Pig Liver Phosphomevalonate Kinase. 2. Participation of Cysteinyl and Lysyl Groups in Catalysis†

Sergio Bazaes, Enrique Beytía,‡ Ana María Jabalquinto,§ Francisco Solís de Ovando, Isabel Gómez, and Jaime Eyzaguirre*

ABSTRACT: Phosphomevalonate kinase from pig liver is inactivated by 5,5′-dithiobis(2-nitrobenzoate) and pyridoxal 5′-phosphate. The substrate phosphomevalonate protects the enzyme against inactivation by these reagents. Inactivation by 5,5′-dithiobis(2-nitrobenzoate) is complete and may be reverted by 2-mercaptoethanol or dithiothreitol. Experiments carried out with partially inactivated enzyme show no change in the k_{cat} or in the apparent K_m for the substrates, as compared with the native enzyme, indicating the existence of two populations of molecules, one intact and the other totally inactive. These results suggest that 5,5′-dithiobis(2-nitrobenzoate) reacts with the only cysteinyl residue of the enzyme and that this residue is located in or near the active site. Inhibition by pyridoxal 5′-phosphate can be reverted, either by dialysis or by the addition of lysine, but not if the partially inactivated enzyme is treated previously with $NaBH_4$, in agreement with the formation of a Schiff base between pyridoxal 5′-phosphate and an amino group of the enzyme. This is further supported by the appearance of an absorption band with a maximum at 325 nm in the enzyme treated with pyridoxal 5′-phosphate and $NaBH_4$. Pyridoxal and pyridoxamine 5′-phosphate are weaker inhibitors than pyridoxal 5′-phosphate, suggesting a specific effect due to the phosphate and aldehyde groups. The enzyme is not completely inactivated by pyridoxal 5′-phosphate, even at a molar ratio of 350, or by a second inactivation treatment after reduction with $NaBH_4$. The partially modified enzyme shows a lower K_m for phosphomevalonate than the native enzyme, suggesting that the reactive group is located near the binding site of phosphomevalonate. The lower K_m may reflect an effect of the positive charge of the pyridoxal 5′-phosphate ring nitrogen, enhancing the binding of phosphomevalonate. Values of 8.15 at 24 °C and 7.95 at 31 °C have been determined for the pK of the reactive group. A ΔH_i of 11.8 kcal/mol has been estimated, in agreement with the values expected for an amino group. One amino group per active site is involved in the enzyme inactivation as shown by kinetic data. Quantification of the number of moles of pyridoxal 5′-phosphate bound per mole of enzyme is not conclusive but supports this assertion. This group may correspond to an ϵ-amino group of lysine.

Pig liver phosphomevalonate kinase has been obtained in homogeneous form as described in the preceding paper (Bazaes et al., 1980). The enzyme has a molecular weight of ~22000, consists of one polypeptide chain, and has only one cysteinyl residue.

The activity of the enzymes from pig liver (Bazaes et al., 1980) and *Hevea brasiliensis* (Skilleter & Kekwick, 1971) seems to be dependent on the integrity of sulfhydryl groups. This is shown by their loss of activity in the absence of 2-mercaptoethanol or other SH-containing reagents. In addition, Hellig & Popják (1961) observed that pig liver phosphomevalonate kinase is inhibited by *p*-(chloromercuri)benzoate.

The presence of sulfhydryl groups has been observed in the active site of several kinases, such as muscle phosphofructokinase (Kemp & Forest, 1968; Schwartz et al., 1976), muscle pyruvate kinase (Flashner et al., 1972), yeast hexokinase (Otieno et al., 1977), and *Escherichia coli* thiokinase (Nishimura et al., 1975).

These findings prompted us to look for the presence of this group in the active site of pig liver phosphomevalonate kinase. For this purpose, DTNB[1] was used as a specific reagent (Ellman, 1959).

Lysine ϵ-amino groups have also been found to participate in the catalysis of several enzymes utilizing phosphorylated substrates, e.g., *Candida utilis* 6-phosphogluconate dehydrogenase (Rippa et al., 1967), rabbit muscle phosphoglucose isomerase (Schnackerz & Noltmann, 1971), pig kidney fructose-1,6-bisphosphatase (Colombo & Marcus, 1974), and, among the kinases, sheep heart phosphofructokinase (Setlow & Mansour, 1972) and muscle pyruvate kinase (Johnson & Deal, 1970).

We have also studied the possible contribution of amino groups to catalysis in phosphomevalonate kinase. Pyridoxal 5′-phosphate, shown to be highly specific for amino groups located at the active site of enzyme requiring phosphorylated substrates, has been used as the modifying reagent.

This work shows that both sulfhydryl and amino groups are important for the activity of pig liver phosphomevalonate kinase.

Experimental Procedure

Chemicals. Nucleotides, pyruvate kinase, lactate dehydrogenase, Tris, 2-mercaptoethanol, dithiothreitol, and PLP and analogues were obtained from Sigma Chemical Co. All other reagents were of analytical grade.

