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ACTIVATION OF RAT LIVER PYRIMIDINE NUCLEOSIDE MONOPHOSPHATE KINASE

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Summary

The activity of the pyrimidine nucleoside monophosphate kinase (ATP:dCMP phosphotransferase, EC 2.7.4.14) from rat liver is dependent upon the presence of sulfhydryl-reducing agents. Addition to the inactive enzyme of 2-mercaptoethanol (5 mM), a reagent specific for cleavage of disulfide bonds, effects a reduction in molecular weight from approx. 53 000 to 17 000, measured by molecular sieve chromatography. This low molecular weight form is partially active in the presence of 2-mercaptoethanol (5 mM). In absence of 2-mercaptoethanol, the low molecular weight form is inactive. Higher concentrations of 2-mercaptoethanol (50 mM) fully reactivate the CMP(ATP) kinase activity followed by dCMP(ATP) and CMP(dCTP) kinase activities in a sequential manner, without further change in molecular weight. Alkylation by iodoacetamide of the enzyme at different stages of reactivation in dithiothreitol suggests an ordered appearance of the various enzyme activities. Furthermore, iodoacetamide inactivates the fully active enzyme. Thioredoxin was found to activate the enzyme in a manner similar to 2-mercaptoethanol and dithiothreitol. These results are consistent with the interpretation that the mechanism of activation of the enzyme involves cleavage of inter- and intramolecular disulfide bonds.

Introduction

Previously, we reported a 2100-fold purification from rat liver of a pyrimidine nucleoside monophosphate kinase (ATP:dCMP phosphotransferase, EC 2.7.4.14) which phosphorylates CMP, UMP and dCMP with ATP or dATP as phosphate donor [1]. dUMP cannot be phosphorylated. It was found also that dCTP is an effective phosphate donor for CMP, but not for UMP or dCMP. All activities appear to be the properties of a single enzyme, and are strictly dependent upon sulfhydryl-reducing agents, although to differing degrees. A natural-

ly occurring protein, thioredoxin, was shown to be the most efficient of any natural or artificial thiol tested. The intriguing phosphate donor and acceptor specificity of the kinase and the strict dependence of activity on sulfhydryl-reducing agents has prompted the study, reported here, of the mechanism of activation by thioredoxin and other thiols. Since thioredoxin is thought to be involved in the in vitro reaction of ribonucleotide reductase [2], it is tempting to attribute an even greater role to thioredoxin in the biosynthesis of pyrimidine nucleotides.

Materials and Methods

Materials

The ^{14}C-labelled nucleotides and 2-mercaptoethanol were purchased from Schwarz-Mann. Lactate dehydrogenase (chicken heart), pepsinogen (swine stomach mucosa), and all other nucleotides were reagent grade products obtained from P-L Biochemicals. Pyruvate kinase (rabbit muscle) was a product of Calbiochem. Dithiothreitol, iodoacetamide, myokinase (rabbit muscle), and sodium phosphoenolpyruvate were purchased from Sigma Chemical Co. Bio-Gel P-100 was a product of Bio-Rad Laboratories. Bovine serum albumin was obtained from Pentex. Thioredoxin [3] and thioredoxin reductase [4] were prepared from rat Novikoff ascites hepatoma cells.

Enzyme purification

Pyrimidine nucleoside monophosphate kinase was purified 2100-fold from rat liver [1]. When required, the enzyme was concentrated in an Amicon Diaflo ultrafiltration apparatus with a Pellicon PS membrane at 40 lb/inch^2 of N_2. When 2-mercaptoethanol removal was necessary, concentrated enzyme (4 ml) was dialyzed 16 h against 5 mM Tris/acetate (pH 7.5)/150 mM KCl, and monitored for complete loss of enzymatic activity by the spectrophotometric method of assay.

