Functional Groups of Diphosphopyridine Nucleotide Linked Isocitrate Dehydrogenase from Bovine Heart. II. Studies of an Active Amino Group by Reaction with Aldehydes†

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ABSTRACT: A number of aldehydes are inhibitors of DPN-linked isocitrate dehydrogenase from bovine heart. The values of the rates of inactivation and of the enzyme–inhibitor association constant show that at pH 7.2 the inhibitory effects decrease in the order 4-nitrosalicylaldehyde, salicylaldehyde, pyridoxal-P, pyridoxal, glyoxal, acetaldehyde, and aldol. Spectrophotometric and fluorometric measurements of the P-pyridoxylidene and the P-pyridoxyl forms of the enzyme indicate that modification of one amino group per subunit of enzyme (42,000 daltons) is directly proportional to loss of activity. The pH–inactivation profile with pyridoxal-P shows that the active amino group has a pK_a of 8. Evidence has been presented for formation of dissociable complexes between enzyme and pyridoxal-P or pyridoxal which are converted subsequently to the covalently linked Schiff base enzyme forms. Such initial addition compounds were not detected with 4-nitrosalicylaldehyde or salicylaldehyde. Manganese isocitrate protects the enzyme against inhibition by a number of aldehydes. With pyridoxal-P a dissociation constant of K_{SP} = 0.069 mM for the manganese isocitrate–enzyme complex has been obtained from substrate protection experiments at pH 7.2. Complete restoration of activity and decomposition of Schiff base by manganese isocitrate have been obtained with enzyme inactivated up to 70% by pyridoxal-P, suggesting that the reactive amino group is located at the catalytic center of the enzyme. Reversal of pyridoxal-P inhibition by substrate indicates a dissociation constant for manganese isocitrate–enzyme complex of K_{SP} = 1.36 mM. When added singly, Mn^{2+}, isocitrate, DPNH, DPN, or ADP does not cause decomposition of the P-pyridoxylidene–enzyme complex suggesting that these substances bind to the enzyme at groups different from the reactive amino group specific for manganese isocitrate.

Reaction of a number of group-specific reagents (*e.g.*, trinitrobenzenesulfonate, ethyl acetimidate ester, potassium cyanate, etc.) with a single reactive amino group per subunit of DPN-linked isocitrate dehydrogenase has been correlated with loss of activity of the enzyme (Fan and Plaut, 1973). The protection against inactivation by substrate (divalent metal–isocitrate chelate) suggested that the reactive amino group may be at or near the catalytic site of the enzyme. However, the irreversible combination of these reagents with the amino group of the enzyme precluded studies on the reversal of the inhibition by substrate and other factors which could define more closely the role of this amino group in enzyme catalysis. Since reaction of an amino group with an aldehyde is reversible, interaction of the enzyme with a number of aromatic aldehydes was studied here.

The combination of protein amino groups with pyridoxal-P[1] as correlated with enzyme activity has been investigated in particular detail (Ronchi *et al.*, 1969; Benesch *et al.*, 1972; Piszkiewicz and Smith, 1971a; Forrey *et al.*, 1971; Rippa *et al.*, 1967; Anderson *et al.*, 1966), and spectral and chemical determination indicate that pyridoxal-P can be bound reversibly by forming either protonated or unprotonated Schiff bases. The latter can be reduced to the corresponding pyridoxamine derivative which possesses characteristic spectral and fluorescence properties which can be used for the further characterization of the nature and the stoichiometry of the interaction of the protein amino group with pyridoxal-P.

The present study shows that inactivation by pyridoxal-P and a number of other aromatic aldehydes is proportional to Schiff base formation at a single amino group per subunit of enzyme and that the activity of aldehyde-inactivated enzyme can be completely restored by manganese isocitrate. An apparent dissociation constant for substrate has been calculated from such an equilibrium system.

Experimental Procedures

Materials

Pyridoxal 5′-phosphate, pyridoxamine 5′-phosphate, and pyridoxal were purchased from Sigma. Glyoxal, aldol, salicylaldehyde, and 4-nitrosalicylaldehyde were from Eastman and acetaldehyde from Baker.

Other materials were of the same quality and were obtained from the same sources cited previously (Fan and Plaut, 1973). DPN-linked isocitrate dehydrogenase was stored at −90° in a solution containing 20% glycerol, 5 mM sodium phosphate at pH 7.2, and 0.1 mM 1,3-dithio-2-propanol.

Methods

Assays. Enzyme activity and protein were determined as described previously (Fan and Plaut, 1973).

Inactivation of Enzyme by Pyridoxal-P and Other Aldehydes. Enzyme (0.4–1.2 mg/ml) was incubated with aldehydes (*e.g.*,

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[1] Abbreviations used are: pyridoxal-P or PLP, pyridoxal 5′-phosphate; pyridoxamine-P or PMP, pyridoxamine 5′-phosphate; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; the 5′-phosphorylated residues containing the pyridoxyl and pyridoxylidene groups are designated P-pyridoxyl and P-pyridoxylidene, respectively.

0.03–0.7 mM pyridoxal-P) in 0.05–0.10 M NaHepes at pH 7.2 and 25°. Aliquots (10 μl or less) were removed from the reaction mixtures at appropriate time intervals and activity was determined in the standard assay (in a final volume of 1.0 ml).

The initial concentrations of pyridoxal-P were determined spectrophotometrically by the method of Peterson and Sober (1954).

Formation and Determination of Pyridoxylidene and Pyridoxyl Derivative of Enzyme. The formation of Schiff base from pyridoxal-P and enzyme was detected spectrophotometrically by the appearance of an absorption maximum at 430 nm. Difference spectra were usually taken at 5-min intervals over a 30-min period after addition of equal amounts of pyridoxal-P to the solution containing the enzyme and that in the reference cell.

