Coenzyme Binding Site of Glutamate Decarboxylase†

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ABSTRACT: Dissociation constants have been measured for the binding of a variety of simple analogues of pyridoxal 5′-phosphate to apoglutamate decarboxylase. Compounds studied have a simple alkyl or aryl group and a negatively charged substituent (phosphate, phosphonate, phosphoramidate, sulfate, sulfonate, or carboxylate). Optimum binding to the phosphate binding site of the enzyme is achieved by compounds having a double negative charge and a tetrahedral geometry. Planar anions and monoanions bind considerably less well. These and previous data are used to derive the magnitudes of the contributions of various coenzyme functional groups to the strength of the apoenzyme–coenzyme interaction.

A variety of analogues of pyridoxal-P[1] have been synthesized and studied both in model systems (Bruice & Benkovic, 1966) and in enzyme systems (Korytnyk & Ikawa, 1970; Korytnyk, 1979) in an attempt to define the roles of the various functional groups of the coenzyme in binding and in catalysis. Roles in catalysis were first defined by studies of model systems (Bruice & Benkovic, 1966). Studies of enzyme systems are consistent with these model studies in that the 3-hydroxyl group, the 4-carboxaldehyde group, and the pyridine ring are the principal contributors to catalytic activity.

Roles in binding, on the other hand, are necessarily studied by use of the enzymes themselves, in conjunction with appropriately designed coenzyme analogues (Snell, 1970). Roles of functional groups in binding are less well established than roles in catalysis because binding has often been ascertained only in connection with measurements of catalytic activity. Most of the coenzyme analogues which have been studied are multifunctional, and it is difficult to separate quantitatively the contributions of the various functional groups to binding.

The enzymes which have been most studied are aspartate aminotransferase (Yang et al., 1975; Mura et al., 1972; Fukui et al., 1969; Bocharov et al., 1968; Mühlradt et al., 1967; Furbish et al., 1969; Evangelopoulos & Sizer, 1965; Morino & Snell, 1967; Fonda, 1971; Hullar, 1969), glutamate decarboxylase (Fonda, 1971; Mekhanik et al., 1972; Snell, 1975; Groman et al., 1972; Morino & Snell, 1967), D-serine dehydratase (Groman et al., 1972; Morino & Snell, 1967; Dowhan & Snell, 1970), arginine decarboxylase (Blethen et al., 1968; Groman et al., 1972), and serine sulfhydrase (Efremova et al., 1974).

The following qualitative picture of binding has emerged from these studies. Different pyridoxal-P-dependent enzymes are tolerant to different degrees of alkyl substitution at the 2 position of the pyridine ring, provided only that the group at the 2 position is not too large to allow proper binding. Methyl substitution at the 6 position likewise has little effect on either binding or catalysis. Although the hydroxyl group at the 3 position of the coenzyme is clearly required for catalytic activity, the role of this group in binding is less well established. Kinetic studies of coenzyme analogue binding (O'Leary & Malik, 1972) suggest that the hydroxyl group may have an important role. Comparison of pyridoxal-P with deoxypyridoxine-P in the case of glutamate decarboxylase (EC 4.1.1.15) (O'Leary & Malik, 1972; Mekhanik et al., 1972) and aspartate aminotransferase (O'Leary & Van Lanen, 1974) suggests that formation of the enzyme-pyridoxal-P Schiff base contributes perhaps a factor of 10^3 to coenzyme binding.

The role of the phosphate ester group in binding is perhaps the most complex and the least understood. Coenzyme analogues lacking an anionic group at the 5 position bind at least 10^6-fold more poorly than the natural coenzyme (O'Leary, 1969; Fonda, 1971; Mekhanik et al., 1971, 1972). Simple phosphates bind to the phosphate binding site, and this binding has been suggested to be the first step in the formation of the protein–pyridoxal-P complex (O'Leary & Malik, 1972). A variety of pyridoxal-P analogues having different anionic substituents at the 5 position have been synthesized and tested for their ability to bind to and reconstitute pyridoxal-P-dependent enzymes. The analogues which bind well and give rise to catalytically competent enzymes generally have dianionic groups with the approximate geometry and charge distribution of the phosphate group, although some geometric flexibility clearly exists. Monoanions (carboxylates, sulfates, and sulfonates) sometimes bind, but they do not support catalytic activity. NMR studies of D-serine dehydratase (O'Leary & Payne, 1976; Schnackertz et al., 1979; O'Leary and Jaworski, unpublished experiments) indicate that the coenzyme is rigidly bound to the protein.

