Binding of Pyridoxal 5-Phosphate to Cystathionase*

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SUMMARY

The binding of pyridoxal 5-phosphate to the apoprotein of the enzyme cystathionase from rat liver was investigated by two independent methods, absorption and fluorescence spectroscopy.

The increase in absorbance at 525 nm associated with Schiff's base formation was used to investigate the binding of pyridoxal-5-P at a protein concentration of \(1 \times 10^{-4}\) M.

A model based on two classes of independent binding sites fits the spectrophotometric data reasonably well. The affinity constant determined for the class of weak binding sites \((K_s = 1.4 \times 10^4\) M\(^{-1}\)) corresponds to extraneous binding of pyridoxal-5-P to lysine residues which are not implicated in catalysis. In order to avoid interference by extraneous binding of the cofactor to the enzyme, binding studies were conducted at a protein concentration of \(2 \times 10^{-5}\) M using the method of protein fluorescence quenching. This method gives a value of \(K_s = 7 \times 10^4\) M\(^{-1}\) for the class of tight binding sites. It is proposed that the catalytic subunits of the enzyme cystathionase display similar affinity constants for the cofactor pyridoxal-5-P.

EXPERIMENTAL PROCEDURE

Methods

Fluorescence and Absorption Spectroscopy—Fluorescence measurements were performed in a fluorimeter built in our laboratory (3). Calibration of the exciting source (Xenon lamp, 150 watts) and detector system (EMI 6256S photomultiplier) was carried out as described in a previous publication (3).

For the fluorescence measurements, the exciting monochromator (Bausch and Lomb, 300 nm, blazed at 300 nm) was operated with slit widths of 1 mm (band width, 3.3 nm). Absorption spectra were recorded in a Cary model 15 spectrophotometer.

Determination of Pyridoxal-5-P and Pyridoxyl-5-P—The pyridoxal-5-P content of cystathionase was determined by the phenylhydrazine method of Wada and Snell (4). For the determination of pyridoxyl-5-P content, samples of enzyme dissolved in 0.1 M phosphate buffer (pH 7.4) to a concentration of 1 mg per ml were allowed to react with sodium borohydride (final concentration 0.5 mg per ml) at 4° for 1 hour. The reduced enzyme was then dialyzed against 0.1 M phosphate buffer (pH 7.4), followed by filtration through a Sephadex G-25 column equilibrated with 0.1 M phosphate buffer (pH 7.4). Pyridoxyl-5-P content was determined by fluorescence spectroscopy (excitation 330 nm, emission 390 nm). Pyridoxyl-ε-lysine was used as standard because its fluorescence yield is identical with that of pyridoxyl-5-P residues bound to cystathionase. The fluorescence emitted at 390 nm, excited at the maximum of absorption of pyridoxyl-ε-lysine (330 nm), is strictly proportional to the concentration of pyridoxyl residues over the concentration range \(2 \times 10^{-4}\) to \(1 \times 10^{-5}\) M.

Resolution of Cystathionase—The enzyme cystathionase at a concentration of 5 mg per ml in 0.1 M phosphate buffer (pH 7.4) was allowed to react with 200-fold molar excess of \(\delta\)-cycloserine at 4° for 1 hour. The incubation mixture was then dialyzed against two changes of 0.1 M phosphate buffer (pH 7.4) for 14 hours at 4°. The absorption spectra of the dialyzed sample was compared to the absorption spectra of native cystathionase. A sample of cystathionase that has been completely resolved by addition of \(\delta\)-cycloserine loses the enzymatic activity and shows negligible absorbance in the spectral region 300 to 500 nm. Samples of cystathionase kept in the freezer for less than 1 week are completely resolved by the procedure described above. However, partial resolution of the enzyme is obtained when the enzyme can be used to gain information about the microenvironment of the catalytic site.
samples of cystathionase are stored in the freezer for longer periods of time.

