Modulation of Heart Muscle Mitochondrial Malate Dehydrogenase Activity. I. Activation and Inhibition by *p*-Mercuribenzoate*

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ABSTRACT: *p*-Mercuribenzoate activation and inactivation of heart muscle mitochondrial malate dehydrogenase was investigated. Peak-stable activation was found when 3–4 rapidly reacting sulfhydryls per mole of enzyme were reacted out of a total of 15–16 found. Reaction of greater than 7–8 sulfhydryls (more slowly reacting) resulted in a still larger but transient activation followed by an inactivation which was proportional to the number of sulfhydryls reacted. Activation was optimal at pH 8.0 and rapidly reversible by excess mercaptoethanol. Substrates and the inhibitor hematoporphyrin partially protected against *p*-mercuribenzoate activation while only reduced nicotinamide-adenine dinucleotide and nicotinamide-adenine dinucleotide completely protected against inactivation by *p*-mercuribenzoate. The pattern of protection suggested that sites involved in activation are not at the catalytic center and differ from those involved in inactivation. Charcoal treatment of enzyme to remove any possible reduced diphosphopyridine nucleotide-X had no effect on activation. Iodoacetic acid, *N*-ethymaleimide, 1-fluoro-2,4-dinitrobenzene, and 5,5′-dithiobis(2-nitrobenzoic acid) inhibited malate dehydrogenase activity at much higher mole ratios than required with *p*-mercuribenzoate but failed to activate enzymatic activity. Iodoacetate inhibition was reversible by *p*-mercuribenzoate. The results suggest that *p*-mercuribenzoate mercaptide formation is responsible for both activation and inactivation and that the activation may be the counterpart of a control process mediated by a naturally occurring activator.

A large body of evidence indicates that sulfhydryl groups influence the catalytic activity of many enzymes (Webb, 1966) and thus are potential sites for action of small molecular modifiers of enzyme activity. While studying the action of *p*-mercuribenzoate on pig and bovine heart muscle mitochondrial malate dehydrogenases, we found the anticipated lack of effect on the activity of crystalline supernatant enzyme of mole ratios of PMB[1]:enzyme as large as 200, corroborating previous studies of Siegel and Englard (1962). However, an unexpected activation of the mitochondrial malate dehydrogenases was observed at low mole ratios of PMB:enzyme instead of the anticipated inhibition (Wolfe and Neilands, 1956; Siegel and Englard, 1962), although inhibition was seen at higher mole ratios.

A number of examples of stimulation of enzymatic activity by sulfhydryl reactants have been reported (Webb, 1966), including myosin adenosine triphosphatase by PMB (Kielley and Bradley 1956), rabbit muscle aldolase and rat liver fructose diphosphatase by PMB and fluorodinitrobenzene (Cremona *et al.*, 1965; Rosen and Rosen, 1966), and liver glutamate dehydrogenase by methyl mercuric chloride and PMB (Hellerman *et al.*, 1958; Bitensky *et al.*, 1965; Silverstein and Sulebele, 1969b). An activation of pig heart M-MDH by PMB which was ascribed to reaction of the PMB with DPNH-X has independently been recently briefly reported (Devenyi *et al.*, 1966). The present communication describes the activation and inactivation process of heart mitochondrial malate dehydrogenase with PMB and other sulfhydryl reagents and some factors affecting it. The results suggest that the sites involved in activation are likely allosteric and different from those involved in inactivation. The succeeding paper describes properties and possible significance of PMB-activated enzyme (Sulebele and Silverstein, 1970).

Materials and Methods

Pig heart mitochondrial malate dehydrogenase was obtained from the Boehringer Mannheim Corporation as an ammonium sulfate suspension. Bovine heart M-MDH (Siegel and Englard, 1961) was kindly supplied by Dr. Lewis Siegel as an ammonium sulfate suspension. Crystalline bovine heart supernatant MDH (Englard and Breiger, 1962) was generously supplied by Dr. Sasha Englard. Oxalacetic acid, malic acid, *p*-mercuribenzoic acid, iodoacetic acid, 1-fluoro-2,4-dinitrobenzene, *N*-ethymaleimide, 5,5′-dithiobis(2-nitrobenzoic acid), and hematoporphyrin were obtained from the Sigma Chemical Co. Oxalacetic acid and malic acid were found by quantitative enzymic conversion with MDH and coenzyme to be entirely pure as determined by the expected formula weight. Titration of mercaptide-forming groups with PMB was done spectrally (Boyer, 1954).

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[1] The abbreviations used are: M-MDH, malate dehydrogenase; PMB, *p*-mercuribenzoate; PHMB, *p*-hydroxymercuribenzoate [the general term PMB and the ion-specific term PHMB have been used interchangeably since the hydroxyl group is thought to be the predominant species under the conditions employed in this work (Boyer, 1954)]; NEM, *N*-ethylmaleimide; FDNB, 1-fluoro-2,4-dinitrobenzene; IAA, iodoacetic acid; OAA, oxalacetic acid.

TABLE I: Effect of Assay Temperature on PMB Activation.[a]

PMB Equiv/Mole of Enzyme	% Increase in Activity (Assay Temperature)	
	(5°)	(25°)
2	78	13
4	80	23

[a] Bovine heart M-MDH (7.14 μμmoles) was activated by incubation with PMB in 0.2 M Tris-NO_3 (pH 8.0), at 0° for 20 hr.

TABLE II: Effect of PMB Treatment of Pig Heart Mitochondrial Malate Dehydrogenase on Enzymatic Activity with Oxalacetate and Malate.[a]

Substrate	Initial Velocity ΔA_{340}/min × 10^3		% Stimulation of Act.
	Native	PMB Treated	
Oxalacetate	54	116	115.0
Malate	181	401	121.5

[a] Solutions containing 0.86 mμmole of pig heart M-MDH in 0.1 M K phosphate, pH 8.0 (final volume, 0.8 ml), were incubated with 4 equiv of PMB/mole of enzyme for 18 hr at 0°, and then assayed for activity with oxalacetate and malate. The rate of oxalacetate reduction was assayed as described in Materials and Methods. The initial rate of malate oxidation was assayed in a solution containing 282 μmoles of glycine-Na (pH 10.0), 100 μmoles of L-malate, 1.45 μmoles of NAD, and 53.3 μμmoles M-MDH in a total volume of 3.05 ml at 5°.