The pyridoxal-P analogues tested to date have limited ability to provide quantitative information about the interaction of the phosphate group with the protein because the other coenzyme–protein interactions mask the protein–phosphate interaction. In addition, coenzyme binding equilibrium is often achieved quite slowly with these compounds (O'Leary & Malik, 1972; Fonda, 1971; Mekhanik et al., 1972), and it is difficult to be sure that the measured dissociation constants are accurate. Alternative approaches to binding make use of simple coenzyme analogues whose principal interaction with the protein is due to their phosphate or other negatively charged group. Past applications of this approach have made use of the ability of such compounds to decrease the rate of binding of pyridoxal-P to glutamate apodecarboxylase (O'Leary & Malik, 1972; Fonda, 1975). The present study makes use of the analogue-induced shift in the equilibrium for binding of deoxypyridoxine-P to glutamate apodecarboxylase. Binding of the latter compound to the apoenzyme is accompanied by a significant spectral change as a result of a protein-induced shift in the pK_a value of the phenolic hydroxyl group. Spectra

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[1] Abbreviations used: pyridoxal-P, pyridoxal 5′-phosphate; pyridoxamine-P, pyridoxamine 5′-phosphate; deoxypyridoxine-P, 4-deoxypyridoxine 5′-phosphate; pyridoxine-P, pyridoxine 5′-phosphate; DTT, dithiothreitol.

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determined at various concentrations of deoxypyridoxine-P provide a titration curve from which the dissociation constant for this compound can be determined. In the presence of a coenzyme analogue, the titration curve is displaced as a result of competition between the analogue and deoxypyridoxine-P. We have used this approach to determine dissociation constants for binding of a number of analogues to glutamate apodecarboxylase. The compounds were chosen to ascertain the role of the 5′-phosphate side chain in coenzyme binding and to eliminate the portions of the pyridoxal-P molecule which obscure the contributions of this group. The data help to define the electronic and geometric factors involved in phosphate binding to the enzyme.

Experimental Section

Materials

L-Glutamic acid, lysozyme (grade 1 from egg white), and protamine sulfate (grade 1 from salmon) were obtained from Sigma. Glutaric acid and DL-α-methylglutamic acid were obtained from Aldrich Chemical Co. Enzyme grade ammonium sulfate was from Mann Research Laboratories. Pyridine was distilled from BaO. Water was purified by using a Millipore Super Q water purification system and had a resistance of 18 MΩ.

Unless otherwise specified, all compounds listed below were purified on Amberlite XE-64 with H_2O as eluant (Peterson & Sober, 1954).

Deoxypyridoxine-P was obtained from Calbiochem. Pyridoxal-P and pyridoxamine-P were from Sigma. Pyridoxine-P was synthesized from pyridoxamine-P (Peterson & Sober, 1954). 4-Pyridoxic acid 5′-phosphate was prepared from pyridoxal-P (Churchich & Oh, 1974).

2-Methyl-4-amino-5-(hydroxymethyl)pyrimidine phosphate (toxopyrimidine phosphate) was prepared from 2-methyl-4-amino-5-(bromomethyl)pyrimidine (Lewin & Brown, 1963).

3-Pyridylmethyl phosphate, 2-(3-pyridyl)ethyl phosphate, and 1-(3-pyridyl)ethyl phosphate were prepared from the corresponding alcohols by condensation with 2-cyanoethyl phosphate (Aldrich) (Tener, 1961).

3-Pyridylmethyl methylphosphonate was prepared by condensation of the corresponding alcohol with methylphosphonic acid (Frank Enterprises, Inc.) by using the method of Tener (1961).

trans-2-(3-Pyridyl)ethenylphosphonic acid and 2-(3-pyridyl)ethylphosphonic acid were prepared from pyridine-3-carboxaldehyde by using the Wittig reaction (Hullar, 1969).

3-Pyridylmethylphosphonic acid was prepared by an Arbuzov reaction (Kosolapoff & Maier, 1973). The free base of 3-(chloromethyl)pyridine (Aldrich) (2.0 g) was dissolved in 25 mL of CH_3CN. Freshly distilled $P(OEt)_3$ (4.0 g) was added dropwise, and the solution was refluxed 4 h. After filtration and rotary evaporation, the sample was dissolved in 20 mL of H_2O, neutralized, and extracted with 3 volumes of $CHCl_3$. Following acid hydrolysis of the esters, the desired compound was purified as described above.

3-Pyridylmethylphosphoramidic acid was prepared from 3-pyridylmethylamine (Chemical Samples, Inc.) and diphenyl phosphorochloridate (Aldrich) (Grayson & Griffin, 1967). After base hydrolysis of the esters and careful neutralization, the compound was purified on a DEAE-cellulose column. After elution with 0.1 M NH_4OAc and lyophilization, 35% of the free amine was present as an impurity.

3-Pyridylmethyl sulfate was prepared from the corresponding alcohol with fuming H_2SO_4 (Yang et al., 1974) and recrystallized from H_2O/EtOH.

2-(3-Pyridyl)ethylsulfonic Acid. Ethyl 3-pyridylacetate (Aldrich) was reduced with lithium aluminum hydride to the alcohol, which was chlorinated (Wagler & Hoyer, 1965). 1-Chloro-2-(3-pyridyl)ethane was converted to the corresponding sulfide (Kasuga & Taguchi, 1965), and the sulfide was then oxidized to the desired sulfonic acid (Bauer & Gardella, 1961). The compound was recrystallized from EtOH and is hygroscopic.