The preparation of substrate, purification of phosphomevalonate kinase, and assay of activity have been described in the preceding paper (Bazaes et al., 1980). The experiments described below were carried out either with the final homo-

† From the Laboratorio de Bioquímica, Instituto de Ciencias Biológicas, Universidad Católica de Chile, Casilla 114-D, Santiago, Chile. *Received December 14, 1978; revised manuscript received December 27, 1979.* This work was supported by Grant No. 64/72 from the Dirección de Investigación, Universidad Católica, by the Regional Program of UNDP–UNESCO RLA 76/006, and by funds from the Organization of American States.

‡ Deceased March 18, 1979.

§ Present address: Departamento de Medicina Experimental, Facultad de Medicina Oriente, Universidad de Chile, Casilla 16038, Santiago, Chile.

[1] Abbreviations used: DTNB, 5,5′-dithiobis(2-nitrobenzoate); PLP, pyridoxal 5′-phosphate; Tris, tris(hydroxymethyl)aminomethane; MVAP, phosphomevalonate; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; CAPS, 3-cyclohexylamino-1,1-propanesulfonic acid.

0006-2960/80/0419-2305$01.00/0

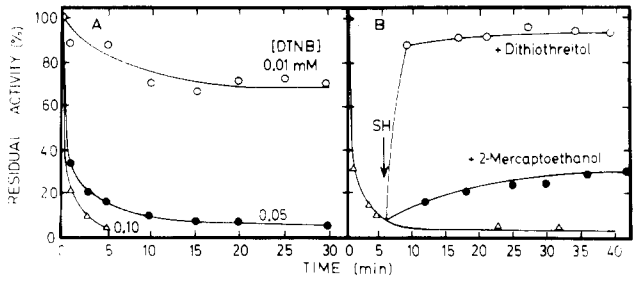

FIGURE 1: (A) Inactivation of phosphomevalonate kinase at different concentrations of DTNB. Enzyme (1.71 μM; sp act. 2.5 units/mg of protein) was incubated with DTNB at the concentrations indicated, as described under Experimental Procedure. No loss of activity was observed in a control in the absence of DTNB. (B) Effect of 2-mercaptoethanol and dithiothreitol on the inactivation by DTNB. Enzyme (2.06 μM; sp act. 2.5 units/mg of protein) was incubated with 0.055 mM DTNB as described under Experimental Procedure. At the arrow, two aliquots were removed for assay with 1 mM dithiothreitol or 1 mM 2-mercaptoethanol.

geneous preparation or with the preparation obtained after the Bio-Gel P-150 chromatography step (which is very stable), when the use of pure enzyme was not essential. Enzyme activity was measured spectrophotometrically (Bazaes et al., 1980).

Reaction with DTNB. The enzyme was dialyzed against two changes, for 1.5 h each, of 1000 volumes of 10 mM phosphate buffer, pH 7.5 (buffer A). The reaction was usually started by adding 0.01 mL of DTNB, dissolved in buffer A of an appropriate concentration, to 0.09 mL of enzyme (also in buffer A). After variable incubation periods at 30 °C, 0.01-mL aliquots were taken for enzyme assay. Controls were performed adding similar volumes of buffer A, instead of DTNB, to the incubation mixture.

Reaction with PLP. Unless stated otherwise, enzyme was incubated at 30 °C with varying concentrations of PLP in buffer A containing 10 mM 2-mercaptoethanol. At appropriate times, aliquots were taken for enzyme assay.

The assay was performed immediately with the usual procedure, without adding PLP to the mixture. No reversal of inhibition was observed in the first 2 min of the assay. Controls were performed by adding buffer A instead of PLP. The reaction of enzyme with the PLP analogues, pyridoxal and pyridoxamine 5′-phosphate, was studied in the same manner. Fresh solutions of reagents were always used. No effect on the activity of the auxiliary enzymes was observed at the concentrations of the inactivating reagents present in the assays.

Determination of Phosphopyridoxyl Residue Bound per Mole of Enzyme. Purified enzyme was dialyzed for 6 h against 10 mM imidazole–1 mM dithiothreitol, pH 7.5, and concentrated by ultrafiltration. Aliquots (final protein concentration 0.5 mg/mL) were incubated for 25 min at 30 °C at different concentrations of PLP (1–20 μM), and the reaction was stopped by the addition of $NaBH_4$ to a final concentration of 1.4 mM. A control lacking PLP was included. All samples were assayed for activity and dialyzed for 20 h in the dark against 10 mM imidazole buffer, pH 7.5. The spectra of the dialyzed samples were recorded with a Cary 118C spectrophotometer, and the molar ratio between phosphopyridoxyl residues and enzyme was estimated from the absorbance at 325 nm (subtracting the control sample) by using an extinction coefficient of 9710 M^{-1} cm^{-1} (Glazer et al., 1975).