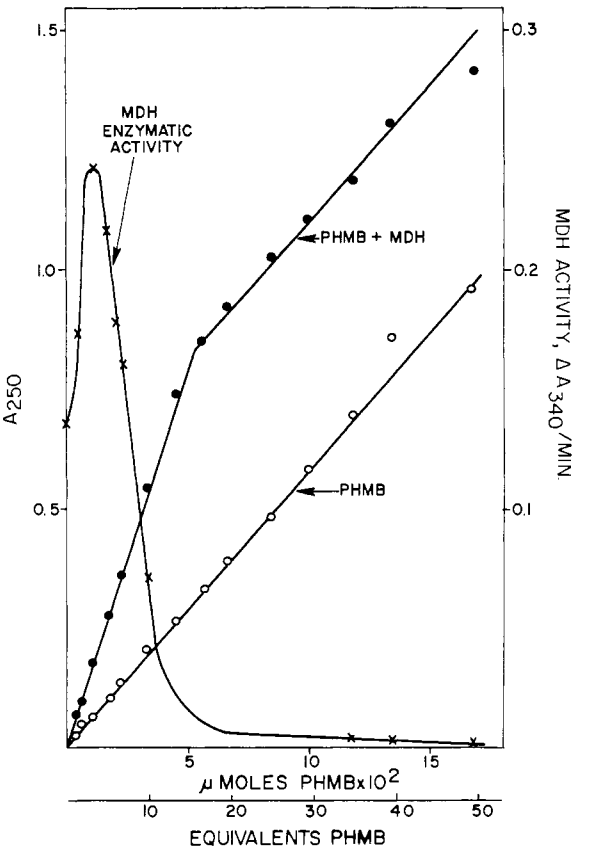

FIGURE 1: Correlation of *p*-mercuribenzoate spectrophotometric titration of sulfhydryl groups with malate dehydrogenase activity. Solutions containing 3.4 mμmoles of pig heart M-MDH were incubated with varying amounts of freshly prepared solutions of PMB in 0.4 ml of 0.1 M K phosphate at 0° for 18–20 hr. Reaction mixtures were then diluted to 0.8 ml with buffer and the absorbance at 250 mμ was read in a Beckman DU spectrophotometer and compared with similar solutions lacking enzyme. Enzymatic activity was determined in duplicate on a sample of the reaction mixture (see Materials and Methods).

Enzymatic activity was routinely assayed, unless otherwise stated, by duplicate measurements of the initial rate of NADH oxidation as reflected in the rate of decrease in absorbance at 340 mμ. The reaction mixture contained 320 μmoles of Tris-Cl (pH 8.0), 0.209 μmole of NADH, and 1 μmole of oxalacetate (freshly prepared and kept at 0°) in a volume of 1.63 ml at 5°. Reaction was initiated by addition of enzyme with an adder–mixer (Boyer and Segal, 1954).

Results

Correlation of Spectrophotometric Titration of Sulfhydryl Groups with Enzymatic Activity. Up to twofold activation of pig and bovine heart M-MDH was initially observed on reaction with 2–5 equiv of PMB instead of the anticipated inhibition (Siegel and Englard, 1962; Wolfe and Neilands, 1956). This activation was further confirmed and quantitated with respect to the number of sulfhydryl groups reacted by spectrophotometric titration with PMB (Figure 1). A total of 15–16 sulfhydryl groups was found compared with 14 reported previously (Thorne and Kaplan, 1963). Activation was performed at 0° and assay at 5° rather than 25° since greater and more stable activation was thus obtained (Table I). Enzymatic activity rose to a peak value with the reaction of three to four sulfhydryl groups (81% activation) and then progressively fell to the level of native enzyme with about seven to eight sulfhydryls reacted, and to about 10% of the initial value when all SH groups were apparently titrated. Further increase in PMB resulted in much smaller decrease in activity; about 1% of original activity remained after the addition of 50 equiv of PMB. The retarded further decrease in activity at more elevated PMB concentrations may be due to reaction of PMB with groups other than sulfhydryl, as has been suggested previously on the basis of binding data with other proteins (Madsen and Gurd, 1956; Benesch *et al.*, 1955).

Kinetics of Activation, Inactivation, and Mercaptide-Bond Formation. Incubation of pig heart mitochondrial MDH with 4 equiv of PMB resulted in a parallel rise in both catalytic activity and in the absorbance at 250 mμ due to mercaptide-

