ester groups, and for coupling the included proteins to the polymer gel backbone using these groups [13].

The particular adenylate kinases (AdK, AMP:ATP phosphotransferase, E.C.2.7.4.3) studied in this work were derived either from porcine or rabbit muscle: these single-subunit enzymes have molecular weights of 21 000, two cysteine groups per molecule, and very similar structures [14, 15]. The mechanism of phosphate transfer for the enzyme from rabbit muscle is random bi bi [16], with Michaelis constants K_{ATP} = 0.3mM, K_{AMP} = 0.5mM, and K_{ADP} = 1.58mM [17 - 19]. The equilibrium constant relating ADP to ATP and AMP varies between 1 and 9, depending on pH and pMg [8, 20], and the rate is relatively insensitive to pH between 7 and 9 [21]. A crystal structure on rabbit muscle myokinase is not available; that of porcine enzyme places one of the cysteine SH groups close to the active site [22, 23] and the second close to the first [24]. It is not clear whether these two SH groups can form an intramolecular disulfide linkage. Their orientation in the porcine enzyme suggests that some strain would be involved, but oxidation of rabbit enzyme is reported not to increase its molecular weight [15]. Acetate kinase (AcK, ATP:acetate phosphotransferase, E.C.2.7.2.1) from *E. Coli* has a molecular weight of 46 000 [25], and one cysteine SH group per molecule [26]. Catalysis proceeds by a random sequential mechanism with Michaelis constants K_{MgADP} = 1.1mM, K_{AcP} = 0.34mM, K_{MgATP} = 0.02mM, and K_{Ac} = 5.8mM [27, 28]. The observed equilibrium constant lies between 50 and 400, depending on pH $\geqslant$ 6 and pMg; the rate is relatively insensitive to pH between 6.5 and 9 [25].

Experimental

Materials were reagent grade, and were obtained from these sources: Tris, Hepes, Tea, Mops, DTT, DTE, 2-mercaptoethanol, ADP, AcP, NADP,

(Sigma); potassium and ammonium persulfate, acrylamide (ultra-pure), Bis (ultra-pure), TMEDA, riboflavin (Polysciences). The nitrogen and argon used as inert gases were purified grade. Water was deionized and distilled using a Corning Model 3B still.

Apparatus

The glassware used with enzymes was washed with distilled water. Volumetric transfers were accomplished using Hamilton syringes, Eppendorf pipettes, and Boralex micropipettes (Fisher Scientific). Dialysis was undertaken using a Bio-Fiber 50 Minibeaker (Biorad) with an 80 cm^2 fiber surface area and a nominal molecular weight cutoff of 5 000. Spectrophotometric determinations employed a Gilford Model 240 spectrophotometer equipped with a thermostatted cell compartment. The u.v. source used to initiate polymerizations was a high-intensity lamp (Polysciences Catalog No. 0222) delivering 8 400 microwatts/cm^2 (measured at 18 in. distance) in the active region for initiation (360 nm).

Enzymes

Adenylate kinase (porcine muscle) was purchased as a suspension in 3.2M ammonium sulfate (Sigma). Its specific activity after treatment with DTT or DTE was 2 050 U/mg (1U = 1 μmole/min): further purification did not increase this activity. Activation was carried out by centrifuging one ml of the commercial suspension (5 mg of AdK/ml) for 20 min at 27 000*g*. The supernatant was discarded and the precipitate was resuspended in degassed Hepes buffer (50mM, pH 7.5) with the final volume adjusted to 1 ml. This suspension was added to 9 ml of degassed Hepes buffer (50mM) containing 20mM of DTT or DTE (pH 7.8); after mixing, the pH dropped to 7.5. The enzymatic activity was monitored during activation at 25 °C; it normally increased to a stable plateau in 2 h. This solution was dialyzed (at 4 °C under positive argon pressure) against two 250 ml changes (1 h each) of degassed 50mM Hepes buffer (pH 7.5). The AdK solution was placed outside the fibers and stirred in a Bio-Fiber 50 minibeaker, while the dialysate was pumped inside the fibers at a flow rate of 7ml/min. Argon was bubbled continuously through the dialysate reservoir. No significant loss of enzymatic activity occurred during dialysis. When dialysis was complete, the dialysed AdK was transferred under argon to a Schlenck tube using a cannula and positive argon pressure. The quantity of DTT remaining in these solutions was not determined, but was undoubtedly small. Differences in the residual DTT concentration may contribute to the small observed differences in the behavior of reduced AdK on autoxidation. The enzyme was stored at 4 °C under argon. An analogous procedure was used for rabbit AdK.

Acetate kinase *(E. Coli)* was obtained as a suspension in 3.2M ammonium sulfate (Sigma). Its specific activity after treatment with DTT was 330 U/mg. This preparation showed two major and one minor band(s) on analytical disk gel electrophoresis [28] and was not purified further. AcK was activated using the same procedure as described for AdK, except

that the mixture of AcK and DTT or DTE required approximately 4 h at 25 °C to reach constant activity.

Hexokinase, G-6-PDH, and bovine serum albumin (Sigma) were used as purchased.

S-*Methyl methanethiosulfonate* (NMTS) was prepared from dimethyl disulfide and hydrogen peroxide in 60% yield by minor modifications of a literature procedure [38], and had a b.p. of 55 - 60 °C (0.04 Torr) (lit b.p. 67 - 68 °C (0.03 Torr)); n.m.r. ($CDCl_3$) δ 2.73 (s, 3) and 3.33 (s, 3).

Caution! This compound appears to be toxic. Although it was handled with rubber gloves in a hood, headache, dizziness, lassitude, and confusion followed very brief exposure to very small amounts of the compound.

Assays

Adenylate Kinase

This was assayed in homogeneous solution by following the rate of production of ATP from ADP. ATP was assayed in turn by reaction with glucose catalyzed by hexokinase, yielding glucose-6-phosphate, followed by oxidation of glucose-6-phosphate with G-6-PDH and NADP, yielding NADPH [10, 16]. The rate of formation of NADPH was followed spectrophotometrically at 340 nm. The following stock solutions were prepared: *Solution 1:* 0.2M Tris-HCl buffer, pH 7.5; 5mM glucose; 30mM $MgCl_2$; hexokinase (2 500 U/l); G-6-PDH (1 250 U/l). The buffer, glucose and $MgCl_2$ were mixed, the pH adjusted to 7.5, and the enzymes added. The resulting solution was stable at 0 °C for several months. *Solution 2:* ADP (disodium salt), 0.5M in water, pH adjusted to 6.8. This solution was stable at 0 °C for several weeks. *Solution 3:* 62.5mM NADP (sodium salt) in water, no pH adjustment. This solution was also stable at 0 °C for several weeks. In a typical assay, 5 ml of *solution 1* was mixed with 100 μl of *solution 2* and 50 μl of *solution 3*. This mixture was equilibrated for 3 min at 25 °C to consume ATP present as an impurity in the ADP. An aliquot of the solution to be assayed was then added; the size of this aliquot was adjusted so that the final solution contained less than 0.01 U/ml of adenylate kinase. The solution was mixed and poured into a 1 cm quartz cuvette, and the rate of appearance of NADPH was followed at 340 nm and 25 °C.