Results

Inactivation with DTNB. As shown in Figure 1A, the incubation of the enzyme with DTNB produces a loss of enzyme activity which depends on the DTNB concentration; almost complete inactivation is observed after 5 min with 0.1 mM DTNB. Controls, incubated under the same conditions in the absence of DTNB, show no loss of activity. The inactivation may be reverted by sulfhydryl compounds like 2-mercaptoethanol and dithiothreitol, as shown in Figure 1B. At identical concentrations (1 mM), the reactivation by dithiothreitol is considerably greater and almost complete.

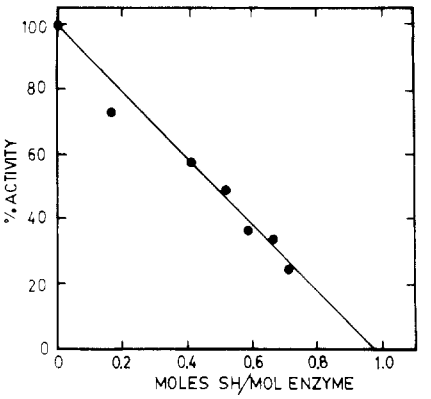

FIGURE 2: Determination of the number of reactive sulfhydryl groups as a function of enzyme activity. 4.48 units of pure enzyme (5.57 nmol) was incubated in buffer A with 0.040 mM DTNB. Aliquots for the assay of enzyme activity were taken at different times during 30 min. Another similarly treated sample was used to determine the change in absorbance at 412 nm for the same period of time. The number of sulfhydryl residues modified was estimated according to Ellman (1959), assuming a molecular weight for the enzyme of 22000.

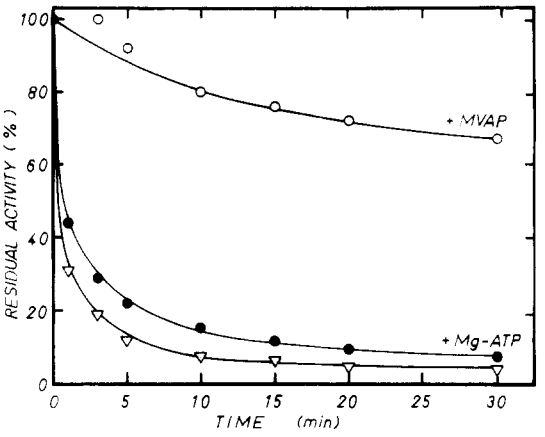

FIGURE 3: Effect of substrates on the inactivation of DTNB. Enzyme (1.71 μM; sp act. 2.5 units/mg of protein) was preincubated for 5 min at 30 °C with 1.5 mM MVAP or 2.3 mM ATP plus 4.5 mM $MgCl_2$ in buffer A. 0.056 mM DTNB was added at time 0. (▽) Inactivation without substrates added.

The number of essential sulfhydryl groups was determined by titrating a sample of the enzyme with DTNB according to Ellman (1959). Another sample of enzyme, identically treated, was used to measure residual enzyme activity. Figure 2 shows that by extrapolating to zero activity, it can be estimated that 0.98 mol of sulfhydryl groups reacts per mol of enzyme, assuming a molecular weight of 22000. Since the enzyme contains one cysteine residue per molecule (Bazaes et al., 1980), these results indicate that this group is modified by DTNB.

If MVAP is added prior to DTNB, the enzyme is partially protected from inactivation. As shown in Figure 3, after 30 min the loss of activity is of about 30%; Mg-ATP, on the other hand, has little protective effect. These results suggest that the cysteine residue of the enzyme is located at or near the binding site for MVAP.

Table I: Kinetic Constants of Native and DTNB-Modified Phosphomevalonate Kinase[a]

	$K_{m,app}$ (mM)		V_{max} [μmol/(min mL)]	
	MVAP	ATP	MVAP	ATP
modified enzyme	0.084	0.256	2.67	2.20
control	0.082	0.266	0.66	0.73

[a] 5.5 units of enzyme (sp act. 5.5 units/mg of protein) was incubated with 0.025 mM DTNB at 30 °C for 15 min and then placed in ice. DTNB was omitted from the control incubation.

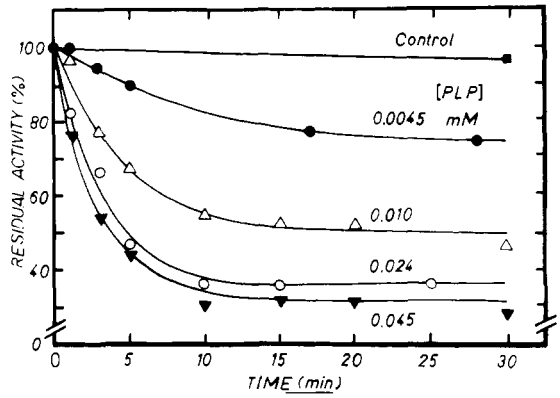

FIGURE 4: Inactivation of phosphomevalonate kinase by different concentrations of PLP. Enzyme (1.17 μM; sp act. 10 units/mg of protein) was incubated at 30 °C in buffer A as indicated under Experimental Procedure at the given concentrations of PLP.