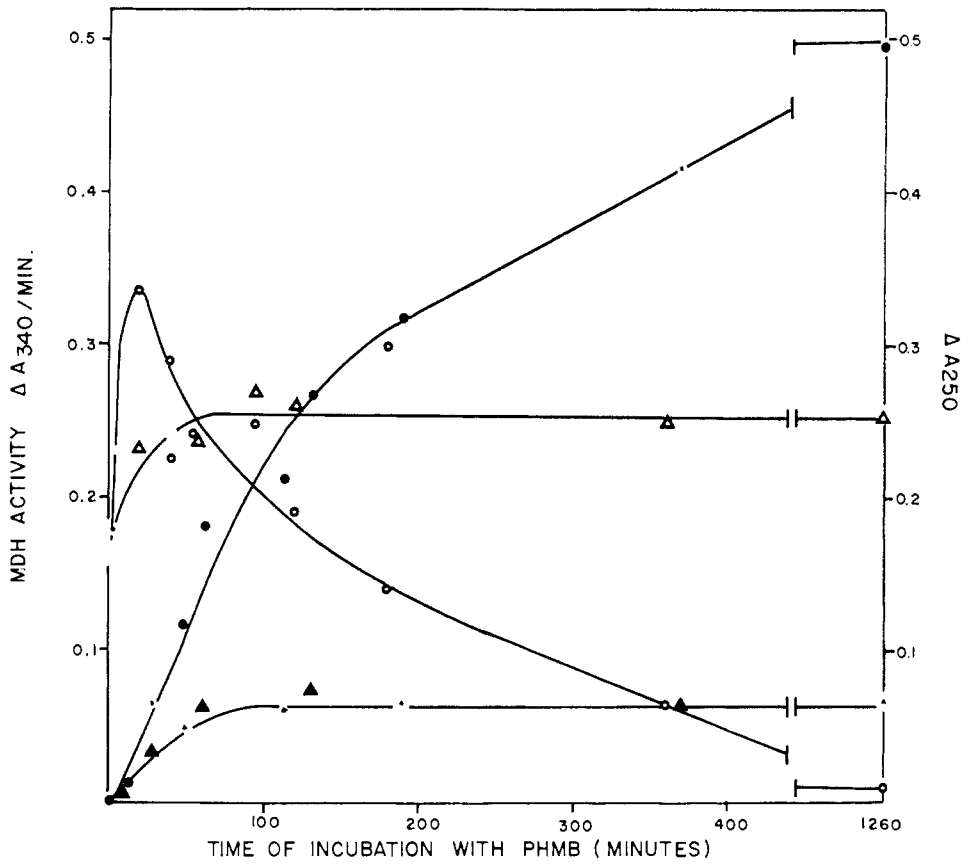

FIGURE 2: Time course of activation and inhibition of mitochondrial malate dehydrogenase by *p*-mercuribenzoate. Solutions containing 3.43 mμmoles of pig heart M-MDH were incubated in 0.1 M phosphate buffer (pH 8.0) in a total volume of 0.8 ml with either 4 or 18 equiv of PMB per mole of enzyme in silica cuvets of 1-cm light path at 0°. Reactions were started by the addition of PMB. At the indicated time intervals the absorbance at 250 mμ was obtained and the activity was determined on a sample containing 2.1 μμmoles of enzyme: (△) enzymatic activity, M-MDH + 4 equiv of PMB; (▲) absorbance at 250 mμ, M-MDH + 4 equiv of PMB; (○) enzymatic activity, M-MDH + 18 equiv of PMB; (●) absorbance at 250 mμ, M-MDH + 18 equiv of PMB.

bond formation (Figure 2). The kinetic similarity of the two increases suggests a direct relationship. Incubation with 18 equiv of PMB resulted in inactivation to about 7.5% of original activity after 21 hr. However, initially there was an activation, the peak of which occurred within 1 hr at about the time that the increase in the absorbance at 250 mμ due to mercaptide-bond formation was equal to the absorbance increase which occurred with the lower, activating level of PMB. The peak level of transient activation was at least twofold higher than the stable activation obtained with activating levels of PMB. Incubation with 5–8 equiv of PMB also resulted in a transient peak of activation which was higher than the stable activation seen with activating levels, and in an apparently stable level of activation after 21 hr which was lower than that obtained with activating levels of PMB (3–4 equiv). The greater the increment in PMB equivalents from 4, the lower was the final enzymatic activity.

The rise in the absorbance at 250 mμ in the presence of 18 equiv of PMB/mole of enzyme had a rapid and a slower component, with a transition occurring after about half the final absorbance was reached. At about this point the enzymatic activity dropped below the initial levels. This finding suggests that activation occurs on reaction of readily accessible sulfhydryl groups with PMB while inactivation may be due to reaction of relatively inaccessible sulfhydryl groups.

Effect of p-Mercuribenzoate on Mitochondrial Malate Dehydrogenase Activity with Other Substrates. The degree of activation of M-MDH catalysis of the reaction of malate with NAD was similar to the activation of the reaction of oxalacetate with NADH (Table II). However, only inhibition rather than activation was found with the reactants mesoxalate (ketomalonate) and NADH, indicating that activation is substrate dependent.

Optimum pH for Activation of Mitochondrial Malate Dehydrogenase with p-Mercuribenzoate. Bovine heart mitochondrial MDH was incubated with PMB from pH 7 to 10 (Figure 3). Best activation was obtained under the conditions studied at pH 8 with 4 equiv of PMB. Activation and inactivation occurred with less equivalents of PMB at pH 7 than at the other hydrogen ion concentrations studied. While different buffer ions were used at the various pH values, and these do affect the PMB reaction (Boyer, 1954), it is likely that pH is the key factor since at pH 8 and 9 quite different results were obtained with the same buffer system.

Effect of Substrates and Inhibitors on Activation by PMB. In order to see whether binding of reactant prior to addition

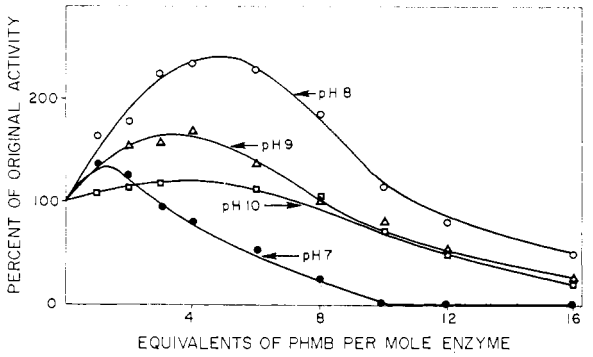

FIGURE 3: Optimum pH for activation of mitochondrial malate dehydrogenase by *p*-mercuribenzoate. Solutions of bovine heart M-MDH (0.4 ml) were incubated at 0° for 18–20 hr with the listed equivalents of PMB per mole of M-MDH, and then assayed for enzymatic activity with the following buffers: 0.09 M K phosphate (pH 7.0), 0.17 M Tris-Cl (pH 8.0), 0.76 M Tris-Cl (pH 9.0), and 0.1 M glycine Na (pH 10.0). PMB addition was made from a solution dissolved in the same buffer.

of PMB could prevent mercaptide formation and activation, PMB activation was carried out in the presence of various reactants. Activation was reduced 44–75% but not eliminated (Figure 4). Coenzyme, particularly NADH, was more effective than substrate in suppressing activation.

If the activating sulfhydryls were situated at the catalytic center, it might be expected that occupation of both substrate and coenzyme binding sites would result in more complete or total suppression of activation. Since recent equilibrium kinetic evidence (Silverstein and Sulebele, 1969a) supports the formation of the unreactive complex enzyme–NADH–malate, both malate and NADH were used in the PMB reaction mixture. However, the presence of both malate and NADH was less effective than coenzyme alone (Figures 4 and 5), while an equilibrium reaction mixture also did not result in suppression of activation beyond that achieved with coenzyme alone.