This assay was a compromise between accuracy and economy because the ADP concentration in the assay mixture (10mM) was only about six-fold higher than the Michaelis constant of AdK for ADP. This concentration gives rates that are approximately 0.9 V_{max}. Values closer to V_{max} could be obtained at higher ADP concentrations, but at greater expense. Since we were interested primarily in relative rather than absolute enzymatic activities, [ADP] = 10mM was chosen as a compromise. The enzyme concentrations of AdK, hexokinase, and G-6-PDH were chosen so that the AdK-catalyzed reaction was overall rate-limiting. Experimentally, we found

the minimum ratio of hexokinase to AdK activities, for AdK to be rate-determining, to be 100; here we used hexokinase/AdK = 250, to provide for losses on storage. The optimum ratio of activities for hexokinase and G-6-PDH is 2. When the final activity of AdK in the assay solution was less than 0.01 U/ml, less than 1% of the total ADP was converted to ATP per min, and the change in absorbance was linear with time. The assay was reproducible to ±5%. Additional details concerning this assay can be found elsewhere [10].

Immobilized AdK was assayed using the same procedure, with care taken that the enzyme-containing polyacrylamide particles were sufficiently small (20 - 50 μm) that diffusion effects were negligible. A dilute suspension of enzyme containing gel particles was added to the assay solution, mixed, and poured into the spectrophotometer cuvette. The particles did not settle appreciably during the time of the assay. The accuracy of the continuous assay for immobilized enzyme was checked against a batch assay. Polyacrylamide particles were suspended in 0.2M Tris buffer, pH 7.5, 10mM ADP, 30mM $MgCl_2$, at 25 °C. Aliquots of 0.1 ml were taken every min, and the reaction was quenched by mixing it with 1 ml of cold 0.1M HCl solution. The suspension was centrifuged at 7 000*g* for 5 min, an aliquot of the supernatant was added to 5.05 ml of ATP assay solution (5 ml of *Solution 1* and 50 μl of *Solution 3*), and the absorbance was read at 340 nm against a control of ATP assay solution. The activities measured by batch and by continuous assay agreed within 5%.

Acetate kinase

The enzyme was assayed using a modification of the AdK assay [10]. A fourth stock solution was prepared (*Solution 4:* AcP in water, 0.5M, stable for several weeks at 0 °C). *Solutions 1*, *2*, and *3* were mixed and incubated as described above, 50 μl of *Solution 4* added, and then an aliquot of AcK-containing solution was added, such that the final activity of AcK in the assay solution was less than 0.01 U/ml. This procedure gave [AcP] ≅ 5mM in the assay solution, in adequate excess over K_{AcP} = 0.34mM [27]. The activities measured with batch- and continuous procedures agreed to within 5%.

Preparation of sulfhydryl-blocked adenylate kinase and acetate kinase

MMTS (5 μl) was added to reduced, fully-activated, dialyzed AdK solution (5 ml), (prepared as described above) at 25 °C. The enzymatic activity of the solution was monitored by taking periodic aliquots: the specific activity of AdK decreased from 1 935 U/mg in the starting solution to one corresponding to 1350 U/mg after *ca.* 15 min. Although we have not examined the composition of the protein in detail at this point, blocking appeared to be complete; the activity was not reduced by a further addition of a 5-μl portion of MMTS. The excess MMTS was removed by dialysis (at 4 °C under positive argon pressure) against eight 250 ml changes (1.5 h each) of degassed 50mM Hepes buffer solution (pH 7.5). The dialysate flow rate was

7 ml/min and argon was bubbled continuously through the dialysate reservoir. When dialysis was complete, the dialysed, blocked AdK was transferred under argon to a Schlenck tube and stored at 4 °C. Contact between the blocked enzyme and rubber tubing should be carefully avoided, since thiols present in rubber as antioxidants or cross-linking agents rapidly regenerate deblocked enzyme.

Blocking of AcK followed an analogous procedure. The activity of this solution decreased during blocking (<1 h, 25 °C) from a value corresponding to 330 U/mg to 96 U/mg for the blocked AcK. Dialysis and storage were carried out as described for blocked AdK.

Blocked AdK or AcK could be deblocked by incubation at pH 7.5 in 20mM DTT or DTE for 1 h at 25 °C, with recovery of greater than 90% of the original activity.

Kinetics experiments

Most data were obtained by periodically sampling the reaction and assaying aliquots. For example, the data for Fig. 1 were gathered using 1 ml

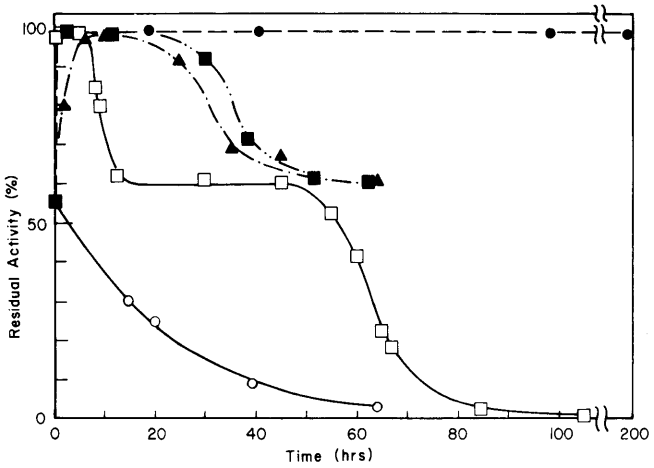


Fig. 1. The deactivation of adenylate kinase in solution depends on its exposure to air and on the concentration of thiol reducing agents. All curves were obtained using stirred solutions containing 0.2M Tris buffer (pH 7.5) and adenylate kinase (98 U/ml), at 25 °C. Experimental variations are: ○, no further additives, exposed to air; ●, 10mM DTT, air excluded by argon; □, 10mM DTT, exposed to air; ■, 20mM DTT, exposed to air; ▲, 20mM 2-mercaptoethanol, exposed to air.

of solution (0.2M Tris buffer) containing 98 U of AdK, stirred magnetically in a 10 ml flask. Aliquots (10 μl) were removed using an Eppendorf pipette, added to 5 ml of assay solution, and analyzed. Other kinetics runs were conducted using similar procedures. When it was necessary to exclude oxygen from the solution, the reaction vessels were closed with serum stoppers and maintained under a slight positive pressure of nitrogen or argon. Aliquots were taken by forced siphon through a stainless steel cannula inserted through

the serum stopper using anaerobic techniques standard in organometallic chemistry [29, 30].

Gel entrapments: adenylate kinase

Six stock solutions (S) were used in forming polyacrylamide gels in solutions containing AdK: **S1**: Hepes buffer, 0.2M, pH 7.5 containing acrylamide (0.475 g/ml) and *N,N'*-methylenebisacrylamide (0.025 g/ml); **S2**: water, containing riboflavin (2 mg/ml as a fine suspension); **S3**: water, pH 7.6, containing potassium persulfate (50 mg/ml); **S4**: Hepes buffer, 0.05M, pH 7.5, containing DTT (10mM), $MgCl_2$ (30mM), and ADP (10mM); **S5**: Hepes buffer, 0.05M, pH 7.5, containing DTT (10mM) and ammonium sulfate (0.5M); **S6**: Adenylate kinase (*ca.* 2 400 U/ml) in Hepes buffer, 0.05M, pH 7.5.