Assay of enzyme

Two types of assay were used. The radiochemical assay measures the conversion of nucleoside monophosphate to nucleoside diphosphate by separation of the reactants and products upon paper electrophoresis. The spectrophotometric assay measures the oxidation of NADH. In the latter assay pyruvate kinase is coupled with lactate dehydrogenase in order to measure ADP. The disappearance of NADH, measured by the decrease in absorbance at 340 nm, is a measure of the kinase activity.

The standard conditions of the radiochemical assay were as follows: for the CMP(ATP) reaction: 40 mM Tris · HCl (pH 7.5), 3.07 mM ATP, 7.50 mM $MgCl_2$, 3.46 mM [^{14}C]CMP (1.3 Ci/mol) enzyme, and water, to a final volume of 50 µl. For the CMP(dCTP) reaction 8.01 mM dCTP, 18.75 mM $MgCl_2$, and 10.05 mM [^{14}C]CMP (1.3 Ci/mol) were substituted. For the dCMP(ATP) reaction 6.07 mM ATP, 15.00 mM $MgCl_2$, and 10.34 mM [^{14}C]dCMP (1.3 Ci/mol) were substituted. The reaction was carried out at 37°C for a length of time which converted less than 10% of substrate to product. The remainder of the assay procedure was carried out as previously described [1].

The standard conditions of the spectrophotometric assay were as follows: 100 mM Tris · HCl (pH 7.6), 3.00 mM ATP, 7.00 mM $MgCl_2$, 3.00 mM sodium phosphoenolpyruvate, 20 mM KCl, 0.131 mM NADH, 7 μg pyruvate kinase (1930 units/mg), 10 μg lactate dehydrogenase (300 units/mg), 1.00 mM CMP, enzyme, and water to a final volume of 1.00 ml. Enzymatic activity was measured at 37°C on a Cary 15 Recording Spectrophotometer. A small blank rate obtained for the enzyme in absence of nucleoside monophosphate was subtracted.

Initial velocities were measured. In all cases the conversion of substrate to product was maintained close to 10% by varying time of incubation.

The concentration of nucleotides was determined spectrophotometrically, using published molar extinction coefficients. Protein was measured with Folin phenol reagents [5], after precipitation with 10% trichloroacetic acid and washing with 5% trichloroacetic acid.

Bio-Gel P-100 chromatography

Bio-Gel P-100 was suspended in 5 mM Tris/acetate (pH 7.5) and hydrated at 20°C for 24 h. After removal of fines from the Bio-Gel P-100, a column (1 × 57.5 cm) was packed and equilibrated at 6°C in 5 mM Tris/acetate (pH 7.5) containing 2-mercaptoethanol, if indicated. Enzyme (1 ml) was applied to the column at a flow rate of 0.2 ml/min. Fractions of 0.45 ml were collected and assayed for CMP kinase activity by the spectrophotometric assay, with and without 30 min preincubation at 37°C in 50 mM 2-mercaptoethanol. The Bio-Gel P-100 column was calibrated with the following molecular weight markers; cytochrome *c* (12 400), myokinase (rabbit muscle; 21 000), pepsinogen (swine stomach mucosa; 41 000), and bovine serum albumin (66 000).

Results

Effect of dithiothreitol concentration on reactivation

The inactive enzyme, preincubated at 37°C for 1 h in increasing concentrations of dithiothreitol, exhibits differential reactivation of CMP(ATP), dCMP(ATP), and CMP(dCTP) activities (Fig. 1). A low concentration of dithiothreitol (0.5 mM) is required for 50% reactivation of CMP(ATP) activity, whereas higher concentrations of dithiothreitol are required for a similar degree of reactivation of dCMP(ATP) (2 mM), and CMP(CTP) (11 mM) activities. All activites are maximal after 1 h of preincubation at 37°C in 50 mM dithiothreitol.