Enzymatic Assays—Cystathionase activity was assayed by measuring α-ketobutyric acid production with either homoserine or cystathionine as substrates (5). Cystathionine cleavage was also followed by measuring cysteine formation according to the method described by Flavin (6). Protein concentration was determined by the method of Lowry et al. (7).

Materials

Cystathionase was prepared by the procedures of Matsuo and Greenberg (1). The pyridoxal-5-P content of cystathionase was 1 mole of pyridoxal-5-P per 45,000 g of enzyme. Pyridoxal-5-P, L-homoserine, DL-cystathionine, and D-cycloserine were obtained from Sigma. Sephadex G-25 and Sephadex G-200 were purchased from Pharmacia. The reagent, 5,5'-dithiobis(2-nitrobenzoic acid), Ellman's reagent, was purchased from Aldrich. Other materials, commercially obtained, were of the highest purity available.

RESULTS

It is well established that the band positions of both absorption and emission spectra of the co-factor pyridoxal-5-P covalently bound to the enzyme cystathionase are not affected when the pH of the medium is decreased from pH 7.5 to pH 5.5 (1, 2). However, the spectral properties of the co-factor are influenced by dissociation of the holoenzyme into apoenzyme and free pyridoxal-5-P. Thus, the reaction of cycloserine with the molecules of co-factor brings about a substantial decrease in the intensity of the absorption band covering the spectral range 360 to 500 nm.

Fig. 1 shows the absorption spectrum obtained after a sample of fresh enzyme was treated with 200-fold molar excess of D-cycloserine at pH 7.4 for 1 hour at 4°C. Under this set of experimental conditions complete resolution of the holoenzyme is attained; the samples of resolved enzyme prepared by reaction with cycloserine were used throughout the binding studies reported in this paper.

Prior to the binding experiments, it was desirable to estimate the macromolecular size of the resolved enzyme at protein concentrations approaching those used in the binding studies. The technique of gel filtration was applied to determine the macromolecular size of the resolved enzyme. The apoenzyme (0.2 ml) at a concentration of 1 mg per ml was applied to a Sephadex G-200 column and eluted with 0.1 M phosphate buffer (pH 7.4). The samples collected were reconstituted by addition of 0.2 mM pyridoxal-5-P, incubated at 25°C for 1 hour, and assayed for enzymatic activity. The elution profile of the resolved enzyme monitored by enzymatic assays and by measurements of protein fluorescence coincided with the elution profile of reduced cystathionase and native cystathionase (mol wt 160,000) (2) (Fig. 2). Furthermore, a comparison with the elution volumes of several standards of known molecular weight (horse alcohol dehydrogenase and glutamate dehydrogenase) indicated that the elution profile of the resolved enzyme would correspond to a molecular weight of approximately 160,000.

In view of these results, it was assumed that the binding of the co-factor pyridoxal-5-P does not induce any association or dissociation of the apoenzyme. Therefore, the number of pyridoxal-5-P binding sites was related to the catalytic unit of 160,000 molecular weight.

Binding of Pyridoxal-5-P—In an effort to determine the affinity of pyridoxal-5-P for the protein, titrations of the apoenzyme with pyridoxal-5-P were performed at two different protein concentrations, 1 × 10^{-5} and 2 × 10^{-6} M, using spectrophotometric and fluorometric techniques, respectively. For the spectrophotometric titrations, samples of apoenzyme at a concentration of 1 × 10^{-6} M were allowed to react in the dark with increasing concentrations of pyridoxal-5-P. After incubation for 1 hour at 25°C, the absorbance was recorded at 425 nm and aliquots were withdrawn from the reaction mixtures for activity assays. Fig. 3 includes the results of both spectrophotometric titrations and enzymatic assays. As may be seen from the spectrophotometric titrations, the major change in absorbance at 425 nm occurs after addition of approximately 4 moles of cofactor per mole of enzyme, but further addition of pyridoxal-5-P still promotes an enhancement of the absorption recorded at 425 nm.