S4 (1.4 ml) and **S2** (100 μl) were placed in a 5 ml beaker containing a small magnetic stirring bar; **S1** (500 μl) and **S3** (50 μl) were transferred into two separate 15 ml centrifuge tubes; containers were capped with serum stoppers and swept with a stream of argon for 20 min to remove molecular oxygen. **S1**, **S2**, and **S4** were stored at room temperature; **S3** was stored at 0 °C. Transfers of less than 10 μl were usually accomplished with a syringe; larger volumes were transferred by forced siphon through a stainless steel cannula under argon. The degassed solutions were carefully protected from contamination by atmospheric oxygen in order that polymerization behavior, gel times, and gel properties were reproducible. Even small quantities of oxygen introduced by accident into these solutions resulted in unacceptably long gel times and poor gel physical characteristics.

The mixture of **S4** and **S2** was cooled to 0 °C by immersion in an ice–salt bath: **S1** and **S3** were transferred rapidly to the beaker. As soon as the mixture in the beaker reached 0 °C (*ca.* 1 min) polymerization was initiated by irradiation. An aliquot of the solution of adenylate kinase (**S6**, 22 μl, 54 U) was added to the polymerizing mixture from a syringe 5 s before the previously determined gel time. (The gel time is defined as the time at which the polymerization has proceeded to the stage at which the stirring bar stops turning. With care it is reproducible to ±10% (*i.e.*, *ca.* ±3 - 4 s). In our experiments, the gel time was 32 ± 4 s.) The mixture was irradiated for a total of 60 s. The gel was then transferred to a mortar precooled to −15 °C and rapidly broken up by grinding with a pestle. Two minutes of vigorous grinding gave irregular particles having an average particle size of approximately 20 - 30 μm. These gel particles were immediately washed into a 50 ml centrifuge tube, using a total of *ca.* 10 ml of **S5**.

The 'yield' of the entrapment reaction was calculated by comparing the enzymatic activity of a solution containing suspended gel particles with the activity of the enzyme present before polymerization, using assays described above. Since the enzyme is not covalently immobilized and rapidly leaks from the gel, this yield is not an immobilization yield: rather, it is a measure of the enzymatic activity that survives the conditions used for the polymerization reactions. Certain of these yields were only moderately re-

producible: variations in the amounts of residual DTT present in solutions of reduced, dialyzed enzyme, differences in the amount of adventitious oxygen present in the solutions, changes in the purity of the enzyme from batch to batch, and idiosyncracies of individual laboratory technique all probably detract from the reproducibility. Reactions carried out by a single operator, using a single batch of enzyme, were, however, reasonably reproducible. The data described in each of the Figures in the text were so obtained, and comparisons within each of these sets of data are reliable; comparisons between data in different series of experiments are less reliable.

Gel immobilizations: acetate kinase

Six stock solutions (S′) were required: S′1: Mops buffer, 0.2M, pH 6.2, containing acrylamide (0.475 g/ml) and *N,N′*-methylenebisacrylamide (0.025 g/ml); S′2: water, containing riboflavin (4 mg/ml as a suspension); S′3: water, pH 7.6, containing potassium persulfate (50 mg/ml); S′4: Mops buffer, 0.05 M, pH 6.2, containing $MgCl_2$ (30mM), ADP (5mM), and acetyl phosphate (5mM); S′5: Hepes buffer, pH 7.5, 0.05M, containing DTT (10mM) and ammonium sulfate (0.5M); and S′6: Acetate kinase (*ca.* 170 U/ml) in Mops buffer, 0.05M, pH 6.2, containing DTT (2mM).

The sequence of steps was analogous to that described for the immobilization of adenylate kinase. Each solution was degassed by sweeping for 20 min with a stream of argon, and stored under argon. S′4 (1.4 ml), S′2 (50 μl) and S′1 (500 μl) were added to a capped 5 ml beaker, and cooled to 0 °C. S′3 (50 μl) was added by syringe, and the resulting solution was stirred for 2 min at 0 °C. Polymerization was initiated by irradiation, and the enzyme-containing solution (S′6, 30 μl, 5.4 U) was injected into the solution 5 s before the gel point. Irradiation was continued for 25 s. The beaker containing the resulting gel (*ca.* 2 ml) was removed from the ice bath. The gel was removed from the beaker, broken up by grinding in a mortar precooled to −15 °C, and transferred to a centrifuge tube with *ca.* 10 ml of S′5.

Results

The principal reaction leading to loss of activity of adenylate kinase and acetate kinase in solution is autoxidation.

Before developing techniques for gel immobilization of AdK and AcK, we examined the factors that determined the stability of these enzymes in solutions that did not contain acrylamide monomer, vinyl-derived polymers, or the radical initiator system. Figure 1 summarizes several representative experiments carried out with stirred solutions of AdK. Preliminary experiments involving variations in the stirring rate established that the data were collected using solutions which were in equilibrium with air, and in which diffusion of oxygen into the solutions was not rate-limiting for autoxidation. AdK, as purchased, is only partially active. Dissolution of this material in pH 7.5 buffer yields a solution whose activity decays further with a half-life of *ca.*

20 h (25 °C) when exposed to air. Treatment of a freshly prepared solution of AdK with DTT results in immediate (<1 min) activation. On exposure to air, the activity of this solution follows a characteristic course: the activity stays constant for a period which depends on the starting concentration of DTT, then falls rapidly to the value characteristic of the original solution before activation with DTT. Addition of DTT at this point results in regeneration of essentially the full activity. If additional DTT is not added, the activity again stays constant for a period, then falls to zero. Addition of DTT after the activity drops close to zero results in only a relatively small increase in activity. If a solution of AdK containing DTT is prepared using degassed buffer, and stored under nitrogen or argon, the activity stays at its maximum value for long periods. We have maintained >90% activity in solutions of AdK at 25 °C by excluding oxygen and periodically renewing DTT, for periods greater than three months.

The principal deduction from these experiments — that AdK is stable in solution for prolonged periods provided that it is protected from autoxidation by exclusion of molecular oxygen and addition of DTT — is useful but not surprising, since the general stability of AdK is well-established [14]. Two features of the data in Fig. 1 do, however, deserve comment. First, fully active AdK exists in the reduced form having two cysteine sulfhydryl groups. The loss of activity of AdK on oxidation proceeds through two distinct phases: one fully reversible with DTT, the second not reversible. Since details of the mechanisms(s) of autoxidation of cysteine sulfhydryl groups have never been fully clarified [31 - 35], it is difficult to define the active species present in solution at the plateau corresponding to 60% activity. They are probably disulfides derived from AdK, either by intramolecular cysteine formation or by intermolecular coupling with DTT, mercaptoethanol or further AdK, although proteins containing sulfinic acid groups [36], or more highly oxidized sulfur-containing species, cannot be excluded. Regardless of the precise course of the oxidation, it is clearly important to prevent the autoxidation of AdK from becoming irreversible if long enzyme lifetimes are required. Second, comparison of the resistance of AdK to autoxidation in solutions containing DTT and mercaptoethanol indicates that the latter is slightly *more* effective than the former as a protective reagent. DTT does reduce partially oxidized AdK to the fully active form more rapidly than mercaptoethanol. The relative widths of the plateaux observed for equivalent concentrations of these two reducing agents (*i.e.*, 10mM DTT and 20mM mercaptoethanol) suggest that DTT is itself more rapidly oxidized than mercaptoethanol. In a situation in which access of oxygen to the AdK-containing solution is not rate-limiting, mercaptoethanol persists for a longer time in solution than DTT, and is thus, apparently, a more effective protective reagent.

Examination of the solution stability of AcK indicated that the rate of autooxidation is also the major determinant of the stability of the enzyme. When used under nitrogen or argon in solutions containing DTT, AcK also shows lifetimes of many months at room temperature. Examination of the activity of AcK in a solution containing DTT during oxidation shows only a hint of the two-plateau profile that characterized AdK (Fig. 2).