Effect of time on reactivation

Differential reactivation is further confirmed in an experiment in which the time course of reactivation is plotted (Fig. 2). The enzyme, preincubated at 37°C in 50 mM dithiothreitol, exhibits different reactivation kinetics for the three activities. The CMP(ATP) activity rapidly reactivates, whereas the dCMP(ATP) and CMP(dCTP) activities require a significantly longer time to reactivate. In some instances the CMP(ATP) activity starts to decline after reaching a maximal activity after 20 min of preincubation. Other experiments showed that the enzyme held at 37°C for 30 min in absence of dithiothreitol is not

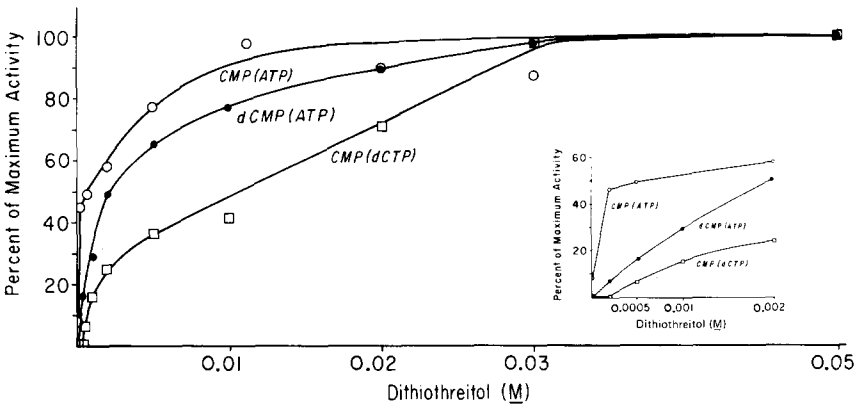

Fig. 1. Effect of dithiothreitol concentration on reactivation. Dialyzed enzyme was preincubated with increasing concentrations of dithiothreitol for 1 h at 37°C prior to assay by the radiochemical method as described in Materials and Methods. 100% maximum activity values are: for CMP(ATP), 194 μmol CDP formed/h per mg; for CMP(dCTP), 340 μmol CDP formed/h per mg; and for dCMP(ATP), 131 μmol dCDP formed/h per mg. Protein concentration was 0.16 mg/ml during preincubation.

capable of reactivating fully when dithiothreitol is added. Thus the effect of temperature appears responsible for the decline in CMP kinase activity.

Bio-Gel P-100 chromatography

Attempts to measure the molecular weight of the enzyme by molecular sieve

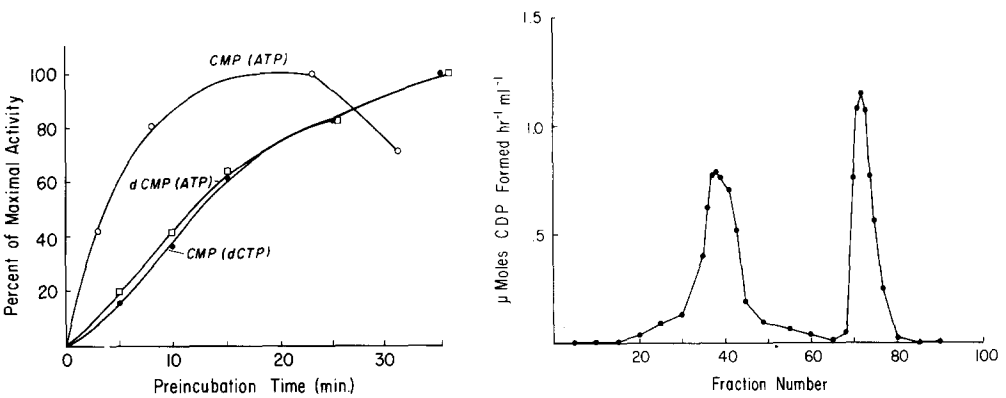

Fig. 2. Effect of preincubation time on reactivation. Dialyzed enzyme was preincubated in 50 mM dithiothreitol at 37°C for increasing lengths of time prior to assay by the radiochemical assay as described in Materials and Methods. To minimize the effect of reactivation during time of assay, initial velocities were obtained from product formation after 5, 10 and 15 min of assay for every preincubation time point plotted. 100% maximum activity values are: for CMP(ATP), 170 μmol CDP formed/h per mg; for CMP(dCTP), 331 μmol CDP formed/h per mg; and for dCMP(ATP), 142 μmol dCDP formed/h per mg. Protein concentration during preincubation was 0.17 mg/ml.

