been added. In sections of mycelia prefixed before incubation in complete ferricyanide medium, a slight deposit is observed on the mitochondrial membranes, and no deposit on the mesosomes or plasma membrane. No deposit at all is seen in sections of prefixed mycelia incubated in ferricyanide medium from which either succinate has been omitted or to which malonate added.

Incubation of fresh mycelia in TNBT reaction mixtures gives results that are essentially similar to those obtained by incubation in ferricyanide medium. However, difficulty is experienced in recognizing the reaction product of the TNBT (formazan) in thin sections in the electron microscope, because of the low electron density of the formazan. Therefore, the ferricyanide technique is preferred, as the reaction product is easily recognized in electron micrographs.

Biochemical assays for SDH activity in fractions isolated from Neurospora mycelia show SDH activity in the microsomal fraction (Fig. 2). This activity is linear with time and protein concentration, and makes up about 8% of the total SDH activity (mitochondrial + microsomal). No SDH activity could be demonstrated in the soluble fraction. Electron microscopic examination of negatively stained samples of the microsomal fractions show a small amount of mitochondrial contamination. However, approximately 98% of the (F_1) ATPase activity is found in the mitochondrial fraction (Fig. 2) and less than 2% of the oligomycin sensitive ATPase activity can be detected in the microsomal fraction. These observations suggest that only about 25% of the SDH activity of the microsomal fraction could be due to mitochondrial contamination.

Discussion

The results of the histochemical investigations indicate the presence of an activity which oxidizes succinate and concomitantly reduces an electron acceptor (ferricyanide or TNBT). This activity is visualized in electron micrographs in association with mesosomes and the plasms membrane, as well as the mitochondrial membranes. The dependence of this activity on the

After exhaustive dialysis to remove excess PLP, the enzyme was found to be totally inactive and spectral analysis of the protein indicated that the inactive protein possessed a new absorption peak with maxima at 325 nm, suggestive of a thiazolidine-like linkage (5).

MATERIALS AND METHODS: Porcine heart mitochondrial malate dehydrogenase was purified from acetone powders of fresh pig hearts (Harrison, to be published). The resultant preparation of enzyme exhibited a specific activity of 1600 units/ml/A_{280}. Protein concentrations were determined spectrophotometrically at 280 nm utilizing a molar extinction coefficient of 17,800. Pyridoxal-5'-phosphate, pyridoxamine-5'-phosphate, pyridoxal, β mercaptoethanol and hippuryl-lysine were obtained from Sigma Chemical Co. $NaBH_4$ was obtained from Fisher. All other reagents were of analytical grade.

The standard pyridoxal-lysine was prepared using hippuryl-L-lysine according to the method of Shapiro et.al. (1). The hippuryl-L-lysine-pyridoxal-5'-phosphate aduct was reduced with $NaBH_4$ and hydrolyzed in vacuo with constant boiling (106^{o}-108^{o}C) 6 N hydrochloric acid for 24 hours at 110^{o}C to yield pyridoxal lysine. The sample was desalted by chromatography on Dowex 50 and taken to dryness. Samples were dissolved in a limited amount of distilled water for thin layer chromatography. Thin layer chromatography was performed on cellulose plates obtained from Eastman utilizing N-butanol: pyridine:acetic acid:water (30:30:6:44) as a solvent system. Pyridoxal lysine was identified by its flourescent property.

RESULTS AND DISCUSSION: When a 300 fold M excess of PLP is incubated with MDH at pH 7.5 and 35^{o}C, enzymatic activity is observed to be lost in a time dependent linear fashion. The addition of 2×10^{-2} M reduced coenzyme, NADH, to the inactivation mixture yielded an enzyme fully protected from inactivation by PLP. Figure 1 represents the effect of PLP on MDH activity in the absence and presence of NADH expressed as log (velocity inhibited sample/velocity control sample) as a function of time in hours. The concentration of PLP

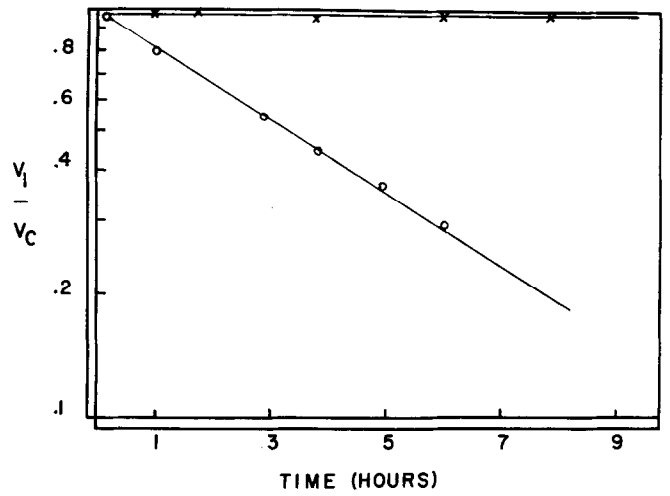

Figure 1. Effect of reduced coenzyme, NADH, on PLP inhibition of MDH. Incubation of MDH and PLP was performed at 35°C and pH 7.5 in 0.1 M sodium phosphate. (-o-) 300 fold molar excess of PLP. (-x-) 300 fold molar excess of PLP plus 20 mM NADH. Control curve and NADH protection curve are identical.

is high with respect to enzyme, therefore, pseudo first order kinetics were observed.