In order to reduce the interference by free pyridoxal-P at 430 nm, readings were made at a slit width of 0.01–0.4 mm in a Cary Model 14 spectrophotometer fitted with a high-intensity light source which was set at 90 V. Even under these conditions no more than 0.4 mM pyridoxal-P could be used without affecting the accuracy of the determination by the pronounced background absorption.

For reduction of Schiff base the pyridoxal-P-inactivated enzyme was cooled to 0° and treated with a drop of octyl alcohol, and 50 mM $NaBH_4$ in 60% alcohol was then added in a 100-fold excess over the amount of pyridoxal-P present in the reaction mixture. After 10 min, the protein was separated from small molecular weight components of the reaction mixture by chromatography on a 0.5 cm × 10 cm column of Bio-Gel P-10, equilibrated previously with a solution of 0.1 mM dithiopropanol in 5 mM sodium phosphate at pH 7.2. The protein fraction was then dialyzed against several portions of the phosphate–glycerol–dithiopropanol buffer to assure complex removal of noncovalently bound reagents. The quantity of pyridoxamine-P bound to the protein was measured spectrophotometrically at 325 nm. The molar extinction coefficient of 10,000 for N^6-phosphopyridoxyllysine (Forrey *et al.*, 1971) was used to estimate the extent of modification of the protein. It was shown in separate experiments that treatment with $NaBH_4$ does not inactivate the enzyme; however, prolonged dialysis following the reduction can lead to loss of activity.

The fluorescence emission and excitation spectra of the P-pyridoxyl–enzyme preparations were measured with the same spectrofluorometer described previously (Harvey *et al.*, 1972) except that it was fitted with a 150-W xenon Hanovia arc lamp and an EMI-6256 phototube. Photodecomposition of phosphopyridoxamine derivatives was minimized by attenuation of the light (Chen, 1965) with two 20-mesh blackened copper screens inserted in front of the second focusing mirror inside of the excitation monochrometer. Fluorescence was measured at 25° in a standard 1-cm quartz fluorescence cuvette usually containing 1 ml of sample.

Calculations. If one assumes that the interaction between an aldehyde and the reactive amino group of the enzyme yields the corresponding Schiff base without significant accumulation of intermediates (*e.g.*, carbinolamines), the reaction can be described by

$$E + I \rightleftharpoons EI \qquad (1)$$

where I represents the aldehyde, E the enzyme, and EI the Schiff base complex of the enzyme. At constant pH, where no significant change in protonation of any group would occur, the equilibrium can be described by

$$K_{pH} = [EI]/[E][I] \qquad (2)$$

The association constant K_{pH} can be calculated according to Piszkiewicz and Smith (1971a) in the presence of total enzyme (E_t) from the enzyme activity before inactivation (Act_0) and from the residual activity after equilibrium with inhibitor has been reached (Act_∞)

$$K_{pH} = \frac{[E_t] - [E_t](Act_\infty/Act_0)}{[E_t][I](Act_\infty/Act_0)}$$

This equation can be simplified to

$$K_{pH} = \frac{1 - (Act_\infty/Act_0)}{(Act_\infty/Act_0)[I]} \qquad (3)$$

For estimation of rates of enzyme inactivation, pseudo-first-order rate constants have been calculated in situations where inhibitor concentration was in excess of enzyme from

$$k_1 = 2.303 \frac{d \log (Act_t)/(Act_0)}{dt} \qquad (4)$$

where Act_t is enzyme activity at time t. The second-order rate constants were calculated from

$$k_2 = \frac{\frac{1}{b - a} \ln \left[\frac{a(b - x)}{b(a - x)}\right]}{t} \qquad (5)$$

where a and b are the initial concentrations of enzyme of 42,000 molecular weight subunit and inhibitory aldehyde, respectively, and x is the concentration of reacted material (EI) at time t.

The concentration of manganese isocitrate$^-$, the predominant species above pH 7, is calculated from known concentrations of total Mn^{2+} and isocitrate added (Plaut *et al.*, 1973) with the stability constant $K = 1.056$ mM^{-1} of Gryzbowski *et al.* (1970).

Results

Enzyme Inactivation

Inhibition. The effect of a number of aliphatic and aromatic aldehydes on the activity of DPN-linked isocitrate dehydrogenase was investigated by incubating the enzyme with the aldehyde in 0.1 M NaHepes at pH 7.2 and 25°. Aliquots of the preincubation mixture were removed and transferred to standard assay media for measurement of initial velocities of DPN reduction. In the absence of the aldehydes the enzyme retained full activity under the conditions of preincubation. Furthermore, the inactivation of the enzyme by aldehydes was not reversed by dilution of aliquots of the preincubation mixture in the standard assay medium during the time required to perform the activity measurements. The activity of aldehyde-inactivated enzyme, however, can be restored by prolonged dialysis against a suitable buffer.

The results obtained with several reagents are shown in Table I as rates of inactivation (second-order rate constants)