Fig. 3. Bio-Gel P-100 chromatography of inactive enzyme. Concentrated, dialyzed enzyme (1 ml; 226 μg) 0 μmol CDP formed/h per ml measured in absence of 2-mercaptoethanol; 9.3 μmol CDP formed/h per ml, measured following 1 h preincubation in 50 mM 2-mercaptoethanol) was applied to the Bio-Gel P-100 column equilibrated in 5 mM Tris/acetate (pH 7.5). Fractions (0.45 ml) were assayed for CMP kinase activity by the spectrophotometric method after preincubation in 50 mM 2-mercaptoethanol for 30 min at 37°C.

chromatography revealed that the molecular weight is dependent upon the presence of a sulfhydryl-reducing agent in the equilibrating buffer.

When the enzyme is dialyzed to remove 2-mercaptoethanol and the resulting inactive enzyme chromatographed on a column of Bio-Gel P-100 equilibrated in 5 mM Tris/acetate (pH 7.5), all fractions emerge completely inactive when assayed in absence of 2-mercaptoethanol. However, when fractions are assayed following preincubation in 50 mM 2-mercaptoethanol, two peaks of CMP kinase activity are generated, corresponding to average molecular weights 53 000 and 17 000 (Fig. 3).

The ratio of activities obtained with CMP, UMP, and dCMP as substrates is identical for fractions of both molecular weight values. In repeating this experiment, the ratio of activity units associated with the small molecular weight varies with respect to the large one. In some experiments only the large molecular weight species is obtained. No explanation is offered. However, in every experiment with dialyzed enzyme, the higher molecular weight peak contains greater than 50% of the total units. It is interesting to note that if the same experiment is performed on dialyzed enzyme obtained from the penultimate step of the protocol of purification, there consistently appears only one peak of activity of the larger molecular weight form.

When the enzyme, partially active by virtue of storage in 5 mM 2-mercaptoethanol, is chromatographed in 5 mM Tris/acetate (pH 7.5)/5 mM 2-mercaptoethanol, a single peak of CMP kinase activity is obtained, corresponding exactly in location to the low molecular weight species presented in Fig. 3. To achieve maximal activity, however, fractions require preincubation in 50 mM 2-mercaptoethanol. UMP and dCMP are also substrates for the fractions found active with CMP. This peaks of activity has an approximate molecular weight of 17 000.

When the enzyme is fully reactivated by preincubation in 50 mM 2-mercaptoethanol for 30 min at 37°C and applied to the column equilibrated in 5 mM Tris/acetate (pH 7.5)/100 mM 2-mercaptoethanol, again a single peak of CMP kinase activity occurs, corresponding to a molecular weight of 17 000. However, preincubation of fractions in 2-mercaptoethanol is unneccessary, as the enzyme emerges in a fully active state.

Since depolymerization appears to be a function of the sulfhydryl-reducing agent, dissociation into subunits must be a step in the activation of the enzyme. However, dissociation alone is not sufficient to generate activity, since the small molecular weight fraction is active only after preincubation in 2-mercaptoethanol.

