The Binding of Cobamides to Ethanolamine Deaminase*

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ABSTRACT: The binding to ethanolamine deaminase of two cobalamin derivatives, 5′-deoxyadenosylcobalamin and hydroxocobalamin, was investigated by kinetic studies and circular dichroism spectroscopy. A change in the circular dichroism spectrum was found to accompany the binding of either of these derivatives to the enzyme. By following the change in the spectrum as a function of the amount of derivative added, it was found that one molecule of enzyme would bind two molecules of cobalamin. This result was confirmed by the kinetic studies, which showed in addition that each molecule of cobalamin was associated with a separate active site. It was further shown that the two active sites do not interact, but appear to function entirely independently.

Ethanolamine deaminase from *Clostridium* sp. is a coenