REVERSIBLE ACTIVATION AND INACTIVATION OF MITOCHONDRIAL MALATE DEHYDROGENASE WITH PARA-HYDROXYMERCURIBENZOATE.[1]

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Summary. Partially photooxidized m-MDH[3] was readily inactivated with PHMB in contrast to the native enzyme. Native m-MDH was activated by PHMB and the degree of activation was correlated with the DPNH-X content of the enzyme. Both the PHMB activation of native m-MDH and the PHMB inactivation of partially photooxidized m-MDH was quantitatively reversed by thiols.

Materials and Methods. Pig heart m-MDH obtained from Seravac Laboratories was purified by CG-50 column chromatography using a modification of the method of Thorne (1960). Activity measurements were carried out in a manner described by Wolfe and Neilands (1956). Photooxidation was carried out in a thermostatted vessel, 3.5 cm in diameter, at 25°. A typical reaction mixture contained 0.3 mg enzyme, 0.01 mg methylene blue in 3 ml of 0.1 M phosphate buffer at pH 7.2. Mercaptide bond formation was determined by the method of Boyer (1954).

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[3] The abbreviations used are: PHMB, para-hydroxymercuribenzoate, m-MDH, mitochondrial malate dehydrogenase.

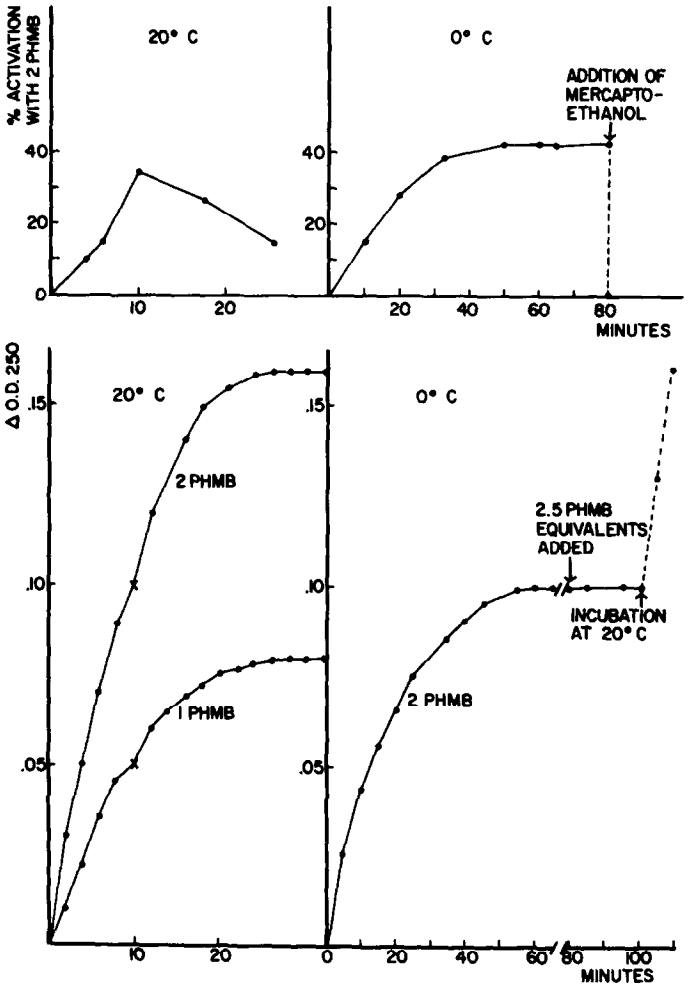

Fig. 1. Activation and mercaptide bond formation at 20° and 0°. (Enzyme concentration 0.4 mg/ml)

Experimental results. Comparison of the effect as a function of time of limited amounts of PHMB (one or two mercurials per subunit)[4] on native enzyme activity and mercaptide bond formation at two temperatures (Fig. 1) revealed (A) enzyme activation[5] that was transient at 20°

[4] The molecular weight of the subunit that was used in all calculations was 32,500.