In contrast to the titration data, the enzymatic assays performed with samples of reconstituted cystathionase indicate a
maximum recovery of enzymatic activity (95%) after the addition of approximately 4 moles of pyridoxal-5-P per mole of enzyme. The subsequent addition of pyridoxal-5-P has no effect on the recovery of cystathionase activity. Thus, the distinct change in slope of the enzymatic activity curve included in Fig. 3 is taken as indication that saturation of the catalytic sites has been accomplished.

In addition, it should be noted that when the concentration of pyridoxal-5-P is larger than 6 x 10^{-6} M, the reconstituted cystathionase shows more than 4 pyridoxyl-5-P residues per mole of enzyme after reduction with NaBH4 and dialysis against 0.1 M phosphate buffer (pH 7.4) (Table I). This increase in pyridoxyl-5-P content, which is also detected when the native enzyme is incubated with 8-fold excess of pyridoxal-5-P, is attributed to extraneous binding of pyridoxal-5-P to lysine residues which are not implicated in catalysis.

The results of the spectrophotometric measurements were analyzed by the method of Scatchard et al. (8):

\[
\frac{\bar{v}}{[L]} = K_A(n - \bar{v})
\]

where \([L]\) is the concentration of free ligand, \(K_A\) is the association constant, \(n\) is the number of binding sites, and \(\bar{v} = \frac{D}{D_0}\) is the average number of ligand molecules per mole of protein.

The fraction (\(\alpha_B\)) of ligand bound to the protein is related to the absorbances \(D_0, D_F,\) and \(D_B\) by means of Equation 2:

\[
\alpha_B = \frac{D - D_F}{D_B - D_F}
\]

where

\[
D = (1 - \alpha_B)[L] + \alpha_B[D_0] = (1 - \alpha_B)D_F + \alpha_BD_B
\]

is the absorbance recorded at 425 nm when both free and bound pyridoxal-5-P are in equilibrium, \([L]_0\) is the total ligand concentration, \(\epsilon_F\) and \(\epsilon_B\) the molar extinction coefficients of free and bound pyridoxal-5-P at 425 nm. \(\epsilon_B = 6,330\) cm\(^2\) mmole for the enzyme of 160,000 molecular weight containing 1 mole of pyridoxal-5-P per 45,000 g was determined by measuring the absorption spectrum of the native enzyme. \(D_B\) and \(D_F\) are the absorbances of bound and free pyridoxal-5-P, respectively.

The data obtained for pyridoxal-5-P binding are not consistent with binding to one class of sites. If this were the case, the plot of \(v/[L]\) versus \(v\) (Fig. 4) would be a straight line intersecting the abscissa at the number of binding sites per mole of enzyme. The simplest model found to fit the spectrophotometric data is based on two classes of independent binding sites, characterized by the association constants, \(K_1 = 3.1 \times 10^5\) M\(^{-1}\) and \(K_2 = 1.4 \times 10^4\) M\(^{-1}\), respectively.

Although one of the association constants \((K_1 = 3.1 \times 10^5\) M\(^{-1}\)) determined by spectrophotometric titrations may correspond to the binding of pyridoxal-5-P to the catalytic site of the enzyme.

**Table I**

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>Pyridoxal-5-P per 160,000 g</th>
<th>Fluorescence maximum</th>
<th>Polarization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apocystathionase + pyridoal-5-P (1:8)</td>
<td>5</td>
<td>390</td>
<td>0.21</td>
</tr>
<tr>
<td>Holocystathionase</td>
<td>3.3</td>
<td>390</td>
<td>0.36</td>
</tr>
<tr>
<td>Holocystathionase + pyridoxal-5-P (1:8)</td>
<td>6</td>
<td>390</td>
<td>0.21</td>
</tr>
</tbody>
</table>

**Fig. 3.** Increase of absorbance at 425 nm when a fixed concentration of apocystathionase is mixed with increasing concentrations of pyridoxal-5-P (○). Aliquots withdrawn from the incubation mixtures were tested for recovery of cystathionase activity. The results of the enzymatic assays are plotted as recovery of enzymatic activity (%) versus concentration of pyridoxal-5-P in the incubation mixture (○).