Ethyl phosphate and methyl phosphate (K and K Industries) were purified to the respective monoesters as barium salts (ethyl, Plimmer & Burch, 1929; methyl, Cramer & Hettler, 1958).

3-Pyridylacetic acid hydrochloride and 3-(3-pyridyl)-propanoic acid (Aldrich) were recrystallized to yield white needles. *3-(3-Pyridyl)propenoic acid* (Aldrich, special collection) and disodium phenyl phosphate (Sigma, phenol free) were used as received.

3-Hydroxypyridine-4-carboxaldehyde was prepared by J. R. Payne (O'Leary & Payne, 1971). *Dicyclohexylammonium benzyl phosphate salt* was prepared by J. Malik (O'Leary & Malik, 1972).

Methods

Ultraviolet spectra were taken on a Cary 15 or Cary 118 spectrophotometer, and fixed wavelength kinetics were followed on a Gilford Model 222 spectrophotometer. All pH measurements were made at room temperature with a Radiometer Model 26 pH meter calibrated by the two-buffer method. A Gilson differential respirometer was used for enzyme assays. Milligram quantities were measured with a Cahn Model M-10 microbalance.

Fluorescence spectra were taken with an Aminco-Bowman ratio spectrophotofluorometer equipped with a thermostated cell holder (the microcell has a brass spacer block to aid thermal equilibration). Entrance, exit, and photomultiplier slits were 1 mm, which provided resolution of approximately 10 nm (peak width at one-half height). A polarizer was used in the excitation beam to reduce the interference from scattered light which occurs even when the solutions have been carefully centrifuged (Udenfriend, 1962). The polarizer was oriented to pass only light polarized with the electrical vector in the plane formed by the excitation beam. Excitation and emission spectra were recorded on a Hewlett-Packard X-Y recorder, and fixed wavelength data were recorded on a Sargent Model SRG recorder.

Glutamate Decarboxylase. L-Glutamic acid decarboxylase was isolated from locally prepared acetone powder of *E. coli* (ATCC 11246) and assayed manometrically (O'Leary, 1969). Addition of 0.1 mg/mL bovine serum albumin improved the linearity of the assays of highly purified enzyme. Protein concentration was determined by absorbance at 280 nm ($OD_{280}^{1\%} = 17$) (Shukuya & Schwert, 1960). Active-site concentration was calculated from the activity, assuming a specific activity of 0.11 mmol min^{-1} mg^{-1} at pH 4.9, 37 °C, for pure enzyme with one active site per subunit of molecular weight 50 000 (Shukuya & Schwert, 1960). Holoenzyme may be stored precipitated in 70% ammonium sulfate containing 0.01 M pyridoxal-P at 4 °C for months with little loss of activity. Before use, the precipitated enzyme was dissolved in buffer, heated at 50 °C for 15 min, and centrifuged to yield enzyme of specific activity greater than 0.08 mmol min^{-1} mg^{-1}.

The enzyme was resolved by adding 1 mL of a saturated solution of DL-α-methylglutamic acid in H_2O (pH 4.9) to 2.0 mL of glutamate holodecarboxylase (>10 mg/mL) in 0.05 M pyridine hydrochloride buffer, pH 4.9, containing 0.135 M chloride, and 10^{-4} M DTT. Additional solid DL-α-methyl-

glutamic acid was added and the pH adjusted to pH 4.9 with NaOH at 1-h intervals until the solution became colorless (about 4 h). The enzyme solution was dialyzed against three 500-mL changes of the same buffer and centrifuged. Dialysis ensures complete removal of the tight binding, competitive inhibitor, sulfate (Fonda, 1975). The apoenzyme routinely had less than 10% residual activity and could be reconstituted to greater than 75% of the original total activity and greater than 95% of the original specific activity. The apoenzyme is stable for weeks at 4 °C at concentrations above 5 mg/mL. Holoenzyme is reconstituted by incubating the apoenzyme in the presence of 0.01 M pyridoxal-P for 10 min at room temperature before assay. In the presence of inhibitors, 30-min incubations were necessary to ensure complete reconstitution.

Equilibrium Binding Experiments. Aliquots of glutamate apodecarboxylase (100 μL, 0.3–1.5 mg/mL) were placed in a series of small conical polyethylene vials. To each was added inhibitor (0–30 mM) and deoxypyridoxine-P (0.25–40 μM) to a final volume of 140 μL. The solutions were mixed, incubated at 20 °C for 8–24 h depending on the experiment, and transferred serially to a fluorescence microcell which had been equilibrated at 20 °C. Between solutions, the microcell was rinsed with H_2O and then CH_3OH, air-dried, and returned to the instrument in the same configuration. The fluorescence intensity of each solution at 380 nm following excitation at 325 nm was recorded for 1 min. After correction for inherent cell differences, the precision of the experimental procedure is ±2.5% of the total fluorescence. Experiments at pH 5.5 measured the fluorescence intensity at 380 nm following excitation at 290 nm.

