Inactivation of Porcine Heart Cytoplasmic Malate Dehydrogenase by Pyridoxal 5'-Phosphate*

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Pyridoxal 5'-phosphate (pyridoxal-5'-P) has been found to act as a bifunctional reagent during the inactivation of porcine heart cytoplasmic malate dehydrogenase (L-malate:NAD⁺ oxidoreductase, EC 1.1.1.37). The biphatic kinetics and X-azolidine-like structure formed were similar to those observed for mitochondrial malate dehydrogenase (Wimmer, M. J., Mo, T., Sawyers, D. L., and Harrison, J. H. (1975) J. Biol. Chem. 250, 710-715). In the cytoplasmic enzyme, however, irreversible inactivation representing X-azolidine formation was found to be the dominant characteristic of the interaction with pyridoxal-5'-P. Spectral evidence indicated that at total inactivation 2 mol of pyridoxal-5'-P were incorporated per mol of enzyme or one pyridoxal-5'-P per enzymatic active site. The presence of NADH protected the enzyme from inactivation suggesting interaction of pyridoxal-5'-P at or near the enzymatic active centers of this enzyme. Fluorometric titrations indicated that pyridoxal-5'-P-inactivated enzyme failed to bind NADH or at least failed to bind NADH in the same fashion as native enzyme.

In recent years pyridoxal-5'-P has been shown to be an effective reagent for the selective chemical modification of numerous proteins (1-6). In all cases pyridoxal-5'-P was found to interact reversibly with the enzymes studied, and Schiff base formation with the ε-amino group of lysine residues was implicated. These reversible interactions could be made irreversible only by sodium borohydride reduction. However, in a preliminary report by Yost and Harrison (7) and a subsequent comprehensive investigation by Wimmer et al. (8) from this laboratory, the interaction of pyridoxal-5'-P with porcine heart mitochondrial malate dehydrogenase (L-malate:NAD⁺ oxidoreductase, EC 1.1.1.37) was found to be quite different. The inactivation of that enzyme demonstrated biphatic kinetics. The first phase was suggested to be caused by reversible Schiff base formation and, hence, was similar to the interaction observed in other enzymes. The second phase was irreversible and was attributed to formation of an X-azolidine-like structure, a further derivative of the Schiff base which involves a 2nd nucleophilic residue of the enzyme. A mechanism for the interaction of the reagent with mitochondrial malate dehydrogenase was suggested by Wimmer et al. (8). Similar results have since been obtained by Chen and Engel (9). Wimmer and Harrison (10) have recently established the existence of an essential lysine residue in mitochondrial malate dehydrogenase. In an attempt to further correlate structure-function relationships between cytoplasmic and mitochondrial malate dehydrogenase, the interaction of pyridoxal-5'-P with the cytoplasmic enzyme has been examined.

EXPERIMENTAL PROCEDURE

Materials—Porcine heart cytoplasmic malate dehydrogenase was purified from acetone tissue powder as previously described by Mathewson et al. (11). NAD⁺, NADH, L-malic acid, oxalacetic acid, AMP, pyridoxal-5'-P, and sodium borohydride were purchased from Sigma Chemical Co. Nicotinamide was purchased from Aldrich Chemical Co., Sephadex G-25 from Pharmacia Fine Chemicals, and sodium cyanoborohydride from Alpha Inorganics.

Enzymatic Assay—The assay conditions and method of determination of protein concentration were the same as those described by Bleile et al. (12). Assays in the direction of NADH oxidation were performed in 50 mM sodium phosphate buffer (pH 7.5) on a Hitachi Perkin-Elmer MFP-2A fluorescence spectrophotometer with a cell compartment thermostatted at 25°. The rate of decrease in emission at 457 nm was monitored upon excitation at 340 nm.

Enzymatic Inactivations with Pyridoxal-5'-P—Cytoplasmic malate dehydrogenase in 50 mM sodium phosphate buffer (pH 7.5) was incubated in the dark with various concentrations of pyridoxal-5'-P. The time course of inactivation was followed by removing aliquots of the incubation mixture at specific intervals and assaying for enzymatic activity. To prepare inactivated enzyme free of excess reagent, the procedure described by Wimmer et al. (8) involving Sephadex G-25 gel filtration and dialysis in 50 mM sodium phosphate buffer (pH 7.0) was employed.