**Fig. 4.** Results of the spectrophotometric titrations of apocystathionase at pH 7.4 in 0.1 M phosphate buffer. Plot of \(v/[L]\) versus \(v\). Two association constants \(K_1 = 3.1 \times 10^5\) M\(^{-1}\) and \(K_2 = 1.4 \times 10^4\) M\(^{-1}\) were determined.
enzyme, it was thought desirable to perform the binding studies under experimental conditions so that the extraneous binding of pyridoxal-5-P would be minimized. To this end, the concentrations of both apoenzyme and ligand were decreased and the binding of pyridoxal-5-P was monitored by measurements of protein fluorescence.

The emission spectra recorded in Fig. 6 show that the binding of pyridoxal-5-P to apocystathionase induces quenching of protein fluorescence. This quenching effect associated with complex formation was used to determine the affinity of the cofactor for the catalytic site of the enzyme. To this end, the fluorescence intensities of samples containing a fixed concentration of protein and varying concentrations of pyridoxal-5-P were recorded after 1 hour of incubation at 25°C. Fig. 5 shows how the protein fluorescence expressed as a fraction of the fluorescence of the apoenzyme in the presence of ligand (F) to that in the absence of ligand (F0) is affected by the addition of pyridoxal-5-P. The plot (F/F0) deviates from linearity at (F/Fe) values smaller than 0.4 when a high degree of saturation has been achieved.

At concentrations of pyridoxal-5-P of 2 × 10^{-5} M, the fluorescence ratio (F/Fe) approaches the limiting value 0.27 observed when the fluorescence of the holoenzyme is compared to the apoenzyme (Fh/Fo).

The fraction of binding sites (θ) occupied by the ligand was determined by Equation 4:

$$\theta = \frac{F_0 - F}{F_0 - F_h}$$  

where F is the fluorescence observed when both free and complexed enzyme are in equilibrium, F0 is the fluorescence intensity of the apoenzyme, and Fh is the fluorescence intensity of the holoenzyme.

Assuming that the binding of each molecule of pyridoxal-5-P causes the same quenching effect on the fluorescence emitted by the protein, it is possible to determine the association constant for any of the ligand binding sites when θ/1 - θ is plotted versus [L] [9]. Application of the law of mass action for a class of intrinsically identical, independent, and indistinguishable binding sites, shows that the concentration of free ligand in solution [L] is related to the fraction of ligand binding sites (θ) by Equation 5:

$$\theta/1 - \theta = K_a[L]$$  

where K_a is the association constant. This model fits reasonably well the fluorometric data included in Fig. 5 up to high degrees of saturation (θ = 0.8). For a model that assumes one class of catalytic binding sites (n = 4), the results of the fluorometric titrations conducted at a protein concentration of 2 × 10^{-6} M yield the association constant K_a = 7 × 10^5 M^{-1}.

Fluorescence of the Enzyme—The fluorescence properties of native resolved and reduced cystathionase examined at pH 7.4 in 0.1 M phosphate buffer are given in Fig. 6.

The emission spectrum of native cystathionase shows a maximum centered at 335 nm when excited in the spectral region coinciding with the absorption of tryptophyl residues. Resolution of the holoenzyme into apoenzyme and free pyridoxal-5-P brings about a substantial increase in the protein fluorescence yield (Fig. 6).