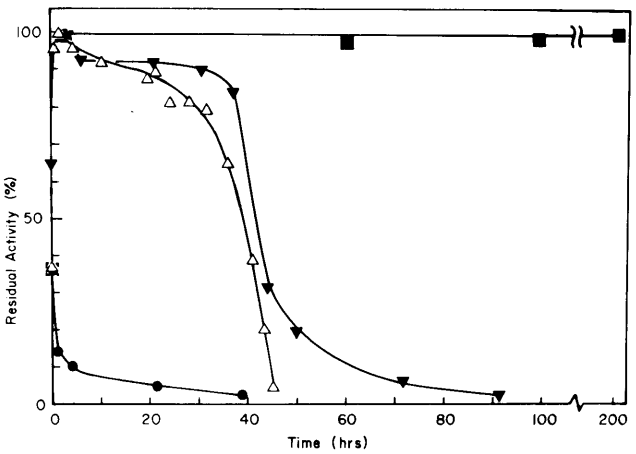


Fig. 2. Acetate kinase shows little evidence of an active partially oxidized intermediate. Solutions contained acetate kinase (3.3 U/ml) in 50mM Hepes buffer (pH 7.5) (25 °C): ●, no additional additives, exposed to air; △, ▼, 10mM DTT, exposed to air (two experiments by different individuals working with different batches of enzyme included to indicate the reproducibility of the data); ■, 10mM DTT protected from air under argon. The DTT was added to the last three solutions at time = 0; the resulting activation was rapid.

Adenylate kinase and acetate kinase are resistant to autoxidation following modification by conversion of cysteine -SH groups to $-SSCH_3$ groups.

The behavior of AdK and AcK on exposure to oxygen established that protection of cysteine sulfhydryl groups against extensive oxidation is important in maintaining enzymatic activity. At the same time, the observation of activity in partially oxidized intermediates suggested that it might be possible to modify these sulfhydryl moieties and still retain useful enzymatic activity. To block the sulfhydryl groups of AdK and AcK, we utilized *S*-methyl methanethiosulfonate (MMTS) [37, 38].

$$\text{Enz-SH} \underset{\text{DTT}}{\overset{CH_3SSO_2CH_3}{\rightleftharpoons}} \text{Enz-SSCH}_3$$

AdK and AcK were reduced to their fully active forms with DTT, treated with excess MMTS, and dialysed against buffer to remove unreacted MMTS. Assay of the blocked enzymes indicated that the modified AdK retained 70% of the activity of the fully active, native enzyme and modified AcK retained 30% of the activity of the native form. Blocking was reversible; treatment of either modified enzyme with DTT for 1 h regenerated 98 - 99% of its starting activity.

Figure 3 compares the activities of unmodified (fully reduced) and modified AdK and AcK on exposure to atmospheric oxygen under comparable conditions. Four conclusions can be drawn from these data. First, in marked contrast to the unmodified enzymes, AdK and AcK modified by conversion of cysteine SH groups to $SSCH_3$ groups are resistant to autoxida-

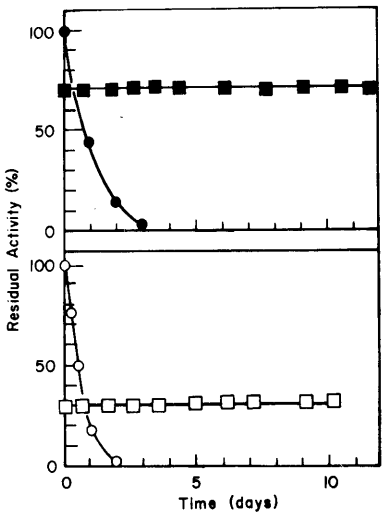


Fig. 3. Adenylate kinase and acetate kinase modified by conversion of cysteine SH groups to $SSCH_3$ groups are more resistant to autoxidation than the modified, fully-reduced enzymes in solution containing no thiol antioxidants. *Upper.* Adenylate kinase, 50mM Hepes buffer (pH 7.5), 25 °C, exposed to air: ●, unmodified enzyme, 34 U/ml; ■, modified enzyme, 24 U/ml. The rate of autoxidation of reduced AdK (●) is not significantly influenced by the presence of 0.5M NaCl. *Lower.* Acetate kinase, 50mM Hepes buffer (pH 7.5) 25 °C, exposed to air: ○, unmodified (fully-reduced) enzyme, 33 U/ml; □, modified enzyme, 9 U/ml.

tion. This conclusion suggests blocking as a practical method of protecting these enzymes against oxidation while they are being used for ATP regeneration. Further, since cysteine is the only amino acid which should be modified by treatment of AdK or AcK with MMTS, the stability of the modified enzymes reinforces the arguments of the previous Section that cysteine autoxidation is responsible for their oxidative instability. Second, since there is no indication of loss of activity of modified AdK and AcK over periods of time in which oxidation of the native enzymes has resulted in complete loss in activity, the extent of modification must be quantitative. Third, since the modified enzymes retain a significant fraction of the activity of the native enzymes, cysteine SH groups are not required for full activity. Fourth, since AdK and AcK modified by conversion of thiol to disulfide moieties are stable toward autoxidation, the mechanism of the *irreversible* steps in the oxidation of the unmodified proteins may involve some oxidizing reagent other then O_2: hydrogen peroxide (generated by autoxidation of SH groups) is a plausible candidate [39].

AdK contains two cysteines. Reaction of the SH groups of these amino acids with silver(I) [40], Ellman's reagent [41], and alkylating agents [42], completely destroys enzymatic activity. Reaction with several derivatives of *p*-hydroxymercuribenzoate yields modified AdK retaining up to 70% of the activity of the native enzyme, with the activity increasing inversely with the size of the mercurial [41]. These observations, combined with the influence

of MMTS blocking on the activity of AdK observed here, and the crystal structure of the porcine enzyme [22, 23], are best rationalized by assuming that the enzyme has two sulfhydryl groups which are sufficiently close to the active site that the enzymatic activity is altered by their modification. Neither of the sulfhydryl groups is, however, required for activity [24].

Myokinase and acetate kinase are deactivated by Michael addition of cysteine SH groups to acrylamide monomer.

Polyacrylamide gel entrapment of an enzyme is carried out by free-radical polymerization of a mixture of acrylamide monomer and cross-linking agent in a solution containing the enzyme. An important step in minimizing loss of activity during the immobilization process is establishing conditions in which the enzyme is stable in this starting solution. Figure 4 shows the loss of activity of AdK and AcK in solutions of acrylamine under several conditions. These experiments, and others described in this Section,