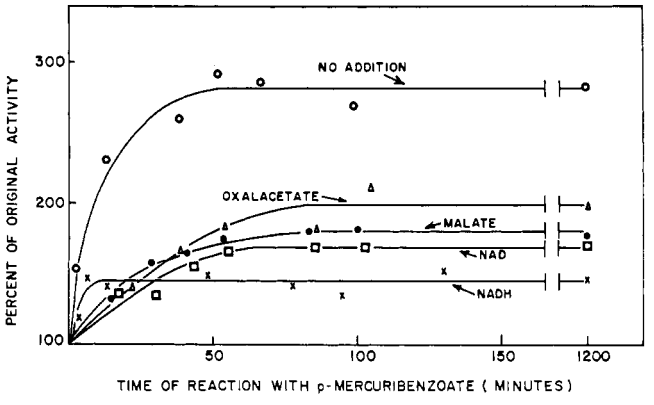

FIGURE 4: Effect of substrates on the activation of mitochondrial malate dehydrogenase by *p*-mercuribenzoate. Solutions containing 0.86 mμmole of pig heart M-MDH and 4 moles of PMB/mole of enzyme in 0.1 M K phosphate (pH 8.0) (final volume, 0.8 ml) were incubated at 0° with the following additions: (○) no addition; (●) 100 mM L-malate; (△) 38 mM oxalacetate; (□) 10 mM NAD; (×) 5.1 mM NADH. Reactions were initiated by PMB addition and samples were assayed for enzymatic activity at the indicated time intervals.

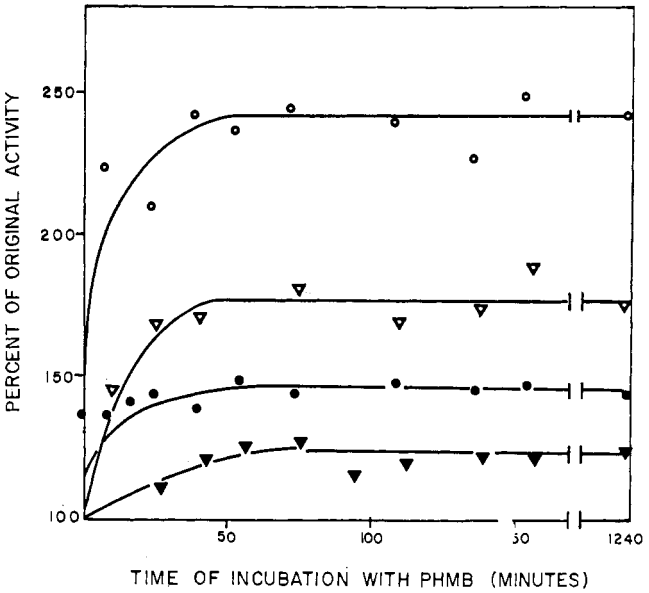

FIGURE 5: Effect of malate, NADH, and hematoporphyrin on activation of mitochondrial malate dehydrogenase by PMB. Pig heart M-MDH was activated by incubation with 4 moles of PMB/mole of enzyme in 0.8 ml of 0.1 M K phosphate at 0°. Prior to addition of PMB, reaction mixtures contained the following: (○) no addition; (▽) 100 mM L-malate + 5.1 mM NADH; (●) 4.9 mM hematoporphyrin; and (▼) 100 mM L-malate + 5.1 mM NADH + 4.9 mM hematoporphyrin. Aliquots of the activation mixture were withdrawn at the indicated time intervals, and diluted in 0.1 M K phosphate (pH 8.0), and enzymatic activity was determined.

Hematoporphyrin inhibits malate dehydrogenase catalysis (Silverstein and Press, 1968). Since hematoporphyrin does not structually resemble substrate or coenzyme, it is quite possible that binding of hematoporphyrin occurs at a regulatory site which may contain some or all of the sulfhydryls which react with PMB during activation. It was thus of interest that hematoporphyrin caused a more marked suppression of activation than did NADH plus malate (Figure 5) or

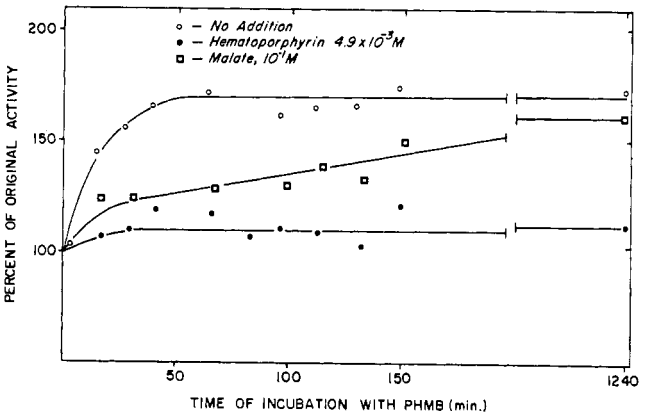

FIGURE 6: Effect of malate and hematoporphyrin on the activation of mitochondrial malate dehydrogenase by *p*-mercuribenzoate. Activation was initiated by addition of 4 equiv of PMB/mole of enzyme to 0.8 ml of 0.1 M K phosphate buffer (pH 8.0), containing 1.15 mμmoles of pig heart M-MDH and either 0.1 M L-malate or 4.9 mM hematoporphyrin. Samples diluted in buffer were assayed for activity.

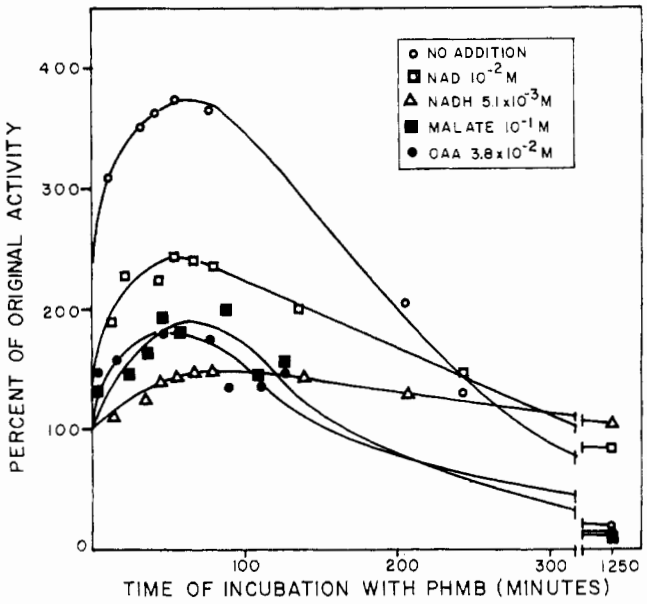

FIGURE 7: Effect of substrates on the enzymatic activity of mitochondrial malate dehydrogenase reacted with inactivating amounts of *p*-mercuribenzoate. Solutions containing 8.6 mμmoles of pig heart M-MDH in 0.1 M K phosphate buffer (pH 8.0) (final volume, 0.8 ml) were reacted at 0° with 38 equiv of PMB in the presence of the indicated substrates. Samples were assayed for activity at the times indicated; (○) no addition, (□) 10 mM NAD, (△) 5.1 mM NADH, (■) 0.1 M malate, and (●) 38 mM oxalacetate.

malate (Figure 6). Addition of NADH and malate to hematoporphyrin caused still further reduction of activation (Figure 5). The incomplete effectiveness of reactant binding and the effectiveness of an inhibitor which does not resemble the reactants suggests that at least some of the sulfhydryls involved in activation may be present at an allosteric site separate from the catalytic center.