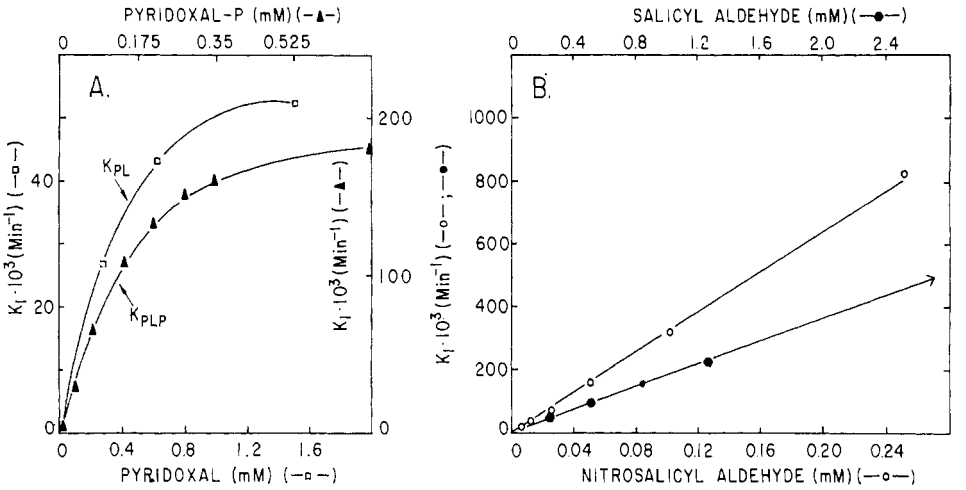

FIGURE 1: The effect of aldehyde concentration on the pseudo-first-order constants of enzyme inactivation. Enzyme (0.0065 mM) was incubated with the aldehydes at the concentrations shown in 0.1 M NaHepes at pH 7.2 and 25°. Aliquots of the preincubation mixture were assayed at appropriate time intervals as described under Methods. (A) Pyridoxal, □; pyridoxal-P, ▲. The lines represent fits of the data to a computer program for a rectangular hyperbola (Cleland, 1963). The apparent half-saturation constants for binding of pyridoxal and pyridoxal-P are indicated by the arrows. (B) Salicylaldehyde, ●; 4-nitrosalicylaldehyde, ○.

TABLE I: Inactivation of Enzyme by Various Aldehydes.[a]

Inhibitor	Concn Range (mM)	Inhibitor Equil Constant, K_{pH} (mM^{-1})	Rate of Inactivn, $k_2 \times 10^2$ (mM^{-1} min^{-1})
Acetaldehyde	44.5	0.085	0.526
Glyoxal	19.3	0.221	4.99
Aldol	31.5–73.4	0.013	0.074
Pyridoxal	0.31–1.55	0.438 ± 0.030	7.02 ± 0.62
Pyridoxal-P	0.21–1.66	2.95 ± 0.24	22.5 ± 0.7
Salicylaldehyde	0.25–1.25	24.8 ± 2.5	25.3 ± 3.0
Nitrosalicyl-aldehyde	0.025–0.25	73.8 ± 4.2	269 ± 33

[a] Enzyme (0.0065 mM) was inactivated with the inhibitors in 0.1 M NaHepes buffer at pH 7.2 and 25°. Aliquots of the preincubation mixture were then assayed after varying time periods as described under Methods. k_2 and K_{pH} were calculated with the aid of eq 5 and 3, respectively.

and at equilibrium (K_{pH}) when with increased time of incubation no further inhibition is obtained. In accord with observations with a number of enzymes (Piszkiewicz and Smith, 1971a; Ronchi *et al.*, 1969) pyridoxal-P is a more effective inhibitor than pyridoxal. However, the salicyaldehyde derivatives, especially 4-nitrosalicyclaldehyde, are at least as potent or more potent inhibitors than pyridoxal-P.

The effects of reagent concentrations with constant enzyme on pseudo-first-order rate constants of enzyme inactivation are shown in Figure 1. The points obtained with pyridoxal or pyridoxal-P can be fitted to a rectangular hyperbola (Figure 1A) suggesting complex formation between these reagents and enzyme before formation of the pyridoxylidene enzyme compounds. Values of apparent K_m of 0.38 and 0.14 mM for pyridoxal and pyridoxal-P, respectively, can be calculated for formation of these intermediary complexes with the computer program for a rectangular hyperbola (Cleland, 1963). In contrast, a linear relationship between concentration and pseudo-first-order rate constants of inactivation was obtained with salicylaldehyde and 4-nitrosalicylaldehyde (Figure 1B), indicating that, at least at the reagent concentrations used, an intermediary enzyme-aldehyde complex is not observed in enzyme–Schiff base formation from these compounds.

Protection against Inhibition. The rates of inactivation of enzyme by a number of aldehydes in the presence of certain cofactors and substrates in the preincubation mixture are shown in Table II. Manganese isocitrate gave significant protection against the effect of all of the inhibitors tested whereas only a slight effect (if any) was obtained with isocitrate or divalent metal ion alone, DPN$^+$, DPNH, or ADP.

TABLE II: Protection against Inhibition by Various Aldehydes.[a]

Inhibitor Protector	Concn (mM)	Inhibition Rate, $k_2 \times 10^2$ (mM^{-1} min^{-1}) Nitro-salicyl-aldehyde[c,d]	Salicyl-aldehyde[d]	Pyri-doxal-P[e]
None		33.5	27.9	19.4
Mn^{2+}	2	20.7	20.9	20.9
DL-Isocitrate	20	24.5	20.1	18.8
Manganese DL-isocitrate[b]	1.9	7.3	3.7	3.7
DPNH	0.2	29.9	26.6	18.0
DPN$^+$	5	24.6	24.7	19.4
ADP	0.25	25.1	19.4	19.4

[a] The enzyme, protectors, and inhibitors were preincubated in 0.1 M NaHepes at pH 7.2 and 25° under the conditions described under Methods. Aliquots were removed at appropriate time intervals and assayed. [b] The concentrations of total manganese and DL-isocitrate were 2 and 20 mM, respectively. [c] To regard the reaction, 0.2 M NaCl was present in the preincubation mixture. [d] The concentrations of 4-nitrosalicyl-aldehyde or salicylaldehyde and enzyme subunit were 0.167 and 0.008 mM, respectively. [e] The concentrations of pyridoxal-P and enzyme subunit were 0.7 and 0.0065 mM, respectively.

The relative ineffectiveness of metal ions as protectors against pyridoxal-P inactivation was confirmed in separate experiments (not shown) in which several levels of Mn^{2+} (2–50 mM) and Mg^{2+} (50–90 mM) were investigated. The results with aldehydes as inhibitors are in marked contrast to experiments with trinitrobenzenesulfonate where bivalent metal ions gave marked protection against inactivation (Fan and Plaut, 1974).