[5] The activity of any given untreated enzyme preparation was arbitrarily expressed as 100%. Activation is expressed relative to this figure since it is not possible to use an absolute scale.

but stable at 0°, showing immediate, total reversal with 0.1 M mercaptoethanol; (B) less complete mercaptide formation at 0° than at 20°, as measured by changes in O.D. at 250 mμ after addition of PHMB; (C) identical kinetics of activation and of mercaptide bond formation at 0°; and (D) deviation points in the time curves of mercaptide formation at 20° that correspond to the time of maximum activation at 20°. Excess PHMB failed to increase mercaptide formation at 0°, although elevation of the temperature caused an immediate rise in the O.D. at 250 mμ. The degree of activation and quantitative difference in the extent of mercaptide bond formation depended on the enzyme preparation in a manner to be explained below; the data in Fig. 1 was taken on a purified "type B" enzyme which had been treated with charcoal. An inverse relationship between PHMB activation and mercaptide bond formation was observed when a number of native enzyme preparations were compared. Enzyme that was activated only slightly (10-15%) with PHMB, formed 1.8-2.0 mercaptide bonds per subunit at 0° while enzyme that was highly activated (80-85%) formed only 0.2-0.4 mercaptide bonds per subunit at 0°.

These preparations of m-MDH were submitted to photooxidation which resulted in a 10-15% loss of activity and a loss of about two histidine residues per subunit[6], and were then incubated with a given amount of PHMB for 90 minutes at 0°. An inverse correlation of PHMB activation with PHMB inactivation of the partially photooxidized enzyme was found as shown in Fig. 2. PHMB titration at 0° by the method of Boyer of

[6] Amino acid analysis was done on enzyme preparations exhibiting only slight PHMB activation. The extent of histidine destruction by partial photooxidation in enzyme preparations containing a large proportion of DPNH-X was not determined.

partially photooxidized "type A" enzyme revealed the formation of four mercaptide bonds. The PHMB inactivation of photo-

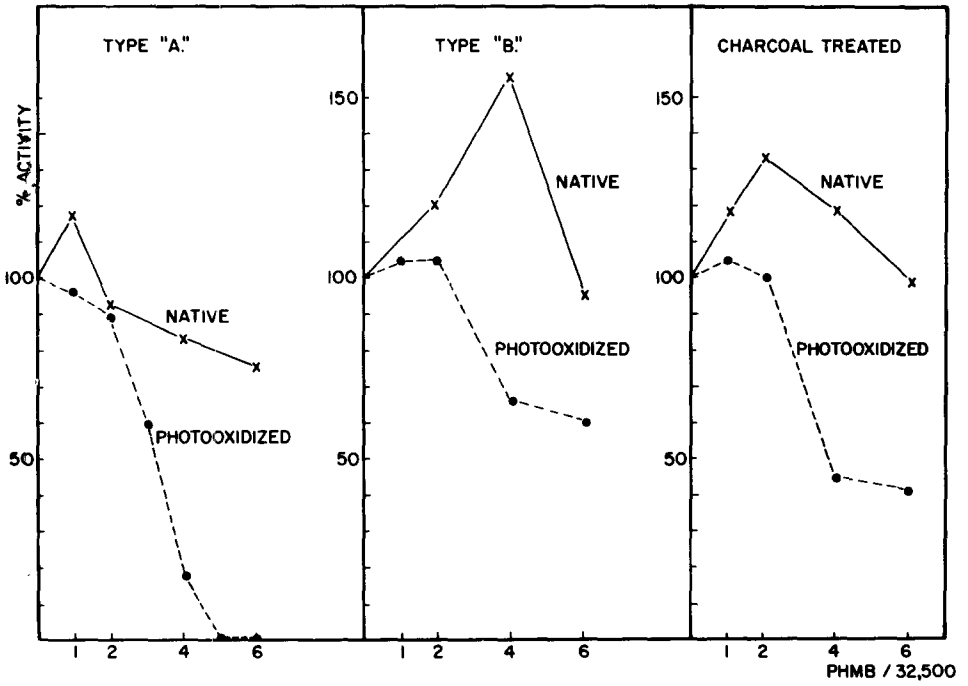

Fig. 2
Effect on PHMB on various types of native and 10-15% photoinactivated m-MDH.

oxidized enzyme was quantiatively reversed by 0.1 M cysteine. The percentage of PHMB activation of a number of native enzyme preparations plotted against PHMB inactivation of photo-oxidized enzyme followed a roughly linear relationship as shown in Fig. 3.

A "nucleotide" spectrally identical to the DPNH-X described by Wieland et al. (1960) was recovered from native enzyme preparations by treatment with charcoal, heat or perchloric acid. It was not reduced my m-MDH and 0.1 M malate and did not inhibit m-MDH in the presence of 0.001 M DPN. The presence of the "nucleotide" in various preparations was correlated with a lower specific activity, activation by PHMB, and a relatively higher absorption at 260 mμ. Calculations based on spectrophotometric measurements using the

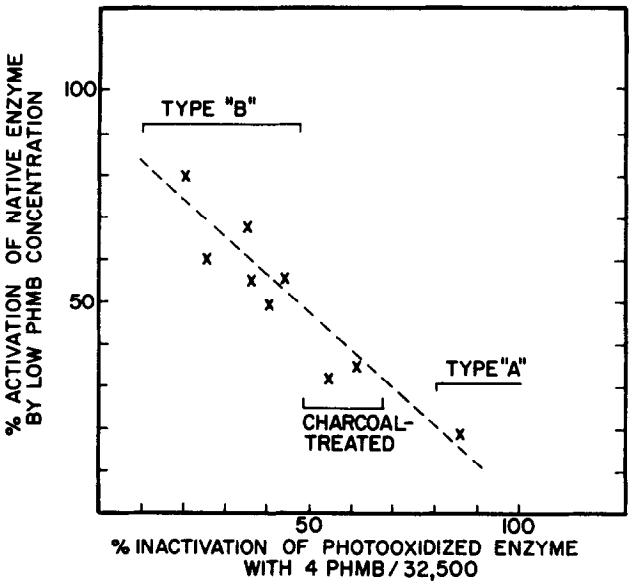

Fig. 3. Correlation between PHMB activation and inactivation. Each X represents a different enzyme preparation (see footnote 5). "Type B" enzyme contains a relatively high proportion of DPNH-X whereas "type A" enzyme has less.

extinction coefficient of DPNH-X given by Wieland suggested that there was one "nucleotide" bound per molecule of m-MDH (MW=65,000) which contained the inhibitor.

Discussion. The discovery of enzyme bound nucleotide allows the tentative identification of types "A" and "B" enzyme preparations (Fig. 3) as containing small and large fractions, respectively, of enzyme molecules binding one molecule of DPNH-X. It seems probable that an enzyme-DPNH-X-mercurial ternary complex represents the activated species which is stable at 0° and unstable at 20°.

The experimental results can be explained by the presence in a heterogeneous enzyme population of different proportions of normal m-MDH (E) and m-MDH with bound DPNH-X (E·DPNH-X). The following reactions of the normal enzyme and DPNH-X containing enzyme are consistent with the data.

$$(1)\quad E \xrightarrow[90 \text{ minutes}]{\text{excess PHMB, } 0^{\circ}} \underset{\text{enzymatically active}}{E(S\text{-}PMB)_2}$$

(2) $E \xrightarrow[\text{photooxidation}]{\text{partial}} E^* \xrightarrow[90 \text{ minutes}]{\text{excess PHMB } 0^\circ} E^*(S\text{-PMB})_4$ enzymatically inactive

(3) E·DPNH-X partially active $\xrightarrow{\text{PHMB}}$ E·DPNH-X(PHMB) activated

E= native enzyme, E* = 10-15% photoinactivated enzyme

Partially photooxidized enzyme (E) forms four mercaptide bonds per subunit by PHMB titration at 0° as compared to two in the native enzyme free of DPNH-X. Apparently limited photo oxidation of the enzyme which is not protected by DPNH-X permits sufficient conformational change in the protein to allow the two "essential sulfhydryls" of each subunit to react with PHMB. The presence of DPNH-X not only prevents the reaction of PHMB with the two nonessential sulfhydryls at 0° but also prevents inactivation by presence of a slight excess of PHMB following photooxidation.

It seems possible that the activation, i.e. enzyme-DPNH-X-PHMB complex formation, is an association phenomenon. Further studies on the nature of this "complex" and the possible regulatory role of DPNH-X are in progress in this laboratory.

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