Effect of Substrate and Inhibitors on the Enzymatic Activity of M-MDH Reacted with an Inactivating Level of PMB. The inactivation resulting from reaction of M-MDH with 38 equiv of PMB/mole of enzyme was prevented completely by NADH, almost completely by NAD, and not affected appreciably by L-malate and oxalacetate (Figure 7). The transient, marked activation seen early in the reaction was inhibited by all substrates, the order of effectiveness being NADH > oxalacetate > malate > NAD. NADH + malate (± hematoporphyrin) completely prevented the inactivation resulting from reaction of 38 equiv of PMB/mole of MDH and markedly lessened the extent but prolonged the duration of the initial activation (Figure 8). The effect of NADH alone (Figure 7) was similar, suggesting that additional binding of malate (Silverstein and Sulebele, 1969a) is ineffective in further altering the reaction. Addition of hematoporphyrin to NADH and malate slightly decreased the transient activation achieved. Hematoporphyrin alone failed to protect against inactivation, but markedly inhibited the transient activation, though somewhat less effectively than NADH + malate.

Effect of Charcoal Treatment of Pig Heart M-MDH on Its Activation with PMB. Since DPNH-X reaction with PMB has been hypothesized to account for PMB activation of M-MDH (Devenyi *et al.*, 1966), M-MDH pretreated with charcoal to remove DPNH-X (Wieland *et al.*, 1962) was compared with native enzyme with respect to activation by PMB (Figure 9). The extent of activation of charcoal-treated MDH was similar and the rate of activation more rapid than activation of enzyme not pretreated with charcoal. Further, no increase in the absorbance at 250 mμ was observed with PMB titration of fully reacted acid-treated NADH which resembles DPNH-X (Wieland *et al.*, 1962). The initial increase in absorbance at 250 mμ which runs parallel with increase in enzymatic activity in PMB activation of MDH would thus

TABLE III: Reversal of PMB-Induced Activation of Bovine Heart Mitochondrial Malate Dehydrogenase by 2-Mercaptoethanol.[a]

Condition of Enzyme	Enzymatic Act. ΔA_{340}/min × 10^3 (% Act.)	
Native	68	60
PMB treated	113 (66.2)	111 (85.0)
PMB treated + 2-mercaptoethanol	82[b] (20.6)	65[c] (8.0)

[a] PMB (4 equiv/mole of enzyme) was incubated for 20 hr at 0° with 0.29 mμmole of bovine heart M-MDH in 0.8 ml of 0.2 M Tris-NO_3 (pH 8.0). Samples of this enzyme solution as well as a similarly prepared control without PMB were assayed for activity. Samples of PMB-reacted M-MDH were also assayed in a duplicate assay mixture, adjusted for volume, to which 2-mercaptoethanol had been added prior to enzyme. [b] 0.14 μmole of 2-mercaptoethanol. [c] 0.29 μmole of 2-mercaptoethanol.

TABLE IV: Effect of Large Concentrations of Iodoacetate, 1-Fluoro-2,4-dinitrobenzene, *N*-ethylmaleimide, and 5,5′-Dithiobis(2-nitrobenzoic Acid) on Pig Heart Mitochondrial Malate Dehydrogenase.[a]

Inhibitor	Concentration (mM)	Mole Ratio Ligand: Enzyme	% Inhibition
Iodoacetate	30	28,000	78.0
1-Fluoro-2,4-dinitrobenzene	3	2,800	100
N-Ethylmaleimide	1	940	93.7
5,5′-Dithiobis(2-nitrobenzoic acid)	0.2	116	95.3

[a] Pig heart M-MDH (0.86 mμmole) was incubated with inhibitor in 0.1 M K phosphate (pH 8.0), for iodoacetate, fluorodinitrobenzene, and 5,5′-dithiobis(2-nitrobenzoic acid) and in 0.1 M K phosphate (pH 7.0), for *N*-ethylmaleimide for 18 hr at 25°. Fluorodinitrobenzene and *N*-ethylmaleimide were added in 5 or 10 μl of ethanolic solution. Enzymatic assays were done on samples diluted in 0.1 M K phosphate (pH 8.0).

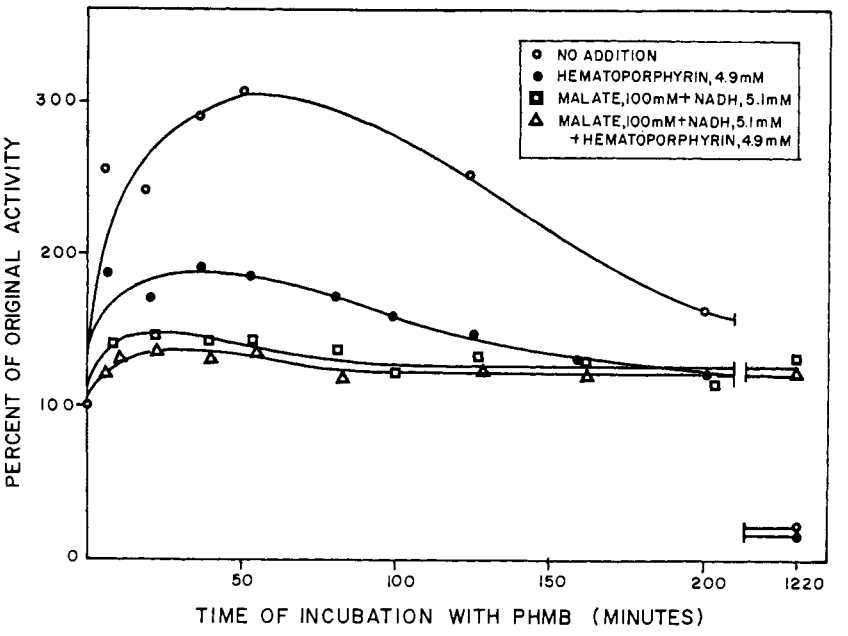

FIGURE 8: Effect of substrates and hematoporphyrin on activity of mitochondrial malate dehydrogenase reacted with inactivating amounts of *p*-mercuribenzoate. Pig heart M-MDH (0.86 mμmole) was incubated at 0° in 0.8 ml of 0.1 M K phosphate (pH 8.0) with substrates and hematoporphyrin as indicated. Samples were assayed for enzymatic activity at the indicated time intervals; (O) no addition, (●) 4.9 mM hematoporphyrin, (□) 0.1 M malate + 5.1 mM NADH, and (△) 0.1 M malate + 5.1 mM NADH + 4.9 mM hematoporphyrin.