For each inhibitor, a series of measurements was made at four concentrations of deoxypyridoxine-P for each of four concentrations of inhibitor. Each measurement was made on at least two, and usually three, independent samples. Concentrations were chosen to cover the range from 10 to 90% binding for all but the most weakly binding inhibitors.

Dissociation constants were calculated by using a program supplied by W. W. Cleland which finds the best weighted least-squares fit to eq 1 where D_T = [total deoxypyridoxine-P],

$$F = [F_{max}[D_T + E_T + K_{ED}(1 + \frac{I_T}{K_{EI}})] - [D_T + E_T + K_{ED}(1 + \frac{I_T}{K_{EI}})]^2 - 4E_TD_T]^{1/2}]/2E_T \quad (1)$$

E_T = [total apoenzyme], I_T = [total inhibitor] ($I_T \gg E_T$), $F = F_{obsd} - F_{E_T} - F_{D_T}$ (the observed fluorescence minus the contributions of apoenzyme and deoxypyridoxine-P taken separately), F_{max} = maximum fluorescence obtained when the apoenzyme is saturated with deoxypyridoxine-P, and K_{ED} and K_{EI} are the dissociation constants for the enzyme–deoxypyridoxine-P and enzyme–I complexes, respectively.

Preliminary estimates were obtained by using the graphical method of Holbrook (1972), which yields both K_{ED} and K_{EI} with eq 2, where α = [ED]/[E_T] = F/F_{max} and [ED] =

$$\frac{K_{ED}(1 + \frac{I_T}{K_{EI}})}{1 - \alpha} = \frac{D_T}{\alpha} - E_T \quad (2)$$

[enzyme–deoxypyridoxine-P complex]. A plot of $1/(1 - \alpha)$ vs. D_T/α gives an apparent K_{ED} as the slope. A plot of slope, $[K_{ED}(1 + I_T/K_{EI})]$, vs. $[I_T]$ yields both true dissociation constants.

In most cases the values obtained by the two methods differed by less than 10%. The data in Table I were determined by using the computer method. Errors given are standard deviations derived from the computer analysis.

Table I: Dissociation Constants for Binding of Coenzyme Analogues to Glutamate Apodecarboxylase at pH 4.9, 20 °C

compd	K_{diss}
deoxypyridoxine-P	0.88 ± 0.18 μM
pyridoxine-P	0.11 ± 0.06 μM
toxopyrimidine-P	39 ± 13 μM
3-PyCH$_2$OPO$_3$H$_2$[a]	0.50 ± 0.13 mM
3-PyCH=CHPO$_3$H$_2$	1.11 ± 0.45 mM
3-PyCH$_2$CH$_2$PO$_3$H$_2$	32.7 ± 6.9 mM
3-PyCH$_2$NHPO$_3$H$_2$	2.24 ± 0.58 mM
3-PyCH(CH$_3$)OPO$_3$H$_2$	10.1 ± 5.4 mM
3-PyCH$_2$OP(CH$_3$)O$_2$H	10.1 ± 1.6 mM
3-PyCH$_2$PO$_3$H$_2$	23.6 ± 9.6 mM
3-PyCH$_2$CH$_2$OPO$_3$H$_2$	9.0 ± 2.1 mM
3-PyCH$_2$OSO$_3$H	2.2 ± 0.4 mM
3-PyCH$_2$CH$_2$SO$_3$H	25.2 ± 5.5 mM
3-PyCH$_2$CO$_2$H	5.14 ± 0.65 mM
3-PyCH$_2$CH$_2$CO$_2$H	no binding
3-PyCH=CHCO$_2$H	no binding
C$_6$H$_5$CH$_2$OPO$_3$H$_2$	1.06 ± 0.13 mM
C$_6$H$_5$OPO$_3$H$_2$	3.21 ± 0.41 mM
C$_2$H$_5$OPO$_3$H$_2$	3.73 ± 0.66 mM
CH$_3$OPO$_3$H$_2$	1.52 ± 0.73 mM
CH$_3$PO$_3$H$_2$	11.5 ± 1.5 mM
HOPO$_3$H$_2$	14.4 ± 5.8 mM
SO$_4^{2-}$	0.032 ± 0.006 mM

[a] 3-Py- = 3-pyridyl.