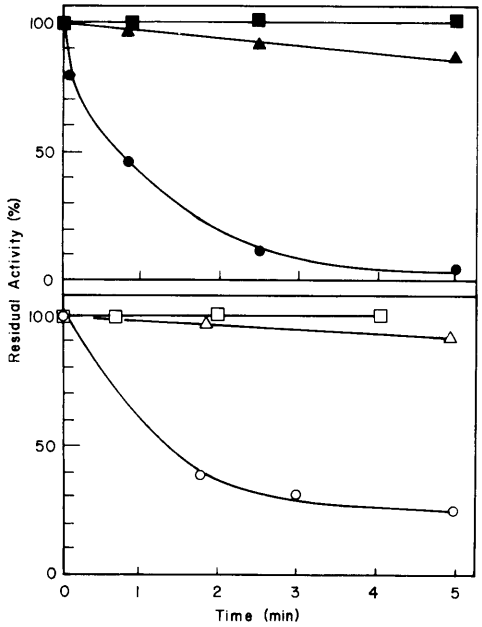


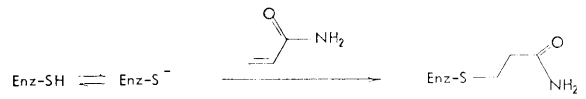
Fig. 4. Adenylate kinase and acetate kinase are protected from deactivation by reaction with 1.7M acrylamide by low temperatures and saturating concentrations of substrates (50mM Hepes buffer, pH 7.5). *Upper.* Adenylate kinase (205 U/ml): ●, 25 °C; ▲, 0 °C; ■, 0 °C, 10mM ADP, 30mM $MgCl_2$. *Lower.* Acetate kinase (20 U/ml): ○, 25 °C; △, 0 °C; □, 0 °C, 5mM ADP, 5mM AcP, 30mM $MgCl_2$.

were carried out using solutions containing 12% (w/v) acrylamide (*ca.* 1.7M) in water, which is the concentration employed in the immobilizations. Oxygen was carefully excluded to minimize autoxidation. Figure 4 yields two conclusions. First, both enzymes are deactivated more rapidly in acrylamide solution at 25 °C than at 0 °C. Second, both enzymes are protected (over the

short spans of these experiments) by including their respective substrates in the solutions at concentrations sufficiently greater than the Michaelis constants for their active sites to be essentially completely occupied.

The rapid deactivation of AdK and AcK observed at 25 °C in the absence of protecting substrates is important for practical reasons. It limits the time that the enzymes may be exposed to acrylamide monomer during the gel formation without unacceptable loss of activity, and encourages the use of polymerization conditions that maximize the conversion of monomer to polymer [43]. Although carrying out the reaction at 0 °C and saturating the active sites with substrate affords good protection against deactivation, it is useful to identify the process(es) leading to deactivation, in order to be able to design protocols that maximize enzymatic stability. Deactivation in concentrated acrylamide solution could result from at least two processes: Michael addition of a nucleophilic group on the enzyme to acrylamide, or disruption of enzyme tertiary structure by this amide. Both protein alkylation by electron-deficient olefins [31, 32, 44 - 47] and denaturation with amides (particularly urea) [48 - 51] are well known.

Three lines of evidence indicate that the deactivation of AdK and AcK is due to Michael addition of cysteine thiolate moiety to acrylamide.



First, the different rates of deactivation of these enzymes in solutions containing equal concentrations of acrylamide, acetamide, and urea demonstrate that acrylamide is significantly more effective than acetamide or urea (Fig. 5). Denaturation by disruption of protein tertiary structure normally is most rapid with urea [48, 49, 52]. Second, the rate of deactivation increases significantly as the pH of the solution is raised for both AdK and AcK (Fig. 6). This pH dependence is qualitatively consistent with a reaction requiring prior ionization of a nucleophile. Third, AdK and AcK modified by conversion of SH moieties to $SSCH_3$ moieties are stable in acrylamide solution for more than 12 h. Removal of acrylamide from solutions of these modified enzymes, followed by reduction of the disulfide moieties with DTT, regenerates the activity of the native enzyme.

Photochemical polymerization initiation using riboflavin sensitizer, if carried out in the presence of molecular oxygen, generates sufficient singlet oxygen to deactivate adenylate kinase and acetate kinase.

Two types of radical initiation systems are commonly used in forming polyacrylamide gels: oxidation–reduction initiation, using, for example, persulfate and TMEDA or Fe(II), and photochemical initiation employing persulfate and riboflavin [53, 54 - 56]. We have used photochemical rather than redox initiation in most of this work because several reactions involving the components of the redox initiation systems complicated the interpretation

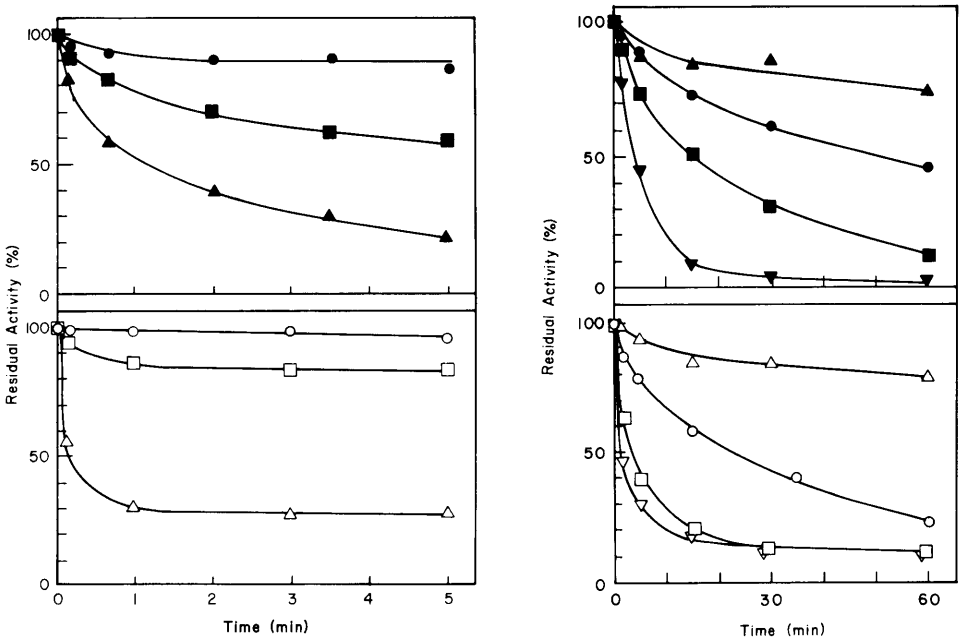


Fig. 5. Acrylamide deactivates adenylate kinase and acetate kinase more rapidly than acetamide or urea (50mM Hepes buffer, pH 7.5, 25 °C, under argon; concentration of amides and urea = 1.7M). *Upper.* Adenylate kinase (205 U/ml): ●, acetamide; ■, urea; ▲, acrylamide. *Lower.* Acetate kinase (66 U/ml): ○, acetamide; □, urea; △, acrylamide.

Fig. 6. Deactivation of adenylate kinase and acetate kinase by acrylamide is more rapid at high than at low pH (50mM Hepes–Mops buffer, 0 °C, under argon). *Upper.* Adenylate kinase (205 U/ml); pH values: ▲, 6.0; ●, 7.0; ■, 8.0; ▼, 8.5. *Lower.* Acetate kinase (66 U/ml), pH values; △, 6.0; ○, 7.0; □, 8.0; ▽, 8.5.

of subsequent experiments designed to maximize yields of enzymes immobilized covalently using modifications of these procedures: in particular, transition metal ions are active catalysts for autoxidation, and tertiary amines significantly catalyze the hydrolysis and aminolysis of the *N*-hydroxysuccinimide esters used [13]. For practical work, redox initiation is, however, often more convenient than photochemical initiation, and in applications of these entrapment and immobilization procedures to problems in which the mechanisms of the deactivation and immobilization procedure were not of direct concern, redox initiation has usually been employed [11].