The reduction of the bound pyridoxal-5-P by addition of NaBH₄ causes drastic changes in the fluorescence properties of cystathionase. As shown in Fig. 6, the emission spectrum of the reduced enzyme differs completely from the resolved enzyme when excited at 290 nm. While the apoenzyme exhibits a maximum of emission centered at 335 nm, the reduced enzyme shows two emission bands centered at around 335 and 392 nm, respectively.

The latter emission band coincides with the emission band due to pyridoxyl-5-P excited at 330 nm and arises through dipole-dipole energy transfer from the tryptophyl residues to the acceptor pyridoxyl-5-P as predicted by Förster's theory (5). The efficiency of this energy transfer process (R = 75%) wa
determined from measurements of the fluorescence yield of the protein in the presence \( Q_d \) and absence \( Q_o \) of pyridoxyl-5-P residues:

\[
E = 1 - \frac{Q_i}{Q_o}
\]

The transfer efficiency \( E \) is also related to the distance \( r \) between the donor-acceptor pairs by:

\[
E = \frac{r^4}{r^4 + R_0^4}
\]

where \( R_0 \) is the distance at which the transfer efficiency is 50\%.

Since the critical distance of transfer for a tryptophyl pyridoxyl-5-P pair randomly oriented (\( K^2 = 3/2 \)) has been previously determined (10) \( R_0 = 25 \) A, it is possible to estimate an average distance of transfer \( r \) (\( r = 21 \) A), assuming arrays of randomly oriented donors (tryptophan) transferring to a single acceptor (pyridoxyl-5-P) per 45,000 g of enzyme. Hence, it is not surprising to find that the presence of approximately 4 pyridoxyl-5-P residues per mole of enzyme (100,000 molecular weight) quenches the fluorescence emitted by all the tryptophyl residues of the macromolecule as shown by the emission spectra recorded in Fig. 6.

The pyridoxyl-5-P residues of reduced cystathionase which are characterized by a quantum yield of fluorescence of 0.14 (\( q = 0.14 \)) display polarization of fluorescence values similar to those of pyridoxyl-e-lysine immersed in a viscous solvent.

The results of the polarization measurements conducted at varying excitation wavelengths with the reduced enzyme in buffer of pH 7.4, together with the polarization measurements of pyridoxyl-e-lysine in glycerol-water (98:2, v/v) and buffer of pH 7.4, are shown in Fig. 7.

It is evident that the covalent binding of pyridoxyl 5P residues to the protein restrict the rotational mobility of the chromophore as evidenced by the remarkable increase in the polarization of fluorescence values (\( \rho = 0.36 \)) of the reduced enzyme when compared to pyridoxyl-e-lysine in buffer (\( \rho = 0.05 \)) (Fig. 7). It appears that the mobility of the pyridoxyl-5-P residues is restricted by strong interaction with amino acid residues at the catalytic sites.

Finally, it should be noted that the pyridoxal-5-P chromophores of the native enzyme also show large polarization of fluorescence values over the wavelength range coinciding with the absorption band of pyridoxal-5-P covalently bound to the enzyme as shown by the results included in Fig. 7. These polarization of fluorescence measurements with the native enzyme were conducted at a protein concentration of \( 5 \times 10^{-8} \) M, but the weak fluorescence yield of the pyridoxyl-5-P chromophores prevented any study designed to monitor structural transitions occurring in the enzyme.

**DISCUSSION**

The aim of the experiments described in this paper was to study the binding of pyridoxal-5-P to the apoprotein of the enzyme cystathionase by two independent methods, absorption and fluorescence spectroscopy.

When the binding of the cofactor to the apoprotein was monitored by measuring the increase in absorbance at 425 nm, it was observed that the plot \( \epsilon/L \) versus \( L \) does not fit the linearity expected for binding sites which are independent and indistinguishable.