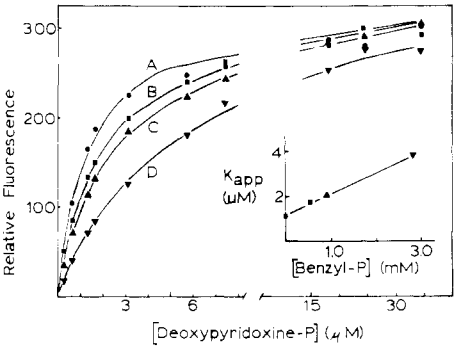

FIGURE 1: Fluorescence intensity (F_{obsd}) at 380 nm (excitation at 325 nm) measured for four parallel titrations of glutamate apodecarboxylase with deoxypyridoxine-P in the presence of benzyl phosphate. The apoenzyme concentration was 0.6×10^{-6} M, and the deoxypyridoxine-P concentration varied from 0.28 to 34.5×10^{-6} M. The benzyl phosphate (dicyclohexylammonium salt) concentration was zero (●), 0.57 mM (■), 0.95 mM (▲), and 2.84 mM (▼). The samples were incubated 8 h at 20 °C in 0.05 M pyridine hydrochloride buffer, pH 4.9, containing 0.135 M Cl^- and 10^{-4} M DTT. All four curves reach a common asymptote at high deoxypyridoxine-P concentrations. The lines represent the computer-generated best fit to the data (see Experimental Section). Inset: Slope of lines found in plots of $1/(1 - \alpha)$ vs. D_T/α at varying inhibitor concentrations vs. total inhibitor concentration.

Results

The binding of deoxypyridoxine-P to glutamate apodecarboxylase results in an increase in fluorescence intensity at 380 nm due to an enzyme-induced change in the apparent pK_a of the phenolic hydroxyl group of deoxypyridoxine-P (O'Leary & Malik, 1972). A titration curve for the binding of deoxypyridoxine-P to glutamate apodecarboxylase can be obtained from measurements at various concentrations of inhibitor. From this titration curve, the proportion of apoenzyme which exists as the apoenzyme–deoxypyridoxine-P complex can be determined and the dissociation constant calculated. A typical set of data is shown in Figure 1, curve A. The dissociation constant, K_{ED}, for the glutamate apodecarboxylase–deoxypyridoxine-P complex is 0.88 ± 0.18 μM at pH 4.9.

The binding curve obtained by using this method shows the proper dependence on deoxypyridoxine-P (Figure 1). In addition, the maximum fluorescence intensity observed at high deoxypyridoxine-P concentration is proportional to the total apoenzyme concentration from 0.85 to 4.25 μM (not shown). The calculated dissociation constant is independent of enzyme concentration and independent of excitation wavelength. The calculated dissociation constant is independent of Cl^- concentration up to 400 mM and independent of pyridine concentration up to 100 mM.

The proportion of the apoenzyme which exists as the apoenzyme–deoxypyridoxine-P complex will decrease in the presence of a competitive inhibitor. As a result, the apparent dissociation constant for the apoenzyme–deoxypyridoxine-P complex will vary with the concentration of the inhibitor. This effect is shown in curves B, C, and D of Figure 1 for the inhibitor benzyl phosphate. All four curves, A–D, show a single asymptote which is parallel to that for deoxypyridoxine-P in the absence of enzyme (not shown). This is expected, as the total apoenzyme concentration is identical in all four cases and at high deoxypyridoxine-P concentrations all apoenzyme will exist as the apoenzyme–deoxypyridoxine-P complex.

An apparent dissociation constant for the apoenzyme–deoxypyridoxine-P complex can be determined at each inhibitor concentration as shown above in the absence of inhibitor. The true dissociation constant of the apoenzyme–inhibitor complex, K_{EI}, can be determined graphically from a plot of the apparent dissociation constant for the apoenzyme–deoxypyridoxine-P complex vs. total inhibitor concentration. A K_{EI} value of 0.95 mM is determined from this graphical method (Figure 1, inset). The computer-weighted least-squares method analyzes the data from all four curves as a single system and yields K_{EI} = 1.06 ± 0.13 mM.

The validity of this competitive method is dependent upon the system being at equilibrium. In all cases, the incubation period represents a minimum of five half-lives for the binding of apoenzyme and the lowest concentration of deoxypyridoxine-P in the presence of the highest concentration of inhibitor used. The existence of a common asymptote for the binding curves at high deoxypyridoxine-P concentrations in the presence of various concentrations of the inhibitor agrees with the theoretical model and is also evidence that equilibrium has been reached.

The method described in detail for the inhibitor benzyl phosphate has been used for a number of inhibitors. Fluorescence data similar to those for benzyl phosphate were obtained for each inhibitor, and dissociation constants were determined by using the computer-fitting method (Table I).

Holoenzyme could be reconstituted by pyridoxal-P to greater than 90% of the initial activity following incubation with deoxypyridoxine-P and all inhibitors listed in Table I, indicating that the apoenzyme is stable and that all inhibitors form reversible complexes. O'Leary & Malik (1972) have shown spectroscopically that the binding of deoxypyridoxine-P to glutamate apodecarboxylase is reversible. The binding of deoxypyridoxine-P can also be reversed by the addition of the inhibitor 3-pyridinemethyl phosphate (data not shown). Thus, the system is reversible, and the requirement that the system be at equilibrium in these studies is fulfilled.

The pH dependences of the dissociation constants for several inhibitors are given in Figure 2. pK_{ED} values for phosphate esters vary linearly with pH from 4.6 to 5.5 with a slope of 1. The unprotonated form of the inhibitor binds more tightly. Inhibition constants for 3-pyridylmethyl sulfate and inorganic sulfate are pH independent.