Since thioredoxin is the best physiological candidate for activation of the enzyme (17 μM thioredoxin gives 1—3 times the activity as 85 mM dithiothreitol), chromatography was repeated in absence of 2-mercaptoethanol using dialyzed enzyme made fully active by preincubation with thioredoxin, thioredoxin reductase, and NADPH (Fig. 4). When fractions are assayed in absence of 2-mercaptoethanol, a single peak of CMP kinase activity occurs with average molecular weight 17 000. However, when fractions are assayed following an additional preincubation in 2-mercaptoethanol (50 mM), two peaks are obtained with average molecular weights of 17 000 and 53 000. A larger heterogeneity of molecular weight of the enzymic species is noted with the thio-

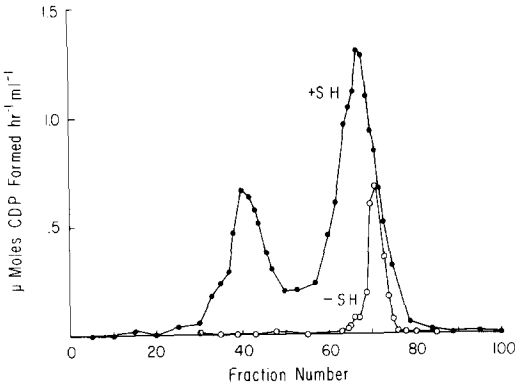

Fig. 4. Bio-Gel P-100 chromatography of thioredoxin-reactivated enzyme. Concentrated, dialyzed enzyme (0.7 ml; 158 μg; 0 μmol CDP formed/h per ml, measured in absence of thioredoxin) was made fully active (14.1 μmol CDP formed/h per ml) by 70 min preincubation at 37°C with thioredoxin (33 μg), thioredoxin reductase (172 μg), and NADPH (1 mg) in a volume of 1.1 ml, then applied to a column of Bio-Gel P-100 equilibrated in 5 mM Tris/acetate (pH 7.5). Fractions (0.45 ml) were assayed for CMP kinase activity by the spectrophotometric assay with and without preincubation in 50 mM 2-mercaptoethanol for 30 min at 37°C.

redoxin-activated enzyme. Thioredoxin thus appears to reactivate the kinase in a manner similar to dithiothreitol and 2-mercaptoethanol; that is, by a reduction in molecular weight of the enzyme, followed by cleavage of intramolecular disulfide bonds, generating activity. The much lower concentration of thioredoxin required to reactivate the enzyme suggests protein-protein interactions, other than those mediated by sulfhydryl groups, which may direct thioredoxin to specific rather than random disulfides on the kinase molecule. Reoxidation of intramolecular disulfides and reassociation to the heavier molecular weight form appear to occur after removal of thioredoxin during gel filtration.

In order to investigate the analogy between activation of the enzyme by thioredoxin and dithiothreitol, K_m values were determined for CMP, UMP, and dCMP with thioredoxin-reactivated enzyme. Enzyme (1 μg) was preincubated for 60 min at 37°C with thioredoxin (30 μg), thioredoxin reductase (26 μg), and NADPH (45 μg) in a volume of 150 μl. The radiochemical assay was used to measure enzymatic activities. K_m values were found to be as follows: for CMP 0.070 ± 0.031 mM, for UMP 0.063 ± 0.019 mM and for dCMP 5.57 ± 0.75 mM. These values are only slightly higher than K_m values obtained with enzyme preincubated for 60 min at 37°C in 50 mM dithiothreitol: for CMP 0.030 ± 0.007 mM, for UMP 0.040 ± 0.009 mM, and for dCMP 2.77 ± 0.39 mM [1].

Alkylation by iodoacetamide

Iodoacetamide was used to irreversibly alkylate available populations of sulfhydryl groups exposed by pretreatment with dithiothreitol. The details of the experimental procedure and results are presented in Table I. Dialyzed, inactive enzyme was treated with 50 mM dithiothreitol for an appropriate length of time to generate different CMP and dCMP kinase activities. This could be achieved as a result of the different in reactivation kinetics (Fig. 2). Iodoaceta-