Upon lowering the temperature of the inhibition mixture from 35° to 25°, it was observed that two distinct portions of the inhibition curve exist (Fig. 2A). During the initial phase, a rapid loss of enzymatic activity is observed at both a 600 and 1200 fold molar excess of PLP. This initial rapid loss of activity, which is PLP concentration dependent, is subsequently followed by a slower time dependent loss of enzymatic activity, which is also PLP concentration dependent (Fig. 2B). The second order rate constant for this slow phase of the inhibition curve was calculated to be 0.31 $min.^{-1}$ $moles^{-1}$ liters.

Contrary to other reported cases of PLP inhibition of enzymatic activity, MDH activity which had been totally lost by incubation with PLP was not regained upon exhaustive dialysis or upon the addition of an excess of L-lysine or β mercaptoethanol. After exhaustive dialysis to remove excess PLP, spectral analysis of the inactive protein (Fig. 3) indicated the presence of a

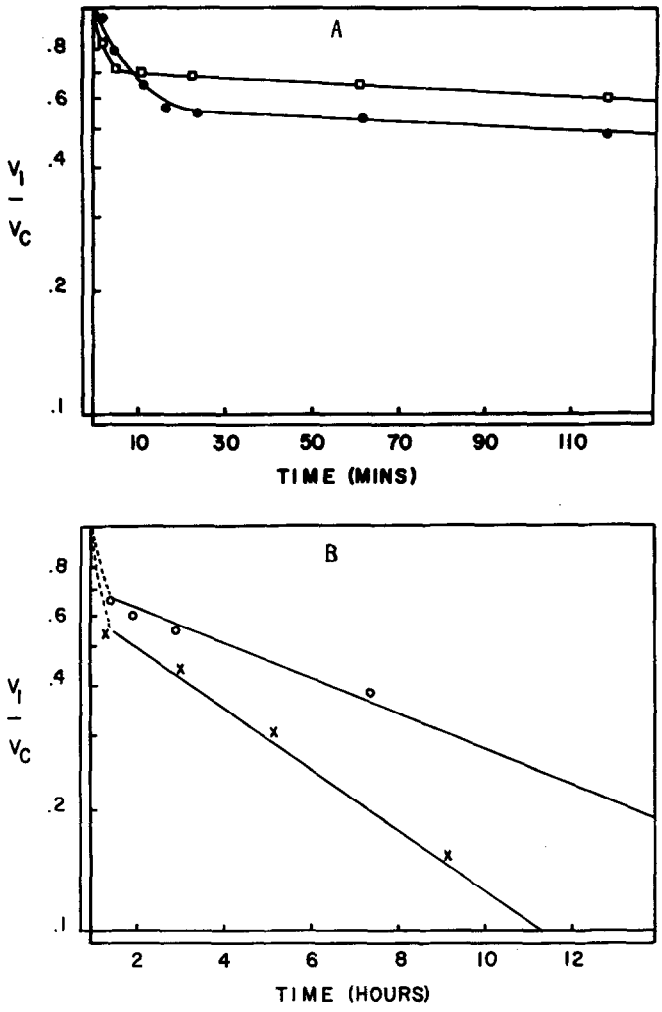

Figure 2. Effect of 600 fold molar excess (-□-) and 1200 fold molar excess of PLP (-●-) on MDH activity. Incubation carried out at 25°C and pH 7.5 in 0.1 M sodium phosphate. Figure 2A represents the initial rate of inactivation as a function of time in minutes. Figure 2B represents both rates of inactivation as a function of time in hours. (-o-) 600 fold molar excess, (-x-) 1200 fold molar excess.

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new absorption peak with maxima at 325 nm. This new absorption peak corresponds generally to that observed by Vallee et.al. (5) for the thiazolidine-like complex formed between PLP and L-cysteine. Using the extinction coefficient determined by Vallee for the thiazolidine-like complex, 3.2×10^3 M^{-1} cm^{-1} at 330 nm, the number of moles of PLP bound to MDH was determined to be 2.02 or approximately one per enzymatic active site.