The simplest model found to fit the spectrophotometric data is based on two classes of independent binding sites that have affinity constants that differ by a factor of 20. However, it should be emphasized that the affinity constant corresponding to the class of tight binding sites (\( K_1 = 3.1 \times 10^2 \) M\(^{-1}\)) was determined at a protein concentration (1 \times 10^{-5} M) which is larger than the dissociation constant (1/\( K_1 \)). Therefore, the affinity constant \( K_1 \) determined by the spectrophotometric method can be considered as an approximate value. Attempts to measure the affinity constant \( K_1 \) at protein concentrations lower than \( 10^{-5} \) M were unsuccessful since we were unable to measure the absorbance changes at 425 nm when increasing concentrations of pyridoxal-5-P were added to a concentration of apoprotein of \( 2 \times 10^{-4} \) M. However, the fluorometric measurements were conducted at protein concentrations approaching the value of the dissociation constant. For this reason, the affinity constant determined by fluorometric titrations can be regarded as a more reliable value (\( K_1 = 7 \times 10^4 \) M\(^{-1}\)) than the value obtained by spectrophotometric titrations.

The data obtained from the fluorometric titrations support the concept that the enzyme cystathionase contains one class of tight binding sites. Although this simple model for the class of tight binding sites (catalytic binding sites) fits reasonably well the fluorometric results up to high degrees of saturation, a more complex model of ligand binding for two classes of tight binding sites of association constants varying in the range \( 3.7 \times 10^5 \) M\(^{-1}\) was also examined. This model, however, did not fit the fluorometric data any better than the model described above.

In the light of these results, it seems reasonable to propose that the catalytic subunits of the enzyme cystathionase display similar affinities for the cofactor pyridoxal-5-P. If there is any difference between the affinity constants, this difference is small and cannot be detected with the method of protein fluorescence quenching. The class of weak binding sites detected by spectrophotometric titrations (\( K_2 = 1.4 \times 10^5 \) M\(^{-1}\)) at pyridoxal-5-P concentrations larger than \( 6 \times 10^{-5} \) M can be ascribed to extraneous binding of pyridoxal-5-P to lysine residues which are not implicated in catalysis. Consistent with this interpretation of the spectrophotometric titrations at large concentrations of cofactor is the finding that samples of reconstituted enzyme...
prepared by incubating the apoprotein with 8-fold molar excess of pyridoxal-5-P, showed the presence of more pyridoxyl-5-P residues than the native enzyme after NaBH₄ reduction.

Fluorescence spectroscopy was also used in this work to gain more information about the environment surrounding the chromophores, pyridoxal-5-P and pyridoxyl-5-P, when they are covalently bound to the protein. The polarization spectra of the reduced enzyme, which is the set of polarization of fluorescence values obtained upon excitation with light of varying wavelengths, were measured in 0.1 M phosphate buffer (pH 7.4) at a protein concentration of 10⁻⁷ M.

The results of the polarization measurements clearly indicate that the degree of polarization of fluorescence (p = 0.36) remains practically constant at exciting wavelengths coinciding with the longest wavelength absorption band of pyridoxyl-5-P residues.

This finding strongly suggests that the rotational mobility of pyridoxyl-5-P is largely suppressed as a result of strong interactions with the binding site of the protein. The presence of pyridoxyl-5-P in reduced cystathionase induces a dramatic quenching of the fluorescence emitted by the tryptophyl residues of the protein. Radiationless energy transfer from tryptophyl to pyridoxyl-5-P residues is responsible for the quenching effect. Judging from the value obtained for the efficiency of energy transfer (E = 75%), it appears that each molecule of pyridoxyl-5-P is favorably oriented for long range interaction with the emitting tryptophyl residues. Unfortunately, due to the existence of conflicting preliminary reports (11, 12) on the number of macromolecular subunits obtained after denaturation with sodium dodecyl sulfate, it is difficult to propose any model dealing with the actual location of the pyridoxyl-5-P residues in the protomeric structure. This problem is currently being investigated in our laboratory.

REFERENCES

Binding of Pyridoxal 5-Phosphate to Cystathionase
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