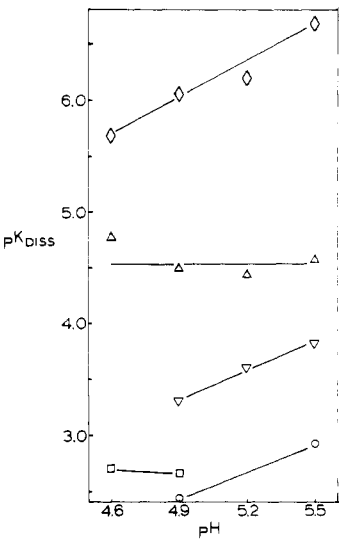

FIGURE 2: The pH dependence of the dissociation constants for ethyl phosphate (○), 3-pyridylmethyl sulfate (□), 3-pyridylmethyl phosphate (▽), inorganic sulfate (△), and deoxypyridoxine-P (◇) binding to glutamate apodecarboxylase in 0.05 M pyridine hydrochloride buffer containing 0.135 M Cl^- and 10^{-4} M DTT at 20 °C.

The dissociation constant for the glutamate apodecarboxylase–pyridoxine-P complex was determined as described previously for deoxypyridoxine-P. The fluoresence intensity at 380 nm following excitation at 290 nm increases upon formation of the apoenzyme–pyridoxine-P complex due to an energy-transfer process (Forster, 1959). The dissociation constant for pyridoxine-P is 0.11 ± 0.06 μM at pH 4.9 (data not shown). No binding is seen for either 4-pyridoxic acid 5′-phosphate or pyridoxamine 5′-phosphate with this direct method; i.e., no enzyme-induced fluorescence change is observed in the presence of 160 μM inhibitor.

Binding of 3-Hydroxypyridine-4-carboxaldehyde to Glutamate Apodecarboxylase. When apoenzyme is mixed with this analogue, the absorbance due to the free compound near 390 nm gradually decreases over a period of 4 h, and a new absorbance appears at 440 nm. This conversion has an isosbestic point at 425 nm. When the apoenzyme–inhibitor complex is incubated with pyridoxal-P under conditions where free apoenzyme recovers complete activity in a few minutes, apoenzyme which has been preincubated in the presence of 3 mM inhibitor recovers only 75% activity, i.e., pyridoxal-P binding is inhibited. The dissociation constant is estimated to be approximately 1 mM. Extensive kinetic studies of the concentration dependence of binding of this inhibitor were not done, and the inhibitor exhibits substantial fluorescence in the region used in this study.

Kinetics of inhibitor binding determined either by ultraviolet spectroscopy or by loss of recoverable activity are first order and yield the same rate. The rate constant and dissociation constant for binding of the inhibitor to the apoenzyme are not affected by the presence of inorganic phosphate. In addition, glutamic acid is not decarboxylated by the apoenzyme–3-hydroxypyridine-4-carboxaldehyde complex in the presence or absence of inorganic phosphate. These results are similar to those previously reported for 3-nitrosalicylaldehyde (O'Leary, 1969) which suggest that phosphate-less analogues of pyridoxal-P bind weakly to the apoenzyme.

Discussion

A number of phosphate esters and other anions bind to glutamate apodecarboxylase. Previous kinetic studies (O'Leary & Malik, 1972; Fonda, 1975) indicate that those examined

to date are competitive against pyridoxal-P and thus presumably bind to the site normally occupied by pyridoxal-P. In this study, a number of additional compounds have been examined and have been shown by equilibrium methods to bind to the coenzyme binding site. Binding in every case shows simple saturation behavior, with no evidence of cooperative interactions. In cases where comparison is possible, kinetic methods and equilibrium methods give similar values for the dissociation constants of the inhibitors. Precise agreement between present and previous data is not expected because of failure to remove traces of sulfate in our earlier enzyme preparations (O'Leary & Malik, 1972; Fonda, 1975) and because of the lack of a full kinetic analysis of the inhibition (Fonda, 1975).

It has been suggested that sulfate and other dianions bind to the chloride binding site on the enzyme (Fonda, 1975). This suggestion was based on kinetic studies of coenzyme binding conducted at a single pyridoxal-P concentration. However, coenzyme binding follows saturation kinetics (O'Leary & Malik, 1972), and kinetic data at a single coenzyme concentration have limited usefulness in determining the nature of the inhibition. Sulfate and other dianions studied here are competitive against pyridoxal-P and deoxypyridoxine-P. Although the concentration of chloride ion does effect the binding of pyridoxal-P in an important but poorly understood way (O'Leary & Malik, 1972), the binding constants determined in this study are independent of chloride ion concentration up to 400 mM. In addition, the chloride binding site exists in both apoenzyme and holoenzyme (Gerig & Kwock, 1973; O'Leary & Brummund, 1974; Shukuya & Schwert, 1960).