Reductions—Reductions by sodium borohydride were performed as described by Wimmer et al. (8). Reductions employing sodium cyanoborohydride were performed at pH 7.5 for 5 min at 37°. Aqueous solutions of sodium cyanoborohydride (2.1 M) were added to reduction mixtures to yield a final concentration of 100 mM.

Fluorescence Measurements—Fluorescence measurements and titrations were performed as described by Bleile et al. (12) in 50 mM sodium phosphate buffer (pH 7.5) at 15°.
RESULTS AND DISCUSSION

Although cytoplasmic and mitochondrial malate dehydrogenase catalyze the same reaction, they are quite distinct enzymes which are coded for by two separate genes and possess different amino acid compositions and physicochemical properties. The ability to make correlations between the enzymes, such as, in their individual interaction with a common modifying reagent, i.e. pyridoxal-5′-P, would be advantageous in evaluation of their homology.

Cytoplasmic malate dehydrogenase (6 mg/ml, 0.081 mM) was incubated with a 250-fold molar excess of pyridoxal-5′-P (20.3 mM). The time course of inactivation is shown in Fig. 1. The time-dependent loss of enzymatic activity was found to be biphasic, exhibiting two pseudo-first order rates. Inactivation was examined at several concentrations of reagent. Plots of observed rate constants versus concentrations of pyridoxal-5′-P for the first phase appeared to be linear. Similar plots for the second phase were not linear and indicated saturation kinetics.

The first phase of inactivation, representing Schiff base formation, was reversible after Sephadex G-25 gel filtration and dialysis. It could be made irreversible by sodium borohydride or sodium cyanoborohydride reduction. However, reduction led to only a small amount (15%) of irreversible inactivation since very little Schiff base was formed with essential residues in the enzyme before equilibrium was reached and the second phase predominated. The second phase was irreversible in the absence of reduction suggesting formation of an X-azolidine-like structure.

In order to identify the residue which initially reacts with pyridoxal-5′-P, it is necessary that sufficient Schiff base be formed so that high yields of inactive, modified enzyme would result after reduction. Therefore, attempts were made to selectively increase the rate of the first phase of inactivation relative to the second. The inactivation was investigated at pH values between 6.5 and 8.0. The effects of temperature on the inactivation by pyridoxal-5′-P were also examined between 0 and 37°C. However, no conditions of pH or temperature could be found which would drastically decrease the rate of the second phase without also decreasing the rate and amount of inactivation during the first phase. Therefore, identification of essential residues involved in pyridoxal-5′-P inactivation of the cytoplasmic enzyme cannot be accomplished by simple Schiff base reduction and will require isolation of those peptides associated with the proposed X-azolidine-like structure.

The fluorescence and absorption spectra of enzyme inactivated by pyridoxal-5′-P in the absence of reduction were examined. A new fluorescence emission maximum at 392 nm appeared upon excitation at 325 nm. This fluorescence peak was characteristic of an X-azolidine-like structure as determined from such model adducts as t-cysteine-pyridoxal-5′-P (13). The absorption spectrum of this inactivated enzyme also indicated a new broad shoulder with a maximum at approximately 325 to 330 nm which was again characteristic of an X-azolidine-like structure (13, 14). These spectral data were very similar to that seen for pyridoxal-5′-P-inactivated mitochondrial malate dehydrogenase (7, 8). Similar spectral data have led to the proposal of an X-azolidine-like structure in pyridoxal-5′-P-dependent glutamate decarboxylase (15). Using the molar extinction coefficient for a thiazolidine-like complex determined by Coombs et al. (16), 3.2 x 10^4 M^-1 cm^-1 at 330 nm, it was determined that 1.7 mol of pyridoxal-5′-P were bound per mol of cytoplasmic malate dehydrogenase at 100% inactivation. Thus, approximately 2 mol of X-azolidine were formed per mol of enzyme or one X-azolidine-like structure per enzymatic active center.