not appear to be due to reaction of PMB with an acid-degraded NADH-like compound or DPNH-X. Although there are some differences between DPNH-X generated by triosephosphate dehydrogenase and the acid-degraded product (Rafter *et al.*, 1953; Meinhart *et al.*, 1956; Bachur and Kaplan, 1955; Gingrich and Schlenk, 1944), it would not seem likely that their reaction with PMB would be different in this regard.

Effect of Mercaptoethanol on PMB Activation. The PMB-induced activation of pig heart M-MDH was rapidly reversed by a large excess of mercaptoethanol in the assay mixture (Table III), as expected from the reversible nature of the PMB–sulfhydryl reaction (Boyer, 1959).

Comparison of the Effect of PMB and Other Sulfhydryl Reagents on M-MDH Activity. With up to 600 moles of sulfhydryl reactant/mole of enzyme at 0° only PMB resulted in activation and inactivation of M-MDH activity; 1-fluoro-2,4-dinitrobenzene, *N*-ethylmaleimide, iodoacetate (Figure 10), and 5,5′-dithiobis(2-nitrobenzoic acid) did not alter activity. Fluorodinitrobenzene was equally ineffective at pH 8 and 10 (0.1 M glycine-Na), while similar inactivation occurred with both fluorodinitrobenzene-treated and native enzyme at pH 6.0 in 0.1 M Na maleate. In this experiment the concentration of enzyme in the reaction mixture was only 71 mμM. Under these conditions much larger mole ratios of PMB are required to cause inactivation than when larger concentrations of enzyme, and hence of PMB, are used. This may be due to a longer time required to complete reaction with slower reacting sulfhydryls when dilute solutions of PMB are used.

Much higher multiplicities of iodoacetate, fluorodinitrobenzene, *N*-ethylmaleimide, and 5,5′-dithiobis(2-nitrobenzoic acid) at 25° resulted in marked inhibition when studied kinetically (Table IV). Under similar conditions no transient activation was observed to precede the inhibition as found with inhibitory levels of PMB.

Since reaction with iodoacetate did indeed occur, it was of interest to see whether such reaction could block activation by PMB. Pig heart M-MDH was inactivated 85% at 25° with iodoacetate and 4 equiv of PMB was then added at 0° to an aliquot of the reaction mixture and to a control solution (Figure 11). Although both activities increased, the percentage activity increase occurred more rapidly and was slightly higher for the iodoacetate-treated enzyme than for the native enzyme control. However, the activity achieved with iodoacetate-treated enzyme was only approximately that of native enzyme. In contrast to the iodoacetate-inhibited enzyme *N*-ethylmaleimide- and fluorodinitrobenzene-inhibited M-MDH were not activatable by PMB (Figure 12).

It was not possible to locate the sites of PMB–mercaptide formation involved in activation and inactivation by labeling pig heart M-MDH with [^{14}C]PMB, peptide mapping (Fondy *et al.*, 1964; Ingram, 1958; Baglioni, 1961), and autoradiography, presumably because reversal of PMB–mercaptide-bond formation was too great despite attempts to minimize it.

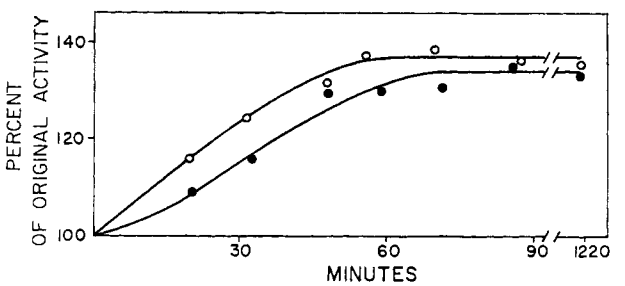

FIGURE 9: Effect of charcoal treatment of mitochondrial malate dehydrogenase on activation with *p*-mercuribenzoate. Solutions containing 3.5 mμmoles of pig heart M-MDH in 1.5 ml of 0.1 M K phosphate (pH 8.0) were gently stirred with 2.5 mg of acid-washed Norit charcoal at 0° for 20 min and centrifuged at 840*g* (0°). Solutions of native and charcoal-treated M-MDH containing 0.86 mμmole of enzyme in 0.8 ml of 0.1 M K phosphate (pH 8.0) were reacted with 4 equiv of PMB/mole of enzyme at 0°. Samples were assayed at the times indicated; (●) native M-MDH; (○) charcoal-treated M-MDH.