As described in the introduction, the binding of pyridoxal-P to glutamate apodecarboxylase arises from separate contributions of the Schiff base linkage, the pyridine nitrogen, the aromatic ring, the 3-hydroxyl group, and the phosphate side chain. Based on the fact that 2-norpyridoxal-P closely approximates the normal coenzyme (Mekhanik et al., 1971), we assume that the 2-methyl group makes a negligible contribution to the binding. In addition, we assume that contributions of the various functional groups are additive. A more quantitative estimate of these separate contributions is given below.

Deoxypyridoxine-P binds reversibly to glutamate apodecarboxylase with a dissociation constant of 0.88 μM at pH 4.9. This binding is at least 10^3 times weaker than binding of pyridoxal-P[2] (O'Leary & Malik, 1972), presumably because of the contribution of the Schiff base linkage to coenzyme binding. Strong binding even in the absence of the Schiff base is consistent with the fact that noncovalent intermediates occurring during enzymatic reactions involving pyridoxal-P do not dissociate from the enzyme. Pyridoxine-P does not form a Schiff base and binds about eightfold more tightly than deoxypyridoxine-P to glutamate apodecarboxylase. This enhanced binding might be due to hydrogen bonding between the 4′-hydroxyl group of pyridoxine-P and the ϵ-ammonium group of the lysine normally involved in Schiff's base formation (Anderson & Chang, 1965). Alternatively, it might be due to differences in the pK_a of the 3-hydroxyl group between the two compounds (Metzler et al., 1973). This hydroxyl group plays an important part in coenzyme binding (see below).

The failure to observe binding of 4-pyridoxic acid 5′-phosphate at 160 μM is not understood but might result from a change in the pK_a of the 3-hydroxyl group due to the presence of the carboxylate anion (Bridges et al., 1966). The very weak binding of pyridoxamine-P to glutamate apodecarboxylase (Mekhanik et al., 1972) and the total lack of binding observed in this study presumably result from unfavorable charge–charge interactions between the ammonium group of the coenzyme and the ϵ-ammonium group of the lysine involved in the Schiff base formation. A similar phenomenon is observed with aspartate aminotransferase (Churchich & Farrelly, 1969).

The binding of pyridoxal-P to glutamate apodecarboxylase (O'Leary & Malik, 1972) and to aspartate apoaminotransferase (O'Leary & Van Lanen, 1974; Fonda & Auerbach, 1976) occurs in two steps, a rapid equilibrium binding followed by a slow conformation change.[3] The Schiff base is formed in or after the second step. The binding of deoxypyridoxine-P shows similar kinetics and presumably follows a similar mechanism. The conformation change is associated with a large change in the pK_a of the phenolic hydroxyl group of deoxypyridoxine-P (O'Leary & Malik, 1972). This hydroxyl group appears to be the key to inducing the conformation change. 3-Pyridylmethyl phosphate binds rapidly, reversibly, and comparatively weakly to glutamate apodecarboxylase (present study and O'Leary & Malik, 1972) whereas deoxypyridoxine-P binds slowly and much more strongly to the enzyme. The hydroxyl group and the attendant conformation change appear to contribute a factor of about 10^3 to the strength of coenzyme binding.

Consistent with this key role of the phenolic hydroxyl group is the observation that otherwise fully functional pyridoxal-P analogues lacking the hydroxyl group bind very weakly to the enzyme. For example, 3-deoxypyridoxine 5′-phosphate has a reported dissociation constant of 0.1 mM for glutamate apodecarboxylase (Mekhanik et al., 1971) while that for 3-*O*-methylpyridoxal 5′-phosphate is 0.8 mM (Fonda, 1971). These compounds are apparently unable to undergo the conformation change and have dissociation constants in the range reported for simple phosphates.

Of the remaining functional groups, the phosphate group makes by far the most significant contribution to the binding of pyridoxal-P. A qualitative appreciation of the importance of this group can be gained from the observation that 3-hydroxypyridine-4-carboxaldehyde, which differs from the natural coenzyme principally in the absence of the phosphate group, differs in binding strength by more than 10^5, even though it does form a Schiff base with the enzyme. It is interesting that unlike some pyridoxal-P enzymes (Fasella, 1968), no synergistic binding effect can be observed with this compound and inorganic phosphate (P_i), and the analogue fails to produce a catalytically competent enzyme, even in the presence of P_i. The binding of 5-deoxypyridoxal is also very weak (Mekhanik et al., 1971; Fonda, 1971).

[2] It should be noted that an accurate value for the dissociation constant of pyridoxal-P with the apoenzyme is not available. At the coenzyme concentrations needed to measure the equilibrium constant, the rate of attainment of equilibrium is exceedingly slow (O'Leary & Malik, 1972). In this paper, we assume that the dissociation constant is near 10^{-8} M, but, in fact, it might be much smaller than this. The arguments presented here are not significantly affected by this uncertainty. Dissociation constants for pyridoxal-P with other enzymes are generally uncertain for the same reason.

[3] It should be noted that the two-step nature of coenzyme binding to aspartate aminotransferase was actually proposed earlier by Churchich & Farrelly (1968). However, the kinetic arguments given in that paper are in error because the kinetics of coenzyme binding (studied spectrophotometrically) and the kinetics of active enzyme formation (studied by observing aminotransferase activity) were not observed under comparable conditions. When proper account is taken of the difference between conditions, the discrepancy between the two rates disappears, thus providing no evidence either for or against the two-step nature of the binding.