The significant protection afforded by manganese isocitrate was studied in more detail at several levels of this substrate and at a fixed concentration of pyridoxal-P. A plot of the second-order rate constants of inactivation against concentration of manganese isocitrate$^-$ (Figure 2) can be fitted to a hyperbola from which the dissociation constant of manganese isocitrate$^-$ at which protection is half-maximal can be calculated to be K_{SP} = 0.069 mM.

Effect of pH on Inactivation.[2] The influence of pH on the rate (second-order rate constants) and extent (K_{pH}, eq 2) of the inactivation by pyridoxal-P was studied. Reasonably parallel curves are obtained between pH 5.60 and 9.0 with a minimum at about pH 6.5. Between pH 6.5 and 9 the inflection points for the best-fit curves describing the effects of pH on inactivation rate and K_{pH} were approximately at pH 8.

The variations in rate and extent of inhibition as a function of pH may relate to the dissociation constants of the various ionic forms of all of the reactive components of the system. Since the dissociation constants of pyridoxal-P (pK_1 = 4.2 and pK_2 = 8.7) and the probable values for the P-pyridoxylidene derivatives (pK_1 = 5.9 and pK_2 = 10.5) (Auld and Bruice, 1967) are quite different from the observed value of around a pK_a of 8, it is likely that the latter represents the apparent dissociation constant of the protonated amino group which is related to the enzyme activity. It is pertinent that the pH–rate profiles of the inactivation of the enzyme by ethyl acetimidate, ethyl benzylimidate, and trinitrobenzenesulfonate also indicate a reactive enzyme amino group with a pK_a of 8 (Fan and Plaut, 1973).

The enhanced rate and extent of inactivation below pH 6.5 might indicate interaction of pyridoxal-P with another functional group on the enzyme with a pK_a below 6.5. While the effect of treatment with pyridoxal-P on sulfhydryl content has not been examined at the lower pH, such a study has been done at pH 7.2.[3] Under this condition, when determined by the method of Ellman (1959), the sulfhydryl contents of unmodified and P-pyridoxyl–enzyme were identical. However, the rate of interaction of 5,5′-dithiobis(2-nitrobenzoate) was somewhat slower with the P-pyridoxyl derivatized than the native enzyme. This may suggest a close proximity of the P-pyridoxal binding site and a sulfhydryl residue on the enzyme.

Ionic Strength. The rate of inactivation of the enzyme by aldehydes is influenced markedly by ionic strength. The rate of inactivation by pyridoxal-P in 0.05 M NaHepes at pH 7.2 is slowed by about 50% in the presence of 0.25 M NaCl.[2] The inhibition by higher salt concentrations of interaction of reagents with enzyme has also been observed with other aldehydes. For example, the rate of inactivation by 4-nitrosalicylaldehyde was larger in the absence (Table I) than in the presence of 0.2 M NaCl (Table II). The effect of high ionic strength seems to reflect a change in the accessibility of the amino group to reaction with a number of reagents. A similar decrease in rate of inactivation with increasing ionic strength has been observed previously with trinitrobenzenesulfonate (Fan and Plaut, 1974).

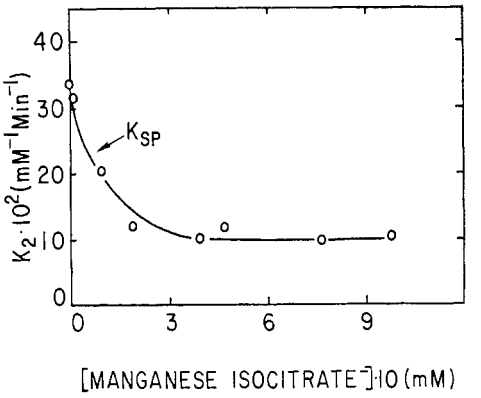

FIGURE 2: Protection by manganese isocitrate against inactivation of enzyme by pyridoxal-P. Manganese DL-isocitrate$^-$ at the concentration indicated was preincubated with the enzyme (0.0065 mM) in 0.05 M NaHepes at pH 7.2 and 25° for 25 min. Inactivation was then initiated by the addition of 0.32 mM pyridoxal-P to the preincubation mixtures. Aliquots were removed and assayed at appropriate time intervals for rate studies. The second-order rate constants and manganese isocitrate$^-$ concentrations were fitted to a rectangular hyperbola by a computer program described by Cleland (1963). The binding constants (K_{SP}) obtained was indicated by arrow.

Enzyme Modification by Pyridoxal-P

Formation of 5′-Phosphopyridoxylidene–Enzyme. Although a number of aldehydes are potent inhibitors of the enzyme the effect of pyridoxal-P was studied in particular detail. The combination of this reagent with amino groups of the enzyme as the pyridoxylidene or pyridoxyl derivatives could be followed conveniently by spectrophotometric or fluorometric methods.

The enzyme in 0.1 M NaHepes at pH 7.2 did not absorb light in the region between 300 and 450 nm. The addition of pyridoxal-P to the enzyme solution resulted in characteristic spectral changes with maxima at 430 and 278 nm, a minimum at 378 nm, and a shoulder in the 340-nm region. Absorption maxima in the 430-nm region have been observed with a number of pyridoxal 5′-phosphate–protein complexes and have been attributed to a form in which a phenolic hydroxyl group is hydrogen bonded to the amino nitrogen of the Schiff base (Mitzler, 1957). The shoulder in the 340-nm region, which is much more pronounced at high pH values, is believed to represent an aldimine without intramolecular hydrogen bonding (Morino and Snell, 1967).

Difference spectra comparing pyridoxal-P with the combination of pyridoxal-P and enzyme are shown in Figure 3A. With constant enzyme concentration absorbance at 430 nm increased with increasing pyridoxal-P concentrations and it was shown in separate experiments that there was a concomitant decrease in enzyme activity. Under the conditions of incubation used, equilibrium between the enzyme and the aldehyde was reached in about 20 min since the intensity of absorption at 430 nm and the inhibition became constant.