TABLE I

SEQUENTIAL ALKYLATION OF SULFHYDRYLS BY IODOACETAMIDE

Concentrated enzyme was preincubated with dithiothreitol (50 mM) for 5, 25, or 60 min at 37°C. Aliquots of 20 μl for CMP kinase and 60 μl for dCMP kinase were taken to measure activity prior to alkylation with the spectrophotometric assay. Iodoacetamide was then added to a final concentration of 155 mM, and incubated for 15 min. Activity after alkylation was measured. Dithiothreitol was then added to a final concentration of 89 mM and incubated with the enzyme at 37°C to reduce remaining disulfide bonds and to generate remaining activities. The time of reexposure to dithiothreitol was 55 min for the sample preincubated 5 min in dithiothreitol prior to alkylation; 35 min for the sample preincubated 25 min; and 15 min for the sample preincubated 60 min. Activity of alkylated enzyme reexposed to dithiothreitol was then measured. Iodoacetamide at a final concentration used in the enzymatic assay mixture does not interfere with the activites of pyruvate kinase and lactate dehydrogenase. The specific activity of the fully active enzyme is 92 $\mu mol \cdot h^{-1} \cdot mg^{-1}$ for CMP(ATP) kinase and 18 $\mu mol \cdot h^{-1} \cdot mg^{-1}$ for dCMP(ATP) kinase.

Time of preincubation in dithiothreitol	Substrate	Activity prior to alkylation ($\mu mol \cdot h^{-1} \cdot mg^{-1}$)	Activity ratio $\frac{CMP^*}{dCMP}$	Activity after alkylation ($\mu mol \cdot h^{-1} mg^{-1}$)	Activity of alkylated enzyme reexposed to dithiothreitol ($\mu mol \cdot h^{-1} \cdot mg^{-1}$)	Activity ratio $\frac{CMP^{**}}{dCMP}$
5 min	CMP	75	11	1	12	2
	dCMP	7		0	9	
25 min	CMP	87	8	1	6	2
	dCMP	11		0	3	
60 min	CMP	92	5	1	4	4
	dCMP	18		0	1	

* Activities were measured during activation in dithiothreitol of the native enzyme.

** Activities were measured during activation in dithiothreitol of the partially alkylated enzyme.

mide was then added to alkylate the available sulfhydryl groups. The partially alkylated enzyme was assayed for kinase activities and reexposed to dithiothreitol in order to reduce the remaining disulfide bonds and to generate remaining activities. If a sequential cleavage of intramolecular disulfide bonds confers an ordered appearance of enzymatic activities, one would expect the CMP/dCMP activity ratio of the enzyme after alkylation and reexposure to dithiothreitol to differ from the CMP/dCMP activity ratio of the enzyme during its normal course of preincubation in dithiothreitol. The results are consistent with this interpretation.

If intermolecular disulfide bonds are responsible for subunit association in the large molecular weight form, alkylation of the small molecular weight form by iodoacetamide should prevent reassociation. The dialyzed, inactive enzyme was treated with a minimal amount of dithiothreitol in order to generate the small molecular weight form, but to reduce as few disulfide bonds as possible. The extent of enzyme activation monitored the effects of this treatment (0% maximal dCMP kinase activity; 17% maximal CMP kinase activity). Iodoacetamide was added to alkylate available sulfhydryl groups. The partially alkylated enzyme was then chromatographed on the same column of Bio-Gel P-100, equilibrated in 5 mM Tris/acetate (pH 7.5) in absence of 2-mercaptoethanol or dithiothreitol. Fractions were assayed following 30 min of preincubation in