An estimate of the contribution of the pyridine ring to coenzyme binding can be gained from the data on simple phosphates given in Table I. The contribution of the pyridine nitrogen to binding must be quite small, as benzyl phosphate and 3-pyridylmethyl phosphate differ in binding constants by only a factor of two. However, studies of *N*-methylpyridoxal 5′-phosphate (Fonda, 1971) suggest that this interaction may become significantly larger following the conformation change in the second step of coenzyme binding.

The data in Table I fail to provide unequivocal evidence for the occurrence of any sort of hydrophobic interaction between the coenzyme and the protein. Benzyl phosphate, methyl phosphate, and ethyl phosphate have quite similar dissociation constants. Any small effects of hydrophobic interactions are probably obscured by the larger effects of phosphate pK_a to be discussed shortly. This conclusion is at variance with previous studies of aspartate aminotransferase based on spectra and other properties of pyridoxal-P and analogues bound to the enzyme (Fasella & Turano, 1970). The difference probably lies not in intrinsic differences between the two enzymes, but rather in the fact that the simple phosphates used in this study are unable to induce the conformation change which occurs in the second step of coenzyme binding. Following this conformation change, there may well be hydrophobic interactions between coenzyme and protein.

Table I shows that a variety of pyridines with anionic substituents in the 3 position bind to glutamate apodecarboxylase. It appears that compounds in which the anionic center is tetrahedral (phosphate esters, phosphonates, and sulfate esters) bind to varying degrees whereas compounds in which the anionic center is planar (carboxylates) bind less well.

The pH dependence of inhibitor binding (Figure 2) indicates that the binding of simple phosphates to the enzyme increases with increasing pH, as if the primary species binding to the enzyme were the phosphate dianion. 3-Pyridylmethyl sulfate, necessarily a monoanion, fails to show such a pH dependence. Sulfate itself, a dianion under all conditions studied, shows no pH dependence and furthermore binds more tightly to the apoenzyme than any of the simple phosphates tested, including inorganic phosphate (which is principally present as a monoanion).

The pH dependence of the binding constant for 3-pyridylmethyl phosphate, together with the known pK_a of this compound (Murakami et al., 1966), enables us to calculate a dissociation constant of 0.025 mM for the dianionic form of this compound and 4 mM for the monoanionic form. Interestingly, the dissociation constant for the dianion is similar to that for the dianion sulfate and that for the monoanion differs by less than a factor of two from that for the corresponding monoanionic sulfate ester.

The pH dependence of the dissociation constant for deoxypyridoxine-P parallels that of the simple phosphates, even though the binding is tighter by more than three orders of magnitude. It is tempting to apply the same analysis to this binding, but such an analysis is problematical because of the possible pH dependence of the conformation change equilibrium.

Thus, the phosphate binding site of glutamate decarboxylase binds tetrahedral dianions preferentially. Chemical modification studies of several pyridoxal-P-dependent enzymes, including aspartate aminotransferase (Gilbert & O'Leary, 1975; Riordan & Scandurra, 1975), D-serine dehydratase (Kazarinoff & Snell, 1976), and glutamate decarboxylase (Cheung & Fonda, 1977), have shown that these apoenzymes are much more rapidly inactivated than the corresponding holoenzymes by arginine-modifying reagents and that the modification is inhibited by P_i. Thus, it is likely that the guanidino group of an arginine residue is one of the sites of binding of the phosphate group of pyridoxal-P. This might provide points of attachment for two of the three terminal oxygens of the phosphate group (Cotton et al., 1973). The conclusion from this study that the site preferentially binds dianions makes it likely that the third terminal oxygen of the phosphate is bound to a cationic group, either a lysine ammonium group or a positively charged histidine ring. Either could provide the kind of geometric specificity implied by the present data.

Conclusion

Coenzyme binding to pyridoxal-P-dependent enzymes results from the accumulation of a large number of relatively modest protein–coenzyme interactions. The largest contributor is the phosphate group, which is preferentially bound in the dianion form. The phenolic hydroxyl group of the coenzyme contributes to the binding and is principally responsible for inducing the conformation change which accompanies coenzyme binding. Schiff base formation also contributes significantly. Hydrophobic interactions between the aromatic ring and the protein and hydrogen-bonding interactions between the pyridine nitrogen and the protein make only minor contributions to the binding.

The analysis of coenzyme binding given here is similar to that given earlier by Fasella & Turano (1970) for aspartate aminotransferase but differs in several respects. In the first place, we are able to include the effects of the coenzyme-induced conformation change in the analysis. Further, we are able to observe an effect of the 3-hydroxyl group which was not apparent in the earlier analysis. We are also able to estimate more accurately the nature of the interaction between the phosphate group and the protein. X-ray diffraction studies provide the next test of this picture.

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