5′-Phosphopyridoxyl–Enzyme. It has been possible to reduce the Schiff base formed from various oxo compounds and enzymes to the corresponding alkyl- or arylamine derivatives. Preliminary experiments indicated that activity was retained

[2] Experimental details on the effect of pH and of various levels of NaCl on inhibition by pyridoxal-P, the proportionality of pyridoxylation of the enzyme and loss of activity, and the absorption (Figure 3B) and fluorescence emission spectra (Figure 3C) of P-pyridoxyl–enzyme are available from the authors on request.

[3] C. Fan and G. W. E. Plaut, unpublished observation, 1973.

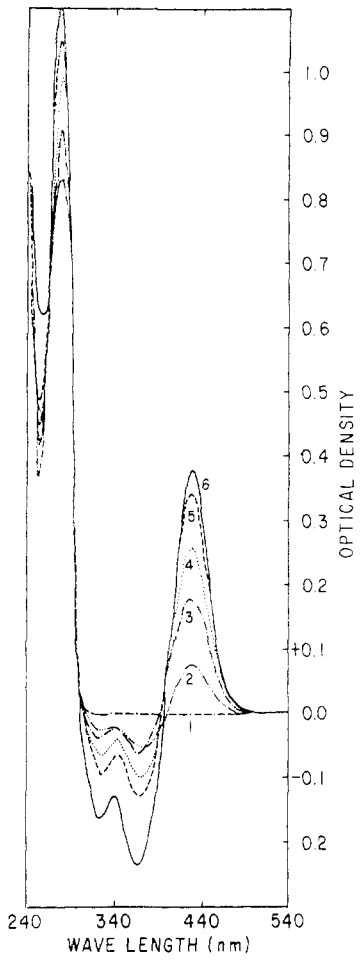

FIGURE 3: Absorption and fluorescence spectra.[2] Difference spectra of P-pyridoxylidene-enzyme. The sample cell contained 34 μM enzyme in 0.1 M NaHepes at pH 7.2 and the reference cell contained an equal volume of buffer. Equal amounts of pyridoxal-P were added to both cells and spectra were taken 30 min after each addition as described under Methods. The final pyridoxal-P concentrations were (1) 0.0 mM (- - -), (2) 0.075 mM (- · · · -), (3) 0.15 mM (- - -), (4) 0.225 mM (· · · · ·), (5) 0.30 mM (- - -), and (6) 0.375 mM (—).

when the native isocitrate dehydrogenase was treated with $NaBH_4$. It seemed possible, therefore, to study the relationship between extent of chemical modification of the enzyme by pyridoxal-P and inhibition of activity by reduction of the Schiff base to the enzyme–pyridoxyl-P derivative. The spectrum of P-pyridoxyl–enzyme preparation has two absorption maxima[2] and it is similar to several other P-pyridoxyl–enzyme complexes (Ronchi *et al.*, 1969; Anderson *et al.*, 1966). Absorption at 280 nm does not differ from that of the untreated enzyme. The second absorption maximum at 325 nm is characteristic of pyridoxamine derivatives, and it is not observed with the protein which had not been treated with pyridoxal-P before reduction.

The correspondence between enzyme modification and inactivation was studied by observing the absorption at 325 nm of different P-pyridoxyl-P preparations. Varying concentrations of pyridoxal-P (0.7–2.1 mM) and enzyme (6–43 μM) were interacted for different periods of time followed by reduction by $NaBH_4$ of the Schiff base formed to the corresponding pyridoxyl derivative. The results indicate that loss of activity (42–70%) is directly proportional to pyridoxylation of approximately one amino group per enzyme subunit.[2]

The properties of the P-pyridoxyl–enzyme were also studied by fluorescence spectroscopy. Excitation of the P-pyridoxyl–enzyme at 327 nm resulted in a single emission band with a maximum at 390 nm which is characteristic of pyridoxamine-P (Churchich, 1965).[2] Furthermore, it was observed in separate experiments that equivalent molecular quantities of free and of the enzyme-bound pyridoxamine-P gave similar quantum yields of fluorescence[4] when excited at 327 nm. However, while free pyridoxamine-P showed practically no fluorescence when excited at 281 nm the P-pyridoxyl–enzyme showed emission over a broad range with maxima near 385 and 338 nm. The emission at the shorter wavelength of the P-pyridoxyl–enzyme corresponds to that of the same protein not treated with the reagent while that at the longer wavelength is due to bound pyridoxamine-P.[2] Energy transfer from an aromatic amino acid residue to the covalently linked P-pyridoxal group is indicated by the appearance of emission near 385 nm and the decrease in intensity of protein fluorescence at 338 nm of the P-pyridoxyl–enzyme.[2] From the critical transfer distance of 23 Å given by Churchich (1965) for the tryptophan–pyridoxamine-P pair and by assuming that all of the tryptophans in the enzyme are involved in the transfer in an identical manner, one can estimate that the actual distance between the donor and acceptor in the enzyme is 24 Å. The radius of the enzyme subunit (42,000 daltons) should be approximately 26 Å; hence the acceptor and donor can be located in the same macromolecule. This becomes even more likely if fewer than the three tryptophans per subunit[5] are involved in the energy transfer; under these conditions the actual distance of the donor–acceptor pair should be less than 24 Å.

Reversal of Pyridoxal-P Inactivation

The inhibition by pyridoxal-P could be reversed partially by gel filtration, dialysis or the addition of 0.05 M lysine in time-dependent reactions. However, the protection by manganese isocitrate against inhibition by pyridoxal-P (Table II and Figure 2) suggested the possibility that the addition of the complex might lead to a substrate-specific decomposition of the P-pyridoxylidene–enzyme compound with restoration of activity.