Table I shows the apparent V_{max} and K_m for the native and DTNB-modified enzyme. No change in apparent K_m is observed for both substrates after an inactivation of 75%. These results suggest that two populations of enzyme molecules are present after partial inactivation, one of native and another of totally inactivated molecules. This interpretation is confirmed by calculating the value of the catalytic constants for the native and partially modified enzyme from the equation

$$V_{max} = k_{cat}[E] \quad (1)$$

The residual concentration of active enzyme after DTNB treatment was estimated by subtracting from the initial enzyme concentration the amount titrated with DTNB, as determined by the method of Ellman (1959). The values obtained for k_{cat} were 609 and 635 mol of MVAP phosphorylated per min per mol of enzyme for the native and partially inactivated enzyme, respectively.

Inactivation by PLP. Incubation of the enzyme with different concentrations of PLP produces inactivation of the enzyme equilibrium being reached after ~15 min (Figure 4). The degree of inactivation observed was concentration dependent, but total inhibition could not be observed even by using concentrations of 0.5 mM PLP. A double-reciprocal plot (not shown) of PLP concentration against the degree of inactivation gives by extrapolation to infinite PLP concentration 23% residual activity. An apparent dissociation constant of 0.0072 mM for the enzyme–PLP complex was estimated from this graph.

The reaction is fairly specific for PLP. Incubation with pyridoxal for 20 min at concentrations of 0.024 and 0.24 mM produced 10 and 25% inactivation, respectively. Pyridoxamine 5′-phosphate at 0.25 mM caused 60% inactivation after 20 min, which is similar to that observed with 0.045 mM PLP, but considerably slower (not shown).

Enzyme inactivation by PLP has been shown to be due to the reaction between primary amino groups and the aldehyde

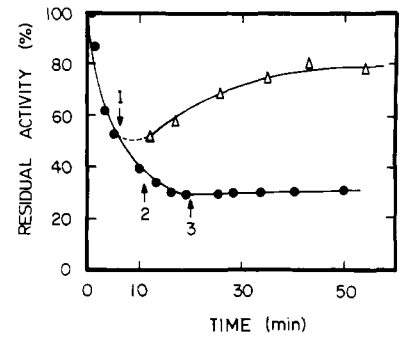

FIGURE 5: Effect of lysine and sodium borohydride on the kinetics of inactivation of phosphomevalonate kinase by PLP. Enzyme (1.17 μM; sp act. 9.9 units/mg of protein) was incubated with 0.025 mM PLP. After 6 min, 2 mM lysine was added (1). To another aliquot, 1 mM $NaBH_4$ was added after 11 min (2) and 2 mM lysine was added after 20 min (3).

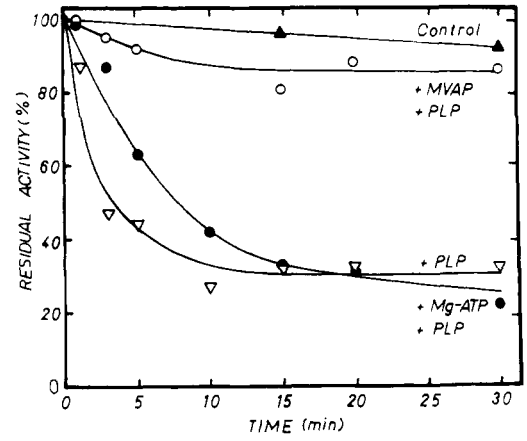

FIGURE 6: Effect of substrate on the inactivation of phosphomevalonate kinase by PLP. Enzyme (1.17 μM; sp act. 9.9 units/mg of protein) was preincubated in the presence of 0.9 mM MVAP or 2.4 mM ATP plus 4.8 mM $MgCl_2$ for 10 min. At time zero 0.024 mM PLP was added.

group of PLP, with the formation of a Schiff base (Glazer et al., 1975). This mechanism was tested, for pig liver phosphomevalonate kinase, as shown in Figure 5, by adding lysine to a sample of PLP-treated enzyme (50% active). This addition produced a partial recovery of the activity, up to 80%. If the treated enzyme was reduced with $NaBH_4$, no reactivation occurred. Besides, phosphomevalonate kinase, inactivated to 45% residual activity, recovered 100% activity when dialyzed against buffer A containing 10 mM mercaptoethanol. This recovery was not observed if the inactive enzyme had been previously treated with $NaBH_4$. Control experiments showed that $NaBH_4$ by itself caused no inactivation of the enzyme at the same concentration (1 mM).

As shown in Figure 6, MVAP at a concentration of 0.9 mM effectively protects the enzyme against inactivation by PLP. Mg-ATP at 2.4 mM, however, slows down only slightly the rate of inactivation, although a similar degree of inactivation as the control (with PLP alone) is observed at equilibrium. These observations suggest that one or more reactive amino groups participate in the MVAP binding site.

The reaction between enzyme and PLP follows pseudo-first-order kinetics, as shown in Figure 7. The apparent velocity constants are proportional to PLP concentration up to at least 0.025 mM, indicating that the overall reaction follows second-order kinetics. The second-order rate constant, obtained from the slope of the graph plotting the pseudo-first-order constants against PLP concentration, has a value of 7.4×10^3 M^{-1} min^{-1}.