We initially explored the stability of AdK and AcK toward a photochemical initiation system based on riboflavin in the absence of acrylamide monomer. The object of these experiments was to answer two specific questions. First, does the electronically excited photochemical sensitizer itself damage these enzymes [57]? Second, is it necessary to exclude oxygen from solution containing enzymes with blocked cysteine sulfhydryl groups during polymerization to maintain enzymatic activity? Figure 7 summarizes experiments which establish that the enzymatic activity of AcK rapidly decreases in air-saturated solution containing riboflavin, on exposure to light. The AcK

used in these experiments was "unactivated": *i.e.*, it was commercial material that had not been treated with DTT. Since this material is relatively slowly deactivated by oxygen in solution, we assume that its sulfhydryl group is incorporated into a disulfide or sulfinic acid moiety. Deactivation requires oxygen, light, and riboflavin, and is slow if any of the three is excluded. The rate of deactivation is essentially independent of pH in either Hepes (indicated in Fig. 7) or phosphate buffers (not shown), but depends on buffer structure: solutions containing Tea or Hepes were more stable than those containing Tris or phosphate. Deactivation is inhibited by 0.1mM β-carotene. Blocking the enzyme cysteine SH group by conversion to a $SSCH_3$ moiety decreases the rate of deactivation substantially compared with that of fully reduced enzyme, but only slightly compared with that of the partially oxidized commercial enzyme; addition of thiols effectively protects the enzyme (Fig. 8).

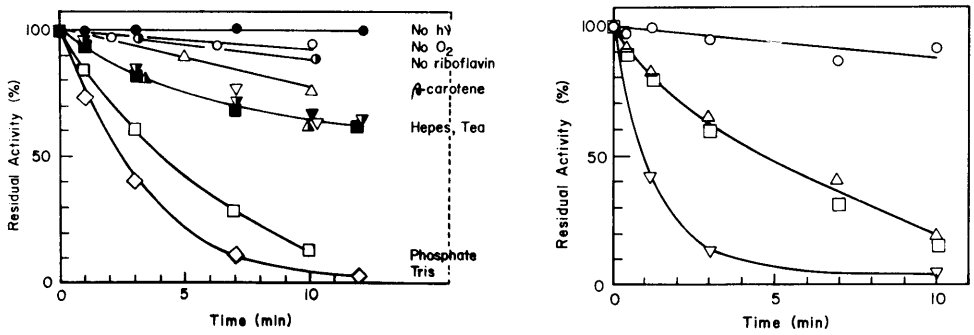


Fig. 7. Acetate kinase is deactivated at 0 °C by irradiation in solution with riboflavin and light. Deactivation is slowed by tertiary amine buffers and β-carotene, but is not influenced by pH. All solutions contain 19.8 U/ml of commercial, partially oxidized enzyme and 0.1 mg/ml of riboflavin and are saturated with air, unless indicated otherwise. Buffer concentrations are 50mM. Other components in solutions, or derivatives from standard conditions are: ●, no hν; ○, air removed and replaced with argon, phosphate buffer, pH 7.5; ◑, no riboflavin; △, 0.1mM β-carotene, Hepes; ▼, Hepes, pH 8.2; ▽, Hepes, pH 8.0; ▲, Hepes, pH 7.5; ▽, Hepes, pH 6.0; ■, Tea, pH 8.0; ◇, Tris, pH 8.0; □, phosphate, pH 8.0. A number of experimental points falling on these curves were omitted from the Figure to avoid clutter.

Fig. 8. Deactivation of acetate kinase by $^1O_2$ is slowed by blocking the cysteine SH group. Thiols added to the solution provide substantial protection. These data were obtained by irradiating an air-saturated solution containing 20 U/ml of acetate kinase, 0.1 mg/ml of riboflavin, 50mM phosphate buffer (pH 8.0) at 0 °C: ○, fully reduced enzyme in solution containing 10mM DTE; △, acetate kinase modified by conversion of the cysteine SH group to an $SSCH_3$ group; □, commercial, partially oxidized acetate kinase; ▽, fully reduced enzyme in solution following dialysis to remove thiol reducing reagent. The small differences between comparable runs in this Figure and Fig. 7 are attributable to differences in the extent of oxidation of the starting enzyme and in the concentration of residual thiol reagent left in solution following dialysis of the reduced enzyme.

The simplest interpretation of these data is that the agent responsible for loss of enzymatic activity under these conditions is singlet oxygen. Ribo-

flavin is a photosensitizing dye capable of generating singlet oxygen by energy transfer [58 - 60]. The high activity of tertiary amines [61, 62] and β-carotene [63] in quenching singlet oxygen rationalizes the protection afforded by tertiary amine buffers (Tea, Hepes) and by β-carotene. The reactivity of many amino acids other than cysteine (particularly histidine, tryptophane, tyrosine, and methionine) toward singlet oxygen is sufficiently high for modification of the cysteine SH group of AcK not to be expected to protect the enzyme completely against attack by singlet oxygen [64]. The protection offered by the presence of excess sulfhydryl reagents can be explained by competitive scavenging of singlet oxygen by these materials.

AdK also deactivates rapidly on irradiation in the presence of riboflavin and oxygen. Although the parameters influencing the rate of this deactivation were not explored in detail, excluding oxygen and adding 2-mercaptoethanol or β-carotene (10^{-4}M) sharply decreased the rate of deactivation. We presume that singlet oxygen is the active agent in this system.

Mercaptans or substrates protect adenylate kinase and acetate kinase from deactivation by radicals generated during initiation.

Enzyme entrapment in a polyacrylamide gel requires the exposure of the enzyme to free radicals during the initiation and polymerization process. We have explored the stability of AdK and AcK to radicals by following the activity of oxygen-free solutions containing enzyme, riboflavin, and persulfate during irradiation (Fig. 9). The activity of both enzymes disappears

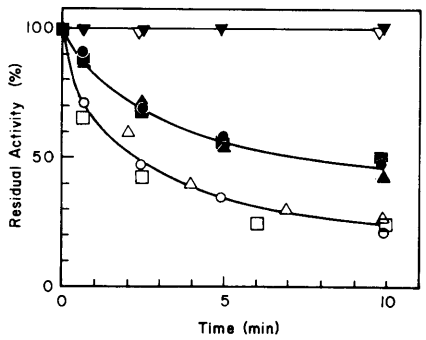


Fig. 9. Radicals generated by a light/riboflavin/persulfate polymerization initiation system deactivate native or cysteine-blocked adenylate kinase and acetate kinase. Data were collected by irradiation of deoxygenated solutions containing Hepes (50mM, pH 7.5), riboflavin (0.1 mg/ml), and $(NH_4)_2S_2O_8$ (6 mg/ml) at 0 °C. Adenylate kinase (*ca.* 4 U/ml): ■, fully active, reduced enzyme; ▲, cysteine-blocked ($SSCH_3$) enzyme; ●, partially oxidized commercial enzyme; ▼, fully reduced enzyme in a solution containing 10mM DTT or 20mM 2-mercaptoethanol. Points for acetate kinase are represented by open symbols (□, △, ○, ▽) having the same meaning as the corresponding solid symbols for adenylate kinase.