Reversal of Inactivation by Manganese Isocitrate. The time course of inactivation of an enzyme by pyridoxal-P leading to a 79% loss of activity in 120 min is shown in Figure 4 (curve a). The addition of manganese isocitrate to aliquots of the reaction mixture at stages of incubation when the enzyme was inhibited by 40, 55, and 70% (Figure 4, curve a), respectively, led to a time-dependent reversal of inactivation and almost complete restoration of activity in 40–50 min (Figure 4, curves b–d).

Relationship of Enzyme Modification and Activity. Enzyme was treated with varying concentrations of pyridoxal-P and incubated until activity had stabilized at the inhibited levels (40–60 min). Modification of the protein by pyridoxal-P was followed spectrophotometrically at 430 nm. As shown in Figure 5 (solid dots) inactivation of the enzyme (in the range between 50 and 80% inhibition) is directly proportional to the P-pyridoxylidene content of the protein. Aliquots of an

[4] C. Fan, L. Tomcho, and G. W. E. Plaut, unpublished observations, 1973. The comparison was based on the assumption that the molecular absorbances at 325 nm of free and enzyme-bound pyridoxamine-P are identical.

[5] J. Fleming and G. W. E. Plaut, unpublished observations (1970).

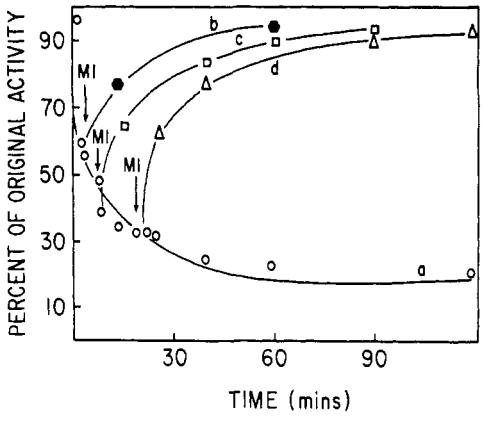

FIGURE 4: Reversal of pyridoxal-P caused inactivation by manganese isocitrate. Enzyme (0.0026 mM) was incubated with 0.4 mM pyridoxal-P in 0.1 M NaHepes at pH 7.2 and 25° (curve a). Samples were withdrawn from the reaction mixture at the time intervals indicated by the arrows, treated immediately with 25 mM manganese isocitrate$^-$ (MI) and incubated (curves b–d). Aliquots were removed from the preincubation mixtures at the times indicated and activities were determined in the standard assay.

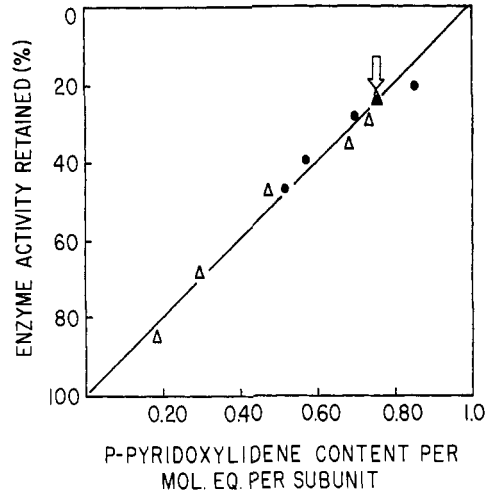

FIGURE 5: Correlation of P-pyridoxylidene content and activity of the enzyme. The incubation conditions were similar to those described in Figure 4. Inactivation: enzyme (7.2–14.1 μM) was incubated with 0.1–0.4 mM pyridoxal-P in 0.1 M Hepes at pH 7.2 and 25° until there was no further change in activity and Schiff base formation, ●. Reversal of inactivation by manganese isocitrate: a sample of the enzyme which had been inactivated 80% with pyridoxal-P (arrow) was incubated with increasing concentrations of manganese DL-isocitrate (10–100 mM) until there was no further change in activity and pyridoxylidene content, △. The P-pyridoxylidene content of the protein was measured at 430 nm and calculated as described in the text. Aliquots of the incubation mixtures were tested for activity in the standard assay.

enzyme preparation which had been inactivated about 70% with pyridoxal-P (Figure 5, arrow) were then treated with varying concentrations of manganese isocitrate and incubated until the activity had stabilized at the new equilibria (30–50 min). As shown in Figure 5 (triangles), increasing the concentrations of manganese isocitrate from 10 to 100 mM led to increasing restoration of activity and a decreasing Schiff base content of the protein. The results obtained either by direct inhibition with pyridoxal-P or its reversal by manganese isocitrate fall on the same straight line showing direct proportionality of enzyme inhibition and P-pyridoxylidene content of the protein (Figure 5). The intercepts of the line indicate that modification of a single amino group per enzyme subunit leads to loss of activity.[6]

In contrast to the reversal by manganese isocitrate of the inhibition caused by pyridoxal-P and the accompanying decomposition of the P-pyridoxylidene–enzyme (Figure 5), substances which did not protect the enzyme against inhibition by aldehydes (Table II) also did not cause reversal of the inhibition. Thus, no change in activity was observed with an enzyme preparation which had been inhibited 50% by pyridoxal-P upon prolonged incubation (1–16 hr) with DL-isocitrate (20 mM), ADP (5 mM), DPN^+ (5 mM), DPNH (0.2 mM), Mn^{2+} (1–14 mM) or Mg^{2+} (14–70 mM).

Equilibrium Constants. The reversal of pyridoxal-P (I) inhibition by manganese isocitrate (S) suggests a competitive relationship of the substrate and the inhibitor for the enzyme

$$\begin{array}{l} E + I \underset{}{\overset{K_{pH}}{\rightleftharpoons}} EI \\ + \\ S \underset{K_{SR}}{\rightleftharpoons} ES \end{array}$$

According to this scheme the concentration of the inactive P-pyridoxylidene–enzyme (EI) will vary depending on the concentrations of I and S. This should make it possible to estimate the dissociation constant (K_{SR}) for the equilibrium (eq 6) from activities obtained with known concentrations of

$$[E][S]/[ES] = K_{SR} \quad (6)$$

enzyme, pyridoxal-P, and manganese isocitrate$^-$ in the preincubation mixture.