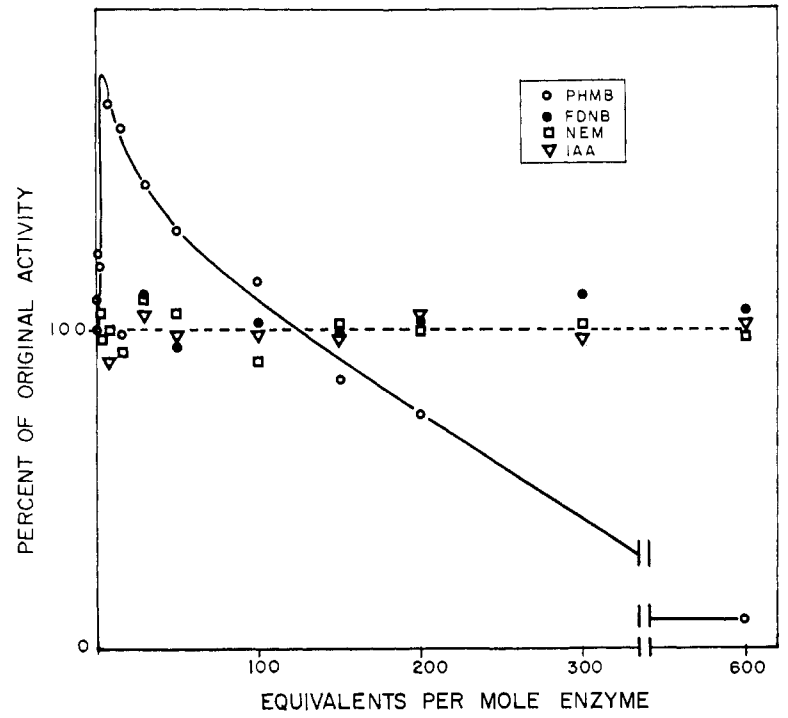

FIGURE 10: Effect of *p*-mercuribenzoate and other sulfhydryl reactants on mitochondrial malate dehydrogenase activity. Solutions of bovine heart M-MDH (71 $\mu\mu$moles) were incubated in 1.0 ml of 0.2 M Tris-NO_3 (pH 8.0) with the indicated equivalents per mole of enzyme of PMB, 1-fluoro-2,4-dinitrobenzene, *N*-ethylmaleimide, and iodoacetate at 0° for 18–20 hr, and samples were assayed for enzymatic activity. Fluorodinitrobenzene and *N*-ethylmaleimide were added as ethanolic solutions in 2 μl.

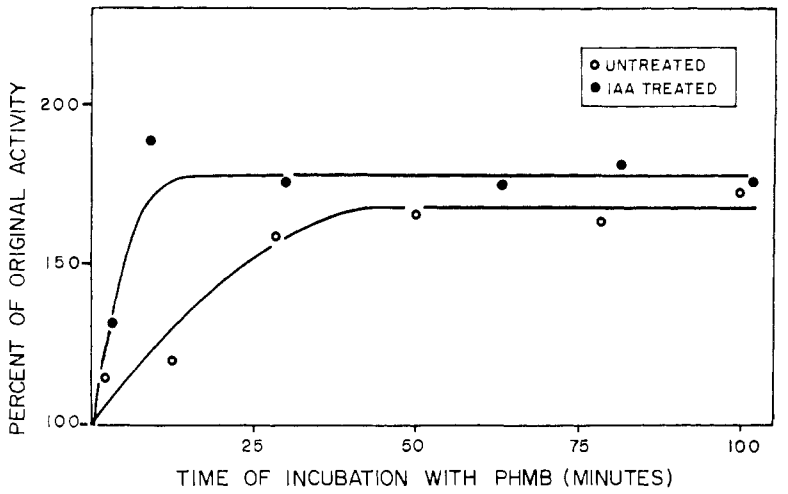

FIGURE 11: *p*-Mercuribenzoate activation of iodoacetate inhibited mitochondrial malate dehydrogenase. Pig heart M-MDH (0.86 mμmole) was incubated in 0.1 M K phosphate (pH 8.0) containing 30 mM iodoacetate in 0.8 ml at 25° for 18 hr, at which time the enzyme had lost 84.5% of its original activity by standard assay as compared with a similarly incubated sample without iodoacetate. Samples (0.4 ml) of iodoacetate reacted and native M-MDH incubation mixtures were transferred to 0° and 4 equiv of PMB/mole of enzyme was added to each. Samples of each reaction mixture were assayed for enzymatic activity at the indicated times.

Discussion

The activity of pig and bovine heart muscle malate dehydrogenase is profoundly affected by reaction with *p*-mercuribenzoate in the direction of activation or inhibition. Rapid, readily reversible activation under conditions of saturating substrate occurs optimally on reaction of 3–4 moles of *p*-mercuribenzoate/mole of enzyme. Inactivation distinctly occurs on reaction of more than 7–8 sulfhydryls out of a total of 15–16 found. The parallel kinetics of activation and mercaptide-bond formation as indicated by increase in absorbance at 250 mμ strongly suggest that PMB–mercaptide formation is responsible for the activation.

Kinetic experiments indicate that the more rapidly formed PMB–mercaptides are responsible for activation of catalytic activity while PMB reaction with more slowly reacting sulfhydryls results in inactivation. This suggests that activation occurs with readily accessible sulfhydryls while inactivation occurs with relatively inaccessible ones, perhaps in hydrophobic or otherwise sterically hindered regions. The rapid, readily reversible nature of activation by reaction of exposed sulfhydryls also suggests a possible mechanism for control of enzyme activity *in vivo* by a naturally occurring reactant. The more sluggish reaction of inactivation makes fine control by this mechanism less likely. The greater number of sulfhydryls reacted and the complex change in activity suggests that inactivation may involve more profound conformational change than does activation.

The sulfhydryls involved in activation appear to be at allosteric sites rather than at the catalytic center. The allosteric in-

hibitor hematoporphyrin, which does not resemble the normal active-site ligands, protects against PMB activation better than substrate or coenzyme, which protect only partially. Ligand protection experiments also indicate that different sulfhydryl sites are involved in activation and inactivation. Unlike the case with activation, hematoporphyrin, as well as malate and oxalacetate, offers no protection agains tinactivation. On the other hand, the binding of coenzyme completely protects against inactivation. It is possible that binding of coenzyme sterically protects a sulfhydryl critical for inactivation. However, since sulfhydryl groups at the coenzyme binding site would be expected to be readily accessible and thus react rapidly with PMB, and the inactivation reaction is relatively slow, it is more likely that binding of coenzyme induces change in conformation which makes sulfhydryls which are important in inactivation inaccessible for PMB reaction.

The protection experiments suggest that of all natural ligands, binding of NADH likely causes the most profound conformational change which results in making most sulfhydryl groups inaccessible for PMB mercaptide formation.

It is of interest that the reaction of activation, which may have a physiological role in metabolic control, is not completely prevented by binding of any reactants, thus making it susceptible of control in the presence of bound ligands, while inactivation is fully prevented by bound coenzyme, thus protecting against loss of catalytic activity.

The transient marked activation produced by mole ratios of PMB which finally result in inhibition is an interesting phenomenon. The amount of activation is invariably greater than that produced by 4 moles of PMB/mole of enzyme which results in stable activation, although the quantity of mercaptide formed at this point is similar to that produced in activation. It is possible that at some early point during the reaction a combination of fast- and slow-reacting sulfhydryls have reacted and result in conformation or environment at the active site which is more favorable for catalysis. It would be of interest to see if this activated state could be stabilized, perhaps by avoiding further PMB reaction. Just as with stable activation, coenzyme, substrate, and hematoporphyrin partially prevent transient activation. In this case, however, NADH was considerably more effective than hematoporphyrin.