The major difference between inactivation of cytoplasmic and mitochondrial malate dehydrogenase appears to be the preponderance of X-azolidine formation in the cytoplasmic enzyme. This predominance suggests that in the cytoplasmic enzyme the 2nd nucleophilic residue involved in X-azolidine formation must either be located in closer proximity to the 1st residue or be more reactive.

Fig. 1 also demonstrates the effects of NADH and oxalacetate on the inactivation of cytoplasmic malate dehydrogenase. NADH (80 mM) produced a large decrease in the rate of both phases of inactivation. Oxalacetate (160 mM) also provided significant protection. A nonproductive ternary complex of NADH (80 mM) and malate (160 mM) was found to protect the enzyme only slightly better than NADH alone (approximately 85% activity remained after 120 min). Malate (160 mM), which has a weaker affinity for the enzyme than oxalacetate, exhibited no effect on the rates of inactivation. The nearly complete protection afforded by NADH suggests that inactivation occurred due to interaction with residues located at or near the enzymatic active centers of the enzyme.

Nicotinamide and AMP, which represent opposite portions of the enzyme molecule and are competitive inhibitors of the enzyme (12), were examined to determine their effects on pyridoxal-5′-P inactivation (Fig 2). Both competitive inhibitors were included in inactivation mixtures at twice their K_i values. AMP provided a small amount of protection from pyridoxal-5′-P inactivation, while nicotinamide sharply increased the rate of inactivation. In the presence of nicotinamide the inactivation was not biphasic under the conditions employed, and loss of enzymatic activity was found to be irreversible. Similar effects have been previously observed for the mitochondrial enzyme; a cooperative effect of nicotinamide and pyridoxal-5′-P binding in simultaneous binding studies.
was reported for that enzyme (8). Recent evidence in our laboratory has suggested that nicotinamide binding caused dissociation of malate dehydrogenase dimers into active subunits. Concomitant with dissociation is the exposure of essential sulfhydryl residues in the enzyme rendering them accessible for subsequent modification by such reagents as N-ethylmaleimide. This exposure of essential residues may account for the enhanced rate of X-azolidine formation.

The $K_m$ and $V_{max}$ values for native and partially inactivated (20% activity remaining) cytoplasmic malate dehydrogenase were determined. X-Azolidine formation did not affect the $K_m$ for NADH (0.036 mM) or oxalacetate (0.093 mM). This suggests that pyridoxal-5'-P inactivation does not result in "partially active" modified enzyme. The $V_{max}$ for native enzyme was observed to be 2540 $\Delta_{457}$/min/mg. The $V_{max}$ for partially inactivated enzyme was determined to be 545 $\Delta_{457}$/min/mg and reflected the presence of 20% residual active enzyme. These results do not, however, remove the ambiguity of the cause for inactivation, i.e., inactivation caused by decreased binding of substrates or inactivation caused by alteration of a catalytic step subsequent to binding.

To explore further the mode of action of pyridoxal-5'-P on cytoplasmic malate dehydrogenase, instantaneous inhibition studies were performed. Noncovalent inhibition was predicted on the basis of the mechanism proposed by Wimmer et al. (8). The reagent was observed to be a noncompetitive inhibitor with respect to NADH and oxalacetate in 50 mM sodium phosphate buffer (pH 7.5) at 25°C. Chen and Engel (5, 9) have reported similar results not only for mitochondrial malate dehydrogenase, but also for several other dehydrogenases. The observation that pyridoxal-5'-P is a noncompetitive inhibitor with respect to both coenzyme and substrate does not permit a clear understanding of the relationship between binding of pyridoxal-5'-P and binding of coenzyme and substrate. Although binding of pyridoxal-5'-P and NADH or pyridoxal-5'-P and oxalacetate are not mutually exclusive, a portion of the inhibition must be due to competition of the reagent for the NADH and oxalacetate binding sites.

**REFERENCES**

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