The equilibrium of the reaction between pyridoxal-P (I) and enzyme in the absence of substrate has been described in eq 2 and the association constant K_{pH} can be determined in experiments where inhibitor is present and substrate absent with the aid of eq 3. The concentration of EI (inactive) can be calculated from the relationship

$$[E_{active}] = [E] + [ES] = [E_t] - [EI] \quad (7)$$

The value of K_{SR} can be calculated from equations 2 and 6.

In the experiments shown in Table III the initial inactivation by pyridoxal-P ranged from 23 to 63% and the extent of reversal of inhibition by manganese isocitrate is indicated by the decrease in concentration of the inactive P-pyridoxylidene–enzyme (Δ[EI]). Under these conditions an average value of K_{SR} of 1.36 ± 0.14 mM was obtained.

Discussion

Inactivation of the enzyme is proportional to modification by pyridoxal-P of a single amino acid residue per subunit of enzyme. This is supported by the correlation of activity and the spectrophotometrically determined P-pyridoxylidene (Figure 5) or P-pyridoxyl[2] content of the protein at varying stages of enzyme inactivation, as well as upon graded reversal of

[6] However, in separate experiments (not shown) it was observed that when inhibition of activity exceeded 75–80%, modification of the protein by further addition of pyridoxal-P (as judged by absorption at 430 nm) was more extensive than enzyme inactivation. Furthermore, while partial reversal of inhibition by manganese isocitrate was still obtained with the more extensively inactivated preparations, complete removal of the modifying groups no longer occurred. The results suggest that with the more highly inactivated preparations further modification of the protein occurs more rapidly at amino acid residues other than that which is a part of the catalytic center.

TABLE III: Apparent Equilibrium Constants for Substrate Reversal of Pyridoxal-P-Inactivated Enzyme.[a]

Pyridoxal-P (mM)	Enzyme Subunit (mM)	K_{pH} (mM^{-1})	Manganese Isocitrate^{-1} (mM)	$-\Delta[EI]$ (%)	K_{SR} (mM)
0.21	0.023	2.66	2.29	11.1	1.01
0.42	0.023	3.14	2.29	19.6	1.92
0.42	0.021	3.14	4.20	32.5	1.35
0.42	0.016	3.14	8.40	46.7	0.81
0.63	0.023	2.73	2.29	23.1	1.45
0.63	0.021	2.73	4.20	29.8	1.70
0.63	0.016	2.73	8.40	44.8	1.25
Average		2.84 ± 0.26			1.36 ± 0.14

[a] Enzyme at the concentrations indicated was incubated with varying pyridoxal-P (0.21–0.63 mM) in 0.1 M NaHepes at pH 7.2 and 25° until the activity was constant (approximately 50 min). Manganese DL-isocitrate$^-$ at the concentrations shown was then added to the equilibrium systems and the incubation was continued until the activity was constant. The difference in activity before and after addition of manganese isocitrate$^-$ is equal to the change in concentration of P-pyridoxylidene–enzyme, $-\Delta[EI]$; it is reported as per cent of E_t. The calculations of the inhibition constant (K_{pH}) and of the binding constant of manganese isocitrate$^-$ for reversal of pyridoxal-P inhibition (K_{SR}) are described in the text.

the pyridoxal-P caused inactivation by manganese isocitrate (Figure 5). These results are in agreement with experiments on inactivation and extent of modification of the enzyme with trinitrobenzenesulfonate and KNCO (Fan and Plaut, 1973).

Studies on inactivation of the enzyme by the group-specific agents KNCO, ethyl acetimate esters, and trinitrobenzenesulfonate (Fan and Plaut, 1973) indicated that an active amino group at or near the substrate binding site was modified. This is confirmed here by the observation that a number of aldehydes cause inactivation (Table I) against which significant protection was found with the substrate manganese isocitrate (Table II). The identity of the reactive residue with an amino group is further strengthened by the spectral characteristics of the P-pyridoxylidene and P-pyridoxyl derivatives of enzyme. For example, the difference spectrum of the P-pyridoxylidene–enzyme with maxima at 430 and 340 nm and with minima at 378 nm and 325 (Fig. 2) is similar to the P-pyridoxylidene derivative of glyceraldehyde-3-phosphate dehydrogenase (Ronchi *et al.*, 1969). The absorption and fluorescence spectra of the P-pyridoxyl–isocitrate dehydrogenase formed by reduction of the P-pyridoxylidene derivative[7] are in accord with those of a number of other protein P-pyridoxyl derivatives (Churchich, 1965; Ronchi *et al.*, 1969; Anderson *et al.*, 1966) and provide additional support for primary Schiff base formation causing inactivation of the enzyme.

It is likely that modification of the enzyme by aldehydes (Table I) and by other amino group specific reagents (Fan and Plaut, 1974) which leads to enzyme inactivation occurs with a common reactive amino group. At low concentrations of trinitrobenzenesulfonate the pseudo-first-order rates of inactivation and trinitrophenylation were linear until 70–80% of activity was lost. Furthermore, the apparent pK_a of this group is around $pK_a = 8$ as determined from rate constants of inactivation with a number of group-specific reagents (Fan and Plaut, 1974). With pyridoxal-P inactivation and equilibrium inhibition studies as a function of pH gave a pK_a of approximately 8.0 for the reactive amino group of isocitrate dehydrogenase. This pK_a value is lower by 1.3–2.5 units than might be expected for an ϵ-lysyl residue in a protein (Edsall, 1943). A similar value of pK_a has been observed in studies of the reaction of lysine-97 of glutamate dehydrogenase with pyridoxal (Piszkiewicz and Smith, 1971b) and pyridoxal-P (Piszkiewicz and Smith, 1971a). These investigators have attributed the lowered pK_a of the amino function to its possible hydrophobic environment and the possibility that formation of an additional positive charge would be retarded by the presence of additional cationic groups near the reactive lysine. Such an explanation could also be applicable to the behavior of the amino group of DPN-linked isocitrate dehydrogenase. However, isocitrate dehydrogenase also shows an increased rate of inactivation by pyridoxal-P below pH 6.5. It may be that this reflects an acid-dependent dehydration of a carbinolamine adduct catalyzed by the phosphate group ($pK_a = 5.5 \pm 0.2$) of pyridoxal-P as has been suggested in other studies (Auld and Bruice, 1967) or it may be due to interaction of pyridoxal-P with enzyme to form a product other than an aldimine.