Devenyi *et al.* (1966) have independently reported activation of native pig heart M-MDH by reaction with 2 moles of PMB/subunit (4 PMB/mole of enzyme) and inactivation of photooxidized M-MDH by reaction with 8 PMB/mole of enzyme at 0°. Activatability of native M-MDH was directly correlated with DPNH-X content (up to one/mole of enzyme) of various enzyme preparations and inversely proportional to mercaptide-bond formation (up to four/mole of enzyme) and inhibitability of photooxidized enzyme. They have suggested that (a) PMB–mercaptide-bond formation (four/mole of enzyme) does not alter enzymatic activity, (b) DPNH-X containing enzyme is activated and fails to form PMB–mercaptide, and (c) unlike the case with native enzyme devoid of DPNH-X which forms only four PMB–mercaptide bonds/mole of enzyme without alteration in activity, photooxidized M-MDH forms eight PMB–mercaptide bonds/mole of enzyme with excess PMB.

The present experiments do not lend support to the possibility suggested by these authors that activation occurs by reaction of PMB with DPNH-X or by the formation of activated enzyme–DPNH-X–mercurial ternary complex. Activation of charcoal-treated and untreated enzyme was similar. Kinetically, activation and mercaptide-bond formation occurred in parallel and PMB failed to give an increase in absorbance at 250 mμ with acid-degraded NADH which is said to resemble DPNH-X (Wieland *et al.*, 1962). Optimum activation was observed with formation of four PMB–mercaptide bonds/mole of enzyme. DPNH-X was not found to inhibit the reaction of malate oxidation (Devenyi *et al.*, 1966). It would thus seem more likely that reaction of PMB with enzyme sulfhydryls is responsible for the activation.

The failure of Devenyi *et al.* (1966) to observe formation of more than four PMB–mercaptide bonds/mole of enzyme at 0° with native enzyme in the presence of excess PMB may be due to lack of sufficient time of observation since the sulfhydryls beyond the four apparently involved in activation may react more slowly; reaction of sulfhydryls beyond 8/mole clearly occurs more slowly. The observation by these workers of formation of eight PMB–mercaptide bonds/mole of photooxidized M-MDH with simultaneous inactivation is likely due to more rapid PMB–mercaptide formation than with native enzyme, which was therefore observable under the conditions used. In the present experiments all sulfhydryls could apparently be ti-

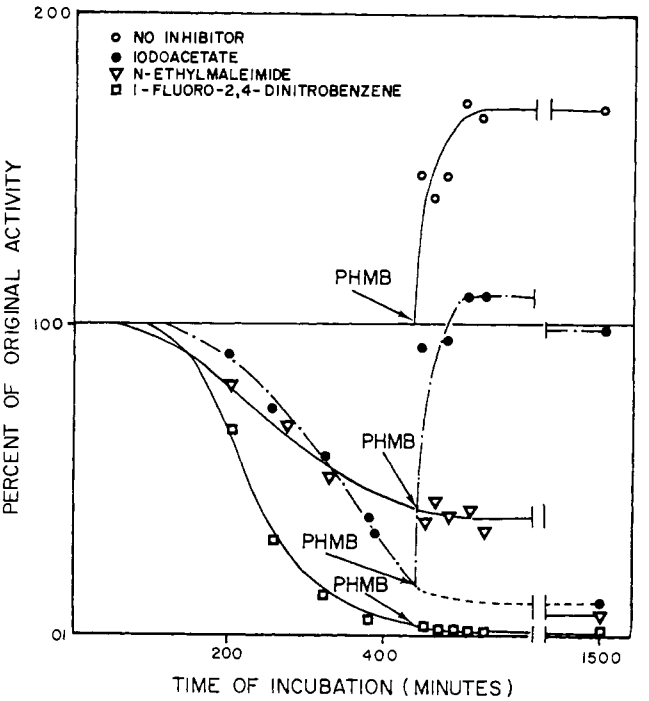

FIGURE 12: Effect of *p*-mercuribenzoate on mitochondrial malate dehydrogenase inhibited with iodoacetate, 1-fluoro-2,4-dinitrobenzene, and *N*-ethylmaleimide. Pig heart M-MDH (0.86 mμmole) was reacted at 25° in 0.1 M K phosphate (pH 8.0) with 30 mM iodoacetate, 4.5 mM fluorodinitrobenzene, or with 1 mM *N*-ethylmaleimide in 0.1 M K phosphate (pH 7.0). Fluorodinitrobenzene was added as an ethanolic solution (5–15 μl); the controls contained a similar amount of ethanol. Tubes containing fluorodinitrobenzene were wrapped in aluminum foil to protect from light decomposition. After about 7-hr incubation 0.4 ml of reaction mixture was reacted at 0° with 4 equiv of PMB/mole of M-MDH and samples were withdrawn at intervals for enzyme assay; (○) no inhibitor, (●) iodoacetate, (▽) *N*-ethylmaleimide, and (□) 1-fluoro-2,4-dinitrobenzene.

trated at 0° by prolonged incubation with PMB. The greater inactivation of photooxidized M-MDH (Devenyi *et al.*, 1966) than native M-MDH (present experiments) by formation of eight PMB–mercaptide bonds/mole of enzyme suggests that the photooxidation contributes to the inactivation.

The reason for lack of activation by sulfhydryl reactants other than PMB is not clear but presumably indicates that simply removing the specific free sulfhydryl function does not confer activation. Perhaps the specific microenvironment conferred by PMB is critical in inducing a proper conformation change. Unlike the case with rabbit muscle aldolase where only the two aromatic sulfhydryl reactants used resulted in activation (Cremona *et al.*, 1965) the other aromatic reactant used in this study, fluorodinitrobenzene, produced only inhibition which was achieved only at elevated mole ratios of fluorodinitrobenzene:enzyme.

The mechanism of rapid reversal by PMB of iodoacetate inhibition is not clear. It may be due to reaction of PMB with groups which have not reacted with iodoacetate, presumably those sulfhydryls involved in activation of the uninhibited enzyme. It is also conceivable, though not likely, that PMB may in some manner displace one or more carboxymethyl groups, perhaps from those sulfhydryls which react rapidly with PMB and are associated with activation.

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