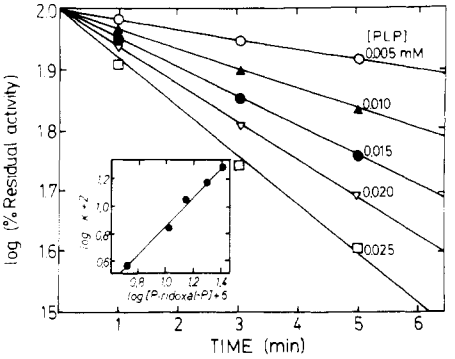

FIGURE 7: Effect of the concentration of PLP on the kinetics of inactivation of phosphomevalonate kinase. Enzyme (0.73 μM; sp act. 3.6 units/mg of protein) was incubated at the concentration of PLP indicated, as described under Experimental Procedure. Inset: plot of log of the pseudo-first-order rate constants against log of PLP concentration.

Table II: Apparent Kinetic Constants of Native and PLP-Modified Phosphomevalonate Kinase[a]

	$K_{m,app}$ (mM)		V_{max} [μmol/(min mL)]	
	MVAP	ATP	MVAP	ATP
control	0.041	0.503	0.58	0.78
plus $NaBH_4$	0.052	0.394	0.69	0.75
plus PLP plus $NaBH_4$	0.021	0.350	0.14	0.16

[a] Phosphomevalonate kinase (sp act. 10 units/mg of protein) was incubated for 30 min with 0.045 mM PLP, reduced with 1.4 mM $NaBH_4$, and exhaustively dialyzed against buffer A containing 10 mM 2-mercaptoethanol. Another sample was similarly treated, except that no PLP was added. The control was incubated with the addition of equivalent volumes of buffer A.

The fact that a second-order kinetics is observed suggests that one molecule of PLP reacts per active site during the inactivation reaction. The relationship (Hollenberg et al., 1971)

$$\log k + \log k' + n \log [\text{inhibitor}] \qquad (2)$$

(where k is the observed pseudo-first-order constant, n is the mean order of the reaction with respect to inhibitor concentration, and k' is the second-order rate constant) can be used to calculate the order of the reaction with respect to inhibitor. This is shown in the inset of Figure 7; the slope obtained has a value of 1.0.

The apparent K_m values for MVAP and Mg-ATP were determined by using enzyme treated with PLP (0.045 mM) to 25% residual activity, followed by reduction with $NaBH_4$. The results obtained are presented in Table II. A significant reduction in the K_m value is observed for the substrate MVAP but not for MgATP. These results, plus the fact that PLP does not totally inactivate the enzyme, suggest that PLP modifies the enzyme, forming a complex with altered catalytic properties. As a consequence, the reactive amino group does not seem to be essential for catalytic activity.

The effect of pH on the inactivation reaction was studied in order to establish the pK of the active group. Aliquots containing a constant concentration of enzyme and PLP were incubated in a pH range from 6.5 to 9.5. The velocity of the reaction is expected to be dependent on the degree of dissociation of the amino group, since the reactive form is the unprotonated species (Streitwieser & Heathcock, 1976). Under the experimental conditions used, the rate of inactivation in all cases followed pseudo-first-order kinetics. Figure 8 shows the results obtained at 24 and 31 °C, when the pseudo-first-order rate constants are plotted against pH. The

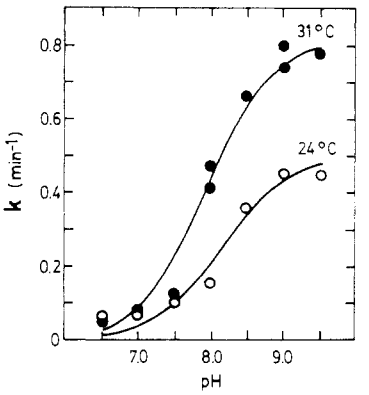

FIGURE 8: Effect of pH on the first-order rate constants of inactivation of pig liver phosphomevalonate kinase by PLP. Inactivation was performed at 24 and 31 °C using the buffers indicated below (at 100 mM) in the presence of 10 mM 2-mercaptoethanol. Enzyme (0.55 μM; sp act. 3.65 units/mg of protein) was incubated with 0.015 mM PLP, and samples were taken at appropriate times, stopping the reaction with 1 mM $NaBH_4$. First-order rate constants were calculated at each pH from the slopes of the graphs of the logarithm of residual activity as a function of time. Buffers used: pH 6.5, Mes; pH 7.0–8.0, Tes; pH 8.5–9.0, Bicine; pH 9.5, CAPS. The points were determined experimentally and the curves were calculated from the equation $k_{obsd} = kK_a/(K_a + [H^+])$, where k_{obsd} is the calculated pseudo-first-order constant, k is the pH-independent rate constant, and K_a is the ionization constant (Cohen, 1970).