The inhibition of DPN-linked isocitrate dehydrogenase by low concentrations of pyridoxal-P can be reversed by lysine, valine, or by dilution. These properties are consistent with formation of an aldimine which affects the activity of the enzyme. The protection by manganese isocitrate against inactivation by aldehydes (Table II) and the restoration of activity with the accompanying decomposition of the P-pyridoxylidene–enzyme by the substrate (Figure 5) cannot be explained by the expected chemical reactivity of the aldimine but must be due to specific binding of manganese isocitrate to the enzyme. One would expect from thermodynamic considerations that the binding constants of manganese isocitrate should be identical when obtained in experiments based on protection against inactivation and from reversal of inactivation by the substrate. However, in experiments with pyridoxal-P the observed dissociation constant for substrate reversal of inhibition (K_{SR} = 1.36 mM, Table III) is approximately 20 times higher than that for protection against inhibition (K_{SP} = 0.069 mM, Figure 2). Even if one estimates the possible effect of differences in ionic strength in these experiments, the discrepancy between these constants would still be about 10-fold.

The marked differences in magnitude of these constants may be due to the molecular properties of the protein. One such major change caused by protein modification may involve alteration of subunit–subunit interaction. Recent studies of the sedimentation velocity of carbamylated DPN-linked isocitrate dehydrogenase show the major component sedimenting at about 7 S indicating a tetramer of mol wt 160,000;[7] this compares to a molecular weight of 320,000 for the active species of the enzyme which is composed of eight apparently identical subunits (Giorgio *et al.*, 1971). Indications that the native enzyme also may disaggregate have come from earlier experiments in which it was found that a marked decline of enzyme activity occurred upon dilution of the enzyme before assay (Plaut, 1970). If a depolymerization also accompanied the interaction of enzyme with pyridoxal-P, it is possible that the reversal of inactivation by manganese isocitrate may in-

[7] C. Fan, R. Roxby, and G. W. E. Plaut, unpublished observation (1973).

clude the additional change(s) of the quaternary structure of the protein, *e.g.*, from the tetramer to the active octamer form. In contrast, in the substrate protection experiments the form of the enzyme exposed to pyridoxal-P will be present mainly as the octamer. In this context, the considerable differences in contact time between reagents and enzyme in the experiments on the reversal of inhibition by substrate compared to protection against inhibition by manganese isocitrate need to be considered in a possible explanation of the differences in the values of K_{SP} and K_{SR}. Thus, inactivation of the enzyme by pyridoxal-P and the restoration of activity by substrate are relatively slow processes (Figure 4) while reaction with manganese isocitrate with the native enzyme is likely to be rapid.

A second major factor which may be involved in the enzyme modification reactions and in binding of substrate is a variation in the environment of the active center, possibly by a change in pK_a of the functional groups. If the binding of the substrate requires a hydrophobic region as well as a cationic site, the pK_a of the reactive amino group could shift to a higher (1–2 units) pH region. If all of the reactive amino groups were also involved in substrate binding they should become inaccessible at pH 7.2 to modifying reagents when the enzyme is saturated with substrate. This is not the case, as complete protection by manganese isocitrate has not been obtained (Figure 2). Nevertheless, these results do not rule out a role of substrate binding on the reactivity of the amino groups. Substrate may bind only to part of the eight reactive centers of the active protomer with the remaining sites vacant but changed in accessibility to modifying agents. The decreased rate of inactivation in the presence of manganese isocitrate (Table II and Figure 2) may reflect a substrate-induced change of protein structure which causes the reactive amino group to shift to a higher pK_a at sites unoccupied by substrate. An accurate estimation of such changes has not been made. However, such a substrate-induced conformational change is consistent with initial velocity studies of the enzyme which gave parabolic plots of $1/v$ *vs.* 1/[MI] and Hill plots for magnesium isocitrate with slopes near 2 in the absence of ADP (Plaut *et al.*, 1973).

With enzyme preparations in which the reactive amino groups have been largely converted initially to the corresponding aldimine derivatives (Figure 5 and Table III) the binding of subsequently added substrate may not exert the type of cooperative effect discussed above. It may be pertinent that while protection by manganese isocitrate against pyridoxal-P inhibition was never complete (Figure 2), complete restoration of activity was obtained with enzyme in which almost one reactive amino group per subunit had been modified by pyridoxal-P (Figures 4 and 5). This implies that more reactive amino groups can be modified by pyridoxal-P than the number of molecules of manganese isocitrate which can be bound maximally to an equal amount of enzyme. Thus, decomposition of the enzyme–pyridoxal-P complex must involve a step(s) other than a direct displacement by manganese isocitrate of covalently bound pyridoxal-P at each subunit.

Differences in quaternary structure and conformation may play a role in the binding of substrate to the native as compared to the modified enzyme. These additional equilibria between different forms of the enzyme, as influenced by ligand binding, may be responsible for the large differences in the binding constants found in experiments on protection (K_{SP}) and reversal (K_{SR}) of inhibition by substrate.

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