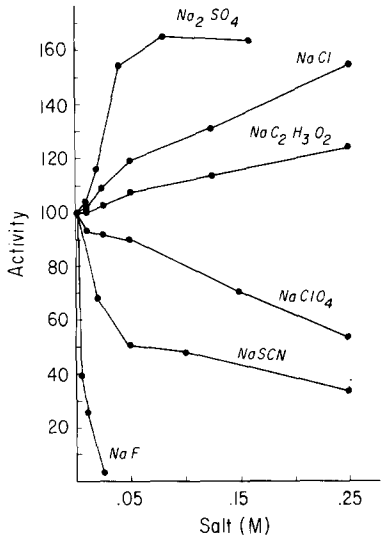

Fig. 5. Effect of neutral salts on CMP kinase activity. The spectrophotometric assay was used to measure activity of CMP kinase, following preincubation with 50 mM dithiothreitol in the presence of varying concentrations of neutral salts. The salt and dithiothreitol concentrations were maintained in the assay. Activities are expressed relative to that obtained after preincubation in 50 mM dithiothreitol in absence of salt (100 = 18.6 μmol CDP formed/h per ml). These salts (0.25 M) were shown to cause no change in the ability of pyruvate kinase and lactate dehydrogenase to oxidize NADH stroichiometrically upon ADP addition, under the conditions described for the assay.

50 mM 2-mercaptoethanol. The enzyme activity corresponded to an approximate molecular weight of 17 000. The inability of the small molecular weight form to reassociate to the large form after partial alkylation by iodoacetamide suggests that intermolecular disulfide bonds may be responsible for subunit association. Alternatively, conformational change occurring upon alkylation of enzyme could prevent reassociation.

Neutral salt effects

Because the enzyme is unstable when fully activated, the possibility was investigated that interactions other than those involved with sulfhydryl groups contribute to the conformational stability of the enzyme. The Hofmeister series of anions is a simple and useful probe of the role of the hydrophobic interactions (Fig. 5) [6]. In general, anions may be listed as a series in increasing degree of strengthening hydrophobic interactions of proteins: SCN^-, ClO_4^- < Acetate$^-$, Cl^- < F^- < SO_4^{2-}. Anions promoting hydrophobic interactions appear to stabilize the active conformation of the enzyme, as evidenced by the apparent activation. The only exception is fluoride, which is inhibitory as such low concentrations that a site-specific, rather than hydrophobic effect is indicated [7].

Discussion

The pyrimidine nucleoside monophosphate kinase from calf thymus [8] and that from rat liver [1] are inactive in absence of sulfhydryl-reducing agents.

Relatively low concentrations of 2-mercaptoethanol (5 mM) effect a reduction in molecular weight of the enzyme from approx. 53 000 to 17 000. This reduction in molecular weight is a necessary but insufficient condition for activation. Because 2-mercaptoethanol and dithiothreitol are reagents specific for cleavage of disulfide bonds, generation of the small molecular weight form of the enzyme may result from cleavage of intermolecular disulfide bonds. Inability of the low molecular weight form, partially alkylated by iodoacetamide, to reassociate to the larger form in absence of a sulfhydryl-reducing agent supports this hypothesis. Subsequent cleavage of disulfide bonds of the low molecular weight form gives rise to the various enzymatic activities in a sequential manner. Either by increasing the time of preincubation for a fixed concentration of dithiothreitol, or by increasing the concentration of dithiothreitol for a fixed time of preincubation, one generates the CMP(ATP) kinase activity, followed by the dCMP(ATP) and CMP(dCTP) activities (Figs. 1 and 2). Sequential alkylation by iodoacetamide (Table I) shows that cleavage of disulfide bonds and presence of free sulfhydryl groups are essential for activity. The alkylated enzyme is inactive. A longer time of incubation in dithiothreitol is required to cleave disulfides responsible for dCMP(ATP) kinase activity than for CMP(ATP) kinase activity, suggesting that the "dCMP" disulfides are less accessible to cleavage. In addition, it was found that the enzyme made fully active by disulfide reduction is unstable. Hydrophobic interactions were shown to be important in maintaining the active conformation of the enzyme (Fig. 5).

Whether this in vitro mechanism of activation has a counterpart in the in vivo regulation of the enzyme activity remains to be elucidated. However, the finding that thioredoxin activates the kinase much more efficiently and in a similar manner to dithiothreitol, would suggest that the concentration of thiols in the cell may be involved in regulation of the kinase activity.

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