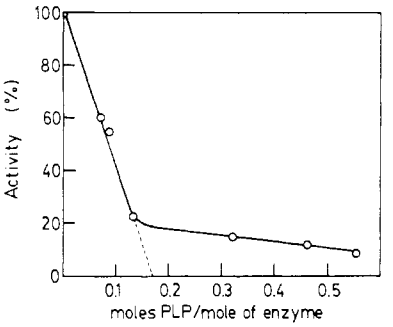

FIGURE 9: Activity of phosphomevalonate kinase as a function of the number of moles of PLP bound to it. The experiment was performed as described under Experimental Procedure.

graphs show a sigmoidal shape, similar to the titration curve of an ionizable group. This can be interpreted as a dependence of the rate of inactivation on the concentration of conjugate base. The estimated pK values are 8.15 at 24 °C and 7.95 at 31 °C. From these values, a heat of ionization (ΔH_i) of 11.8 kcal/mol has been calculated for the reactive group (Barnard & Stein, 1958).

The absorption spectrum of the enzyme treated with PLP and reduced with $NaBH_4$ shows a maximum at 325 nm, as expected for the formation of a pyridoxyl derivative of lysine (Glazer et al., 1975) (not shown).

Quantitation of the number of pyridoxyl groups bound per mole of enzyme is shown in Figure 9. Extrapolation to zero activity gives a value of about 0.2 mol of PLP per mol of enzyme. This low value may be due to the extinction coefficient used (9710 M^{-1} cm^{-1}). Blackburn & Schachman (1976) have found ϵ = 5800 M^{-1} cm^{-1} for aspartate transcarbamylase, and Paech & Tolbert (1978) have found ϵ = 4800 M^{-1} cm^{-1} for ribulose-1,5-bisphosphate carboxylase, suggesting that an extinction coefficient should be determined for each enzyme–PLP derivative. This determination could not be performed in our case, due to lack of enough pure enzyme. Our results suggest, however, that it is very unlikely that more than 1 mol of PLP reacts per mol of enzyme to account for the rapid inactivation phase of Figure 9.

Discussion

The experiments presented above indicate that pig liver phosphomevalonate kinase is inactivated by the reaction of DTNB with the only cysteinyl residue of the enzyme (Bazaes et al., 1980). DTNB produces complete inactivation, and this occurs when an average of 0.98 molecule of DTNB has reacted per molecule of enzyme. The enzyme is protected by MVAP against inactivation, but little by the other substrate, Mg-ATP.[2] The apparent K_m values of the partially inactivated enzyme with respect to both substrates are similar to those of the native enzyme, and no differences in k_{cat} are apparent. These results suggest that the enzyme sulfhydryl group is essential for its catalytic activity. The protection afforded by MVAP indicates that this group may be involved in the binding of MVAP to the enzyme or in a subsequent step of the catalytic process involving this substrate. The weak protection against inactivation observed with Mg-ATP supports this idea, although it may be an indication that MVAP binding must precede that of Mg-ATP. Kinetic data may help decide among these two alternatives. It has been shown that the enzyme follows a sequential mechanism (Bazaes et al., 1980), but the type of sequential mechanism is not known.

Pig liver phosphomevalonate kinase is also inactivated by PLP. This reagent is shown to inhibit the enzyme rapidly at low concentrations: 60% inactivation is obtained after 30 min with 0.024 mM PLP. Total inactivation is not observed. The inactivation can be reverted by the addition of lysine or by dialysis, but this reactivation does not occur if the enzyme is previously treated with $NaBH_4$. These results are compatible with the formation of a reversible Schiff base between an enzyme amino group and the aldehyde group of PLP, which is reduced to a stable linkage by $NaBH_4$.

The PLP analogues, pyridoxal and pyridoxamine phosphate, produced inhibition but were considerably less effective than PLP. These results suggest a specific role for the aldehyde and phosphate groups in the molecule.

Our kinetic data show that one amino group per enzyme active site is involved in the inactivation process. However, the fact that total inactivation with PLP is not obtained even when incubating the enzyme with a 350-fold excess, or using a second inactivation cycle, suggests that the modified amino group is not essential for catalysis. The protective effect of MVAP against this inactivation may be an indication that the reactive amino group is involved in the binding of this substrate.

The pK values for the reactive group obtained in Figure 8 (7.95 at 31 °C and 8.15 at 24 °C) are rather low if compared to that estimated for the ϵ-amino group of a polypeptide chain [9.5–10.6 (Segel, 1975)]. However, it may correspond to that of a highly reactive ϵ-amino group with lower pK value. This has been observed in oxaloacetate decarboxylase [pK = 5.9 (Schimdt & Westheimer, 1971)] or glutamate dehydrogenase [pK = 7.7 (Veronese et al., 1972)]. The ΔH_i obtained corresponds to that of an amino group [10–13 kcal/mol (Barnard & Stein, 1958)].