rapidly on irradiation under conditions representative of those used to initiate polymerization. The rate at which activity disappears is independent of the oxidation state of the cysteine moieties: reduced, fully active enzyme, commercial 'unactivated' enzyme, and enzyme containing $SSCH_3$ moieties

all deactivate at the same relative rates. The free radical formed initially in these systems is $SO_4^{\cdot-}$, generated by riboflavin-sensitized homolysis of persulfate. In Hepes buffer, radicals derived by hydrogen abstraction from buffer may also be present. Complete protection of the enzymes over spans of 3 - 5 min is again afforded by adding a large excess of a thiol to the solution. Protection is also obtained by adding substrates (25mM ADP and 50mM $MgCl_2$ for AdK, 20mM ADP, 20mM AcP, and 50mM $MgCl_2$ for AcK). Details of the mechanism(s) through which thiols protect the enzyme are not entirely clear. Thiols are, of course, effective radical scavengers [31, 65], and also useful chain transfer agents in vinyl monomer polymerizations [66]. Thus, the major functions of the added thiols are almost certainly to scavenge $SO_4^{\cdot-}$ (and buffer) radicals before they can react with the enzyme, and to reduce radical centers on the enzyme once formed. The resulting thiyl radicals would still be capable of initiating acrylamide polymerization, and would only moderately increase the gel time. The major uncertainty in this picture is the identity of the group(s) on the proteins whose reaction with radicals leads to deactivation. Cysteine SH groups are obvious candidates for the attack site. The experimental observation that the rate of deactivation of AdK and AcK takes place equally rapidly when cysteine sulfur atoms are present as thiols or disulfides is compatible with the high reactivity of disulfide groups toward $SO_4^{\cdot-}$ radicals [67], but it does not exclude attack of this radical on other groups, or attack by other radicals.

Polyacrylamide gels can be formed by free-radical polymerization in solutions containing adenylate kinase and acetate kinase with good preservation of enzymatic activity.

The functionalities in AdK and AcK that are most easily attacked during polyacrylamide gel immobilization are the cysteine SH groups, although other groups must certainly be attacked to some extent. By choosing the immobilization conditions to minimize the reactions that deactivate these enzymes, it is possible to form gels which physically entrap these enzymes, with preservation of 50 - 90% of the activity of AdK and 20 - 60% of the activity of AcK. The resulting enzyme-containing gels are of little practical value, because the enzymes escape from them rapidly on washing: washing a suspension of gel containing AdK with 15 times its volume of buffer solution results in 90% loss of enzyme from the gel after 40 min. The most practical method of retaining the enzyme in the gel is to bond it covalently to the polymer, and techniques which accomplish this objective are described in the accompanying paper [13]. Identification of the process responsible for enzyme deactivation during radical polymerization of acrylamide in enzyme-containing solutions is, nonetheless, an important foundation for techniques leading to covalent immobilization in polyacrylamide gels.

In order to preserve enzymatic activity during gel formation, five experimental conditions must be met. First, oxygen must be carefully excluded from the reaction mixture. Second, the enzyme should be introduced last

into a solution containing the other components, to minimize its reaction with monomer and other reactive species. In practice, we normally carry the polymerization almost to the gel point in the *absence* of enzyme, and then interrupt irradiation, introduce the enzyme, and resume irradiation. Third, the enzyme active site should, if possible, be protected by concentrations of substrates and cofactors well above their respective Michaelis constants. Added thiol reagents provide additional protection, although their presence during polymerization may lengthen the gel time and decrease the mechanical strength of the gel. Fourth, the immobilization should be carried out at low temperature (4 °C), and preferably at the lowest practical pH, to minimize Michael additions. Fifth, unreacted acrylamide monomer and peroxidic groups [68] should be destroyed after the polymerization is completed by treating the gel with a solution containing a thiol and ammonium sulfate.

The stability of AdK and AcK in the presence of these polyacrylamide gels is excellent, provided that oxygen is excluded and an adequate concentration of a reducing thiol is maintained. If a polymer gel is formed in a solution containing either enzyme, and the gel is then broken mechanically into small pieces and re-suspended in buffer solution, much of the enzyme rapidly leaks from the gel. The loss in activity of either enzyme in the resulting solution containing suspended gel particles is, however, less than 10% over three months at room temperature, provided that enzyme autoxidation is prevented. Thus, the gels themselves provide innocuous environments for these enzymes.

Discussion

Four types of reactions participate in the deactivation of adenylate kinase and acetate kinase during photochemically-initiated polyacrylamide gel entrapment: alkylation of protein by Michael addition to acrylamide monomer and other electrophilic vinylic species; autoxidation by molecular oxygen ($^3O_2$), probably catalyzed by transition metal ions; oxidation by singlet oxygen ($^1O_2$) generated during irradiation by energy transfer from excited riboflavin; attack on protein by other species, including $SO_4^{\cdot-}$ radicals, persulfate itself, and possibly other radicals (*e.g.*, polymer radicals, radicals derived from buffer, adventitious oxygen, or added thiols). Most of these reactions will almost certainly be important for any enzyme containing structurally or catalytically important cysteine residues, and probably also for many enzymes containing other essential nucleophilic or reducing amino acids: in particular, since the rates of alkylation of thiolate and amino groups by electrophilic olefins may differ by less than a factor of 10 [46], Michael addition may provide a generally important deactivation mechanism for proteins in the presence of electrophilic vinylic monomers.

There are two effective strategies for minimizing deactivation of AdK and AcK by these reactions. The first, applicable to work with unmodified

enzyme, decreases the rates of the possible deactivating reactions by excluding oxygen, maintaining low temperatures, adding thiols and substrates, and employing conditions that result in a short gel time. The second strategy modifies the enzyme to render sensitive amino acids resistant to these reactions. The conversion of cysteine sulfhydryl groups to mixed disulfides proved effective in protecting AdK and AcK against alkylation by acrylamide and oxidation by triplet oxygen, partially effective in preventing deactivation by singlet oxygen, and ineffective in slowing deactivation by $SO_4^{\cdot-}$ (or buffer-derived) radicals. The use of modified enzymes to improve storage and use-lifetimes seems particularly attractive, since a major contributor to deactivation of immobilized enzymes is often autoxidation. Although AdK and AcK modified to contain mixed disulfide moieties were less active than the unmodified enzymes, they were so much more stable toward autoxidation that their usefulness would be greater than unmodified enzymes in applications in which exclusion of oxygen would be difficult or impossible (*e.g.*, in clinical analyses).

Acknowledgments

Drs. Armin Ramel and Hiro Nishikawa (Biopolymers Laboratory, Hoffmann-LaRoche) provided invaluable advice and assistance throughout this work. Professor G. Kenyon (Berkeley) provided information about the preparation and use of $CH_3SSO_2CH_3$. This research was supported by the National Science Foundation (RANN), Grant No. GI 34284. O.A. acknowledges a fellowship from Halcon International, Inc.

References

1 O. Zaborsky, Immobilized Enzymes, CRC Press, Cleveland, Ohio, 1973, p. 83 ff.
2 I. Chibata, T. Tosa and T. Sato, Appl. Microbiol., 27 (1974) 878.
3 H. Nilsson, R. Mosbach and K. Mosbach, Biochem. Biophys. Acta, 268 (1972) 253.
4 H. H. Weetall and S. Suzuki (eds.), Immobilized Enzyme Technology, Plenum Press, New York, 1975.
5 D. C. MacWilliams, in R. H. Yocum and E. B. Nyquist (eds.), Functional Monomers, Vol. I, Marcel Dekker, New York, 1973, p. 1.
6 C. R. Gardner, C. K. Colton, R. S. Langer, B. K. Hamilton, M. C. Archer and G. M. Whitesides, in K. E. Pye and L. B. Wingard (eds.), Enzyme Engineering 2, Plenum Press, New York, 1974, p. 109.
7 R. S. Langer, C. R. Gardner, B. K. Hamilton and C. K. Colton, AIChE J., 23 (1977) 1.
8 C. K. Colton, M. I. Nemet and R. Y. K. Yang, AIChE Symp. Ser., 74 (1978) 8.
9 G. M. Whitesides, A. Chmurny, P. Garrett, A. Lamotte and C. K. Colton, in K. S. Pye and L. B. Wingard (eds.), Enzyme Engineering 2, Plenum Press, New York, 1974, p. 217.
10 G. M. Whitesides, A. Lamotte, O. Adalsteinsson and C. K. Colton, Methods Enzymol., XLIV, 1976, p. 887.
11 A. Pollak, R. L. Baughn and G. M. Whitesides, J. Am. Chem. Soc., 99 (1977) 2 366.
12 J. M. Lewis, S. L. Haynie and G. M. Whitesides, J. Org. Chem., 44 (1979) 864.