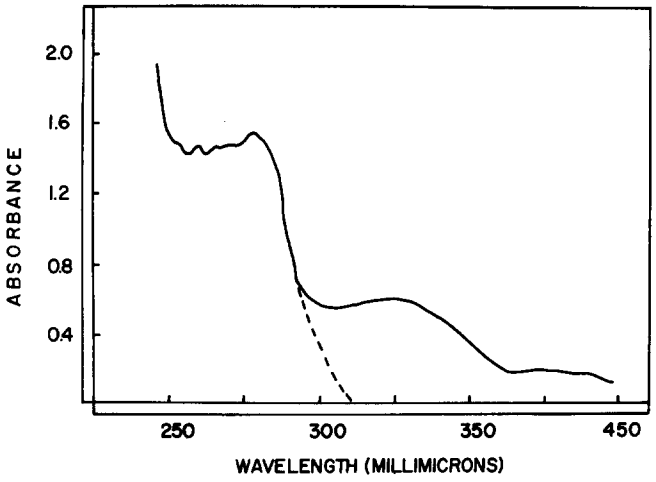

Figure 3. Spectra of inactive MDH after incubation with PLP as described in text, followed by dialysis vs. 0.05 M sodium phosphate pH 7.0 to remove excess PLP. Dashed line represents spectra of native MDH.

The 325 nm absorption peak present in MDH after exhaustive dialysis was found to be stable to dialysis conditions of pH 5.0 and 9.0 and to dialysis at neutral pH against 4 M guanidine hydrochloride. Reduction of this inactivated MDH, after exhaustive dialysis, with sodium borohydride at pH's between 5 and 9 failed to produce any measureable amounts of PLP lysine as determined by thin layer chromatography after acid hydrolysis. In this respect, the stability of the PLP-MDH complex appears to be quite similar to the stable product formed between PLP and bovine serum albumin which also exhibited an absorption maxima at 330 nm and was not reduced with sodium borohydride (6).

The inhibition observed with PLP can be completely prevented by the presence of 2×10^{-2} M NADH in the inactivation mixture. This prevention of inactivation suggests that the interaction of PLP with MDH must be in the general area of the NADH binding site and hence at or near the enzymatic active center of the enzyme.

Two analogs of pyridoxal-5'-phosphate, pyridoxal and pyridoxamine-5'-phosphate, were observed to produce no effect on the enzymatic activity of MDH when incubated with the enzyme at a 600 fold molar excess and pH 7.5 and 35°C.

The lack of inhibition by these analogs would suggest that the interaction of MDH and PLP was dependent not only on the aldehyde function of the inhibitor, but also the phosphate moiety.

The unique factor of this interaction between PLP and MDH is that the inhibition produced is irreversible to procedures which would normally be expected to easily reverse the formation of a Schiff base intermediate between PLP and the ε amino group of a lysine present in the enzyme (i.e., exhaustive dialysis or the addition of free amino groups as in L-lysine). The rapid inactivation of MDH upon addition of PLP, as observed in the early portion of the inactivation curves, appears to be similar to losses of activity observed in similar cases with studies on enzymes such as aldolase (1), glyceraldelyde-3-phosphate (2), glutamic (3), and 6-phosphogluconic dehydrogenases (4) (i.e., the formation of an easily reversible Schiff base). In each of these above mentioned systems, reversal of inhibition is possible prior to the subsequent reduction of the enzyme with sodium borohydride. In MDH the relatively slow rate of inactivation observed in the latter part of the inactivation curve appears to be unique to this particular enzyme. The observation of a new absorption peak (325 nm) present in MDH after total inactivation and prior to $NaBH_4$ reduction is consistent with the formation of a complex between PLP and MDH in a form other than as a Schiff base (absorption max 415) and may, in part, explain the tenatious binding of PLP to the protein. Pyridoxal-5'-phosphate has previously been shown to react with amino thiols, by incorporating the nitrogen and sulfur atoms of these compounds into a thiazolidine ring (5,7,8, 9,10). This interaction is accompanied by a characteristic change in the ultraviolet spectrum at 325-330 nm and is similar to spectral changes observed upon interaction of PLP and muscle phosphorylase (11). It appears quite reasonable that the formation of a thiazolidine-like complex, as has been proposed for the interaction of PLP and phosphorylase, might explain both the observed spectra and irreversibility of the lost enzymatic activity of MDH. In this respect, the PLP reaction with MDH may involve an intermediate Schiff

base formation with a specific lysine amino group, followed by a second slower reaction to form a more stable product.

The spectral changes observed in this system and the rather tight binding of the PLP moiety to MDH without reduction with sodium borohydride, might render PLP suitable as a site-specific reagent for the identification of a nitrogen-sulfur site in MDH. Work is currently in progress to determine the residues involved in the binding of PLP to MDH.

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