A low pK value may be due to the presence of one or more positive charges in the microenvironment surrounding the group. Another explanation for the high reactivity of an amino group may be a microenvironment of low polarity, which would favor the interaction between the negatively charged phosphate group of PLP and a positively charged group located in the vicinity of the amino group. Such a group may be the guanidino group of an arginine residue. Several enzymes with anionic substrates (substrates possessing carboxyl or phosphate groups) present both lysine and arginine residues at the binding site of these substrates, such as lactate dehydrogenase (Yang & Schwartz, 1972), fructose-1,6-bisphosphate phosphatase (Colombo & Marcus, 1974; Marcus, 1976), and pyruvate kinase (Cardemil & Eyzaguirre, 1979). Fructose-1,6-bisphosphate aldolase presents a lysine residue involved in the binding of the C_6-phosphate, as shown in studies with PLP (Shapiro et al., 1968; Anai et al., 1973), while an arginine residue is involved in the binding of the C_1-phosphate, as shown with chemical modification with butanedione (Lobb et al., 1975). Preliminary experiments performed in pig liver phosphomevalonate kinase using butanedione in borate buffer (S. Bazaes, unpublished experiments) indicate that 7 mM butanedione inactivates the enzyme with pseudo-first-order kinetics; MVAP protects the enzyme against inactivation and, to a lower extent, so does Mg-ATP. This suggests that an arginine residue may also be necessary for MVAP binding.

The fact that the enzyme is not totally inactivated by PLP suggests that the residual activity may correspond to that of a modified enzyme. Another possible explanation is the existence of two lysine residues reactive with PLP being mutually exclusive, so that the reaction of one residue produces an inactive enzyme while that of the other produces a modified but active molecule, as has been proposed for horse liver alcohol dehydrogenase (McKinley & Morris, 1972). This is unlikely, however, in view of the results presented in Figure 9.

The fact that the modified enzyme presents a lowered $K_{m,app}$ for MVAP may be interpreted as an increase in affinity for this substrate. At the pH of inactivation (7.5), the PLP pyridinium nitrogen is charged [pK = 8.69 (Piskiewicz & Smith, 1971)], and this charge may favor the binding of MVAP to the enzyme.

Acknowledgments

We thank Dr. Emilio Cardemil for helpful discussions during the course of this work and Dr. Catherine C. Allende for revision of the manuscript.

References

Anai, M., Lai, C. Y., & Horecker, B. L. (1973) *Arch. Biochem. Biophys. 156*, 712.

Barnard, E. A., & Stein, W. D. (1958) *Adv. Enzymol. Relat. Areas Mol. Biol. 20*, 51.

Bazaes, S., Beytía, E., Jabalquinto, A. M., Solís de Ovando, F., Gómez, I., & Eyzaguirre, J. (1980) *Biochemistry* (preceding paper in this issue).

Blackburn, M. N., & Schachman, H. K. (1976) *Biochemistry 15*, 1317.

Cardemil, E., & Eyzaguirre, J. (1979) *Arch. Biochem. Biophys. 192*, 533.

Cohen, L. A. (1970) *Enzymes, 3rd Ed. 1*, 147.

Colombo, G., & Marcus, F. (1974) *Biochemistry 13*, 3085.

Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70.

Flashner, M., Hollenberg, P. F., & Coon, M. S. (1972) *J. Biol. Chem. 247*, 8114.

Glazer, A. N., De Lange, R. J., & Sigman, D. S. (1975) in *Chemical Modification of Proteins* (Work, T. S., & Work, E., Eds.) p 131, North-Holland Publishing Co., Amsterdam.

Hellig, H., & Popják, G. (1961) *J. Lipid Res. 2*, 235.

Hollenberg, P. F., Flashner, M., & Coon. M. S. (1971) *J. Biol. Chem. 246*, 946.

Johnson, G. S., & Deal, W. C. (1970) *J. Biol. Chem. 245*, 238.

Kemp, R. G., & Forest, P. B. (1968) *Biochemistry 7*, 2596.

[2] Since MVAP protects in the absence of Mg^{2+}, it can be inferred that MVAP rather than MVAP–Mg^{2+} is the true substrate.

Lobb, R. R., Stokes, A. M., Hill, H. A. O., & Riordan, J. F. (1975) *FEBS Lett. 54*, 70.
Marcus, F. (1976) *Biochemistry 15*, 3505.
McKinley-McKee, J. S., & Morris, R. L. (1972) *Eur. J. Biochem. 28*, 1.
Nishimura, J. S., Kenyon, G. L., & Smith, R. J. (1975) *Arch. Biochem. Biophys. 170*, 461.
Otieno, S., Bhargava, A. K., Serelis, D., & Barnard, E. A. (1977) *Biochemistry 16*, 4249.
Paech, C., & Tolbert, N. C. (1978) *J. Biol. Chem. 253*, 7864.
Piskiewicz, D., & Smith, E. L. (1971) *Biochemistry 10*, 4544.
Rippa, M., Spanio, L., & Pontremoli, S. (1967) *Arch. Biochem. Biophys. 118*, 48.
Schmidt, D. E., & Westheimer, F. H. (1971) *Biochemistry 10*, 1249.
Schnackerz, K. D., & Noltmann, E. A. (1971) *Biochemistry 10*, 4837.
Schwartz, K. J., Nakagawa, Y., & Kaiser, E. T. (1976) *J. Am. Chem. Soc. 98*, 6369.
Segel, I. H. (1975) in *Enzyme Kinetics*, p 885, Wiley, New York.
Setlow, B., & Mansour, T. E. (1972) *Biochim. Biophys. Acta 258*, 106.
Shapiro, S., Enger, M., Pugh, E., & Horecker, B. L. (1968) *Arch. Biochem. Biophys. 128*, 554.
Skilleter, D. N., & Kekwick, R. G. O. (1971) *Biochem. J. 124*, 407.
Streitwieser, A., & Heathcock, C. H. (1976) in *Introduction to Organic Chemistry*, p 380, Macmillan, New York.
Veronese, F. M., Piskiewicz, D., & Smith, E. L. (1972) *J. Biol. Chem. 247*, 754.
Yang, P. C., & Schwartz, G. W. (1972) *Biochemistry 11*, 2218.