13 O. Adalsteinsson, A. Lamotte, R. F. Baddour, C. L. Colton and G. M. Whitesides, J. Mol. Catal., submitted (1977) (accompanying paper).
14 L. Noda, in P. D. Boyer, (ed.), The Enzymes, (1972) 8, chapt. 8, Academic Press, New York.
15 O. H. Callaghan, Biochem. J., 67 (1957) 651.
16 D. G. Rhoads and J. M. Lowenstein, J. Biol. Chem., 243 (1968) 3 963.
17 O. H. Callaghan and G. Weber, Biochem. J., 73 (1958) 473.
18 J. M. Blair, Eur. J. Biochem., 13 (1970) 384.
19 P. DeWeer and A. G. Lowe, J. Biol. Chem., 248 (1973) 2 829.
20 R. S. Langer, B. K. Hamilton, C. R. Gardner, M. Archer and C. K. Colton, AIChE J., 22 (1976) 1079.
21 L. Noda, J. Biol. Chem., 232 (1968) 237.
22 G. E. Schultz, K. Biedermann, W. Kabsch and R. H. Schirmer, J. Mol. Biol., 80 (1973) 857.
23 G. E. Schultz, M. Elzinga, F. Marx and R. H. Schirmer, Nature (London), 150 (1974) 120.
24 N. C. Price, M. Cohn and R. H. Schirmer, J. Biol. Chem., 250 (1975) 674.
25 R. S. Anthony and L. B. Spector, J. Biol. Chem., 246 (1971) 6 129.
26 I. A. Rose, M. Grunberg-Manago, S. T. Korey and S. Ochoa, J. Biol. Chem., 211 (1954) 734.
27 C. A. Janson and W. W. Cleland, J. Biol. Chem., 249 (1974) 2 567.
28 R. S. Langer, Jr., Ph. D. Thesis, Mass. Inst. Technol., Cambridge, MA, 1974.
29 D. F. Shriver, The Manipulation of Air-Sensitive Compounds, McGraw-Hill, New York, N.Y., 1969, Ch. 7.
30 H. C. Brown *et al.*, Organic Synthesis via Boranes, Wiley, New York, N.Y., 1975.
31 P. C. Jocelyn, Biochemistry of the SH Group, Academic Press, New York, N.Y., 1972.
32 M. Friedman, The Chemistry and Biochemistry of the Sulfhydryl Group in Amino Acids, Peptides, and Proteins, Pergamon Press, Oxford, 1973.
33 Yu. M. Torchinskii, in H. B. F. Dixon (trans.), Sulfhydryl and Disulfide Groups of Proteins, Plenum Press, New York, N.Y., 1974.
34 J. E. Taylor, J. F. Yan and J. Wang, J. Am. Chem. Soc., 88 (1966) 1 663.
35 J. E. Taylor and M. S. Swaisgood, Biochim. Biophys. Acta, 284 (1972) 268.
36 W. S. Allison, Acc. Chem. Res., 9 (1976) 293.
37 D. J. Smith and G. L. Kenyon, J. Biol. Chem., 249 (1974) 3 317.
38 D. J. Smith, E. T. Maggio and G. L. Kenyon, Biochemistry, 14 (1975) 766.
39 P. P. Trotta, L. M. Pinkus and A. Meister, J. Biol. Chem., 249 (1974) 1 915.
40 L. Noda and S. A. Kuby, J. Biol. Chem., 226 (1957) 551.
41 L. F. Kress, V. H. Bono and L. Noda, J. Biol. Chem., 241 (1966) 2 293.
42 T. A. Mahowald, E. A. Noltmann and S. A. Kuby, J. Biol. Chem., 237 (1962) 1 535.
43 A. Chrombach and D. Rodbard, Sep. Sci., 7 (1972) 663.
44 G. E. Means and R. E. Feeney, Chemical Modification of Proteins, Holden-Day, San Francisco, 1971.
45 J. F. Cavins and M. Friedman, Biochemistry, 6 (1967) 3 766.
46 J. F. Cavins and M. Friedman, J. Biol. Chem., 243 (1968) 3 357.
47 M. Friedman, J. F. Cavins and J. S. Wall, J. Am. Chem. Soc., 87 (1965) 3 672.
48 C. Tanford, The Hydrophobic Effect, Wiley-Interscience, New York, N.Y., 1973.
49 C. Tanford, Adv. Protein Chem., 24 (1970) 1.
50 Y. Nozaki and C. Tanford, J. Biol. Chem., 238 (1963) 4 074.
51 W. P. Jencks, Catalysis in Chemistry and Enzymology, McGraw-Hill, New York, N.Y., 1969, p. 323 ff.
52 W. P. Jencks, Federation Proc., Fed. Am. Soc. Exp. Biol., Suppl. No. 15,24(2), Pt. 3 (1965) 50.
53 N. M. Bikales, in N. M. Bikales, (ed.), Water Soluble Polymers, Plenum Press, New York, N.Y., 1973, p. 213.
54 A. Chrambach and D. Rodbard, Science, 172 (1971) 440.

55 D. Rodbard and A. Chrambach, Anal. Biochem., 40 (1971) 95.
56 M. L. Dirksen and A. Chrambach, Sep. Sci., 7 (1972) 747.
57 J. Brant, M. Fredriksson and L.-O. Anderson, Biochemistry, 13 (1974) 4 758.
58 D. R. Kearns, R. A. Holling, A. U. Khan and P. Radlick, J. Am. Chem. Soc., 89 (1967) 5 456.
59 T. Wilson and J. W. Hastings, Photophysiology, 5 (1970) 49.
60 I. R. Politzer, G. W. Griffin and J. L. Laseter, Chem.-Biol. Interact., 3 (1971) 73.
61 E. A. Ogryslo and C. W. Tang, J. Am. Chem. Soc., 92 (1970) 5 034.
62 C. Ouannis and T. Wilson, J. Am. Chem. Soc., 90 (1970) 6 527.
63 P. B. Merkel and D. R. Kearns, J. Am. Chem. Soc., 94 (1972) 7 244.
64 J. D. Spikes and R. Livingston, Adv. Rad. Biol., 3 (1969) 29.
65 W. A. Pryor, Free Radicals, McGraw Hill, New York, N.Y., 1966.
66 J. Flory, Principles of Polymer Chemistry, Cornell Univ. Press, Ithaca, New York, 1953, Ch. IV.
67 M. Bonifacic and K.-D. Asmus, J. Phys. Chem., 80 (1976) 2 426.
68 D. Rodbard and A. Chrambach, Sep. Sci., 7 (1972) 663.