Some Sulfhydryl Properties and Primary Structure of Human Erythrocyte Superoxide Dismutase†

Jack R. Jabusch, David L. Farb, Daniel A. Kerschensteiner, and Harold F. Deutsch*

ABSTRACT: Human Cu–Zn superoxide dismutase prepared by different methods shows varying properties relevant to its sulfhydryl chemistry. A cysteine residue not found in the analogous bovine enzyme appears to be responsible for its unusual lability. Alkylation of this cysteine results in a marked increase in stability, and this form of the protein may be readily crystallized. The primary structure of the 153 amino acid residues found in the human protein has been determined, and 82% of the residues are identical with those of the bovine enzyme. A significant variation is seen in the portion of those proteins comprising residues 17–36, with eleven changes being noted.

Proteins possessing superoxide dismutase activity have been isolated from a wide variety of sources (Fridovich, 1976) since the first isolation of this protein by Mann & Keilin (1939). Properties of the bovine and human erythrocyte forms in particular have been extensively delineated (Weser, 1973). Although the bovine enzyme was originally reported to be made up of two disulfide-linked subunits (Keele et al., 1971), subsequent studies have demonstrated that these are not covalently linked either in the human (Hartz & Deutsch, 1972) or in the bovine (Evans et al., 1974; Richardson et al., 1975) forms. The former workers indicated that the subunits of the human protein, unlike the bovine enzyme, showed differences. However, more recent studies (Farb, 1977; Briggs & Fee, 1978a) have shown that the human protein also has two identical subunits.

In contrast to bovine superoxide dismutase, the human enzyme appears to undergo various transformations during isolation by methods employing the widely used ethanol–chloroform procedure (Stansell & Deutsch, 1965). This appears to be due to the presence of an additional and fairly reactive cysteine residue in the human enzyme (Hartz & Deutsch, 1972). The alkylation of this cysteine appears to markedly stabilize the protein.

Preliminary studies from our laboratory have revealed portions of the primary structures of human superoxide dismutase which included four cysteine-containing peptides separated by affinity chromatography (Farb, 1977). More recently, Barra et al. (1978) have presented data on the sequence of about two-thirds of this protein. We have now completed the sequence studies initiated by Farb (1977), and the results are presented along with the sequence for the bovine enzyme reported by Steinman et al. (1974). These two Cu–Zn forms of superoxide dismutase have sequence identities of 82%.

Experimental Section

Enzyme Preparation. Superoxide dismutase was obtained from blood provided by the Badger Regional Laboratory of the American Red Cross. Three methods were employed to isolate the enzyme from freshly washed erythrocytes. One method utilized only the chromatographic procedures employed previously by Hartz & Deutsch (1972). A second method used an initial ethanol–chloroform fractionation step to remove hemoglobin (Tsuchihashi, 1923) in a manner similar to that employed by McCord & Fridovitch (1969) in their isolation of the bovine erythrocyte enzyme. A third method used the ethanol–chloroform procedure on hemolysates obtained from 1 L of erythrocytes following a thiol–disulfide exchange reaction at 0 °C with 1.2 g of CPDS.[1] Enzymes modified in this manner could be readily crystallized. The material isolated by the method of McCord & Fridovich

† From the Department of Physiological Chemistry, University of Wisconsin, Madison, Wisconsin 53706. *Received August 2, 1979.* This investigation was supported in part by U.S. Public Health Service Research Grant CA-1786 from the National Cancer Institute.

[1] Abbreviations used: CPDS, 6,6′-dithionicotinic acid; EDTA, ethylenediaminetetraacetate; BNPS-Sk, 2-(2-nitrophenylsulfenyl)-3-methyl-3-bromoindolenine; Tos-Phe-CH_2Cl, 1-tosylamido-2-phenylethyl chloromethyl ketone; PTH, phenylthiohydantoin; pMA, *p*-mercurianiline; $NaDodSO_4$, sodium dodecyl sulfate; Cm, carboxymethyl.

0006-2960/80/0419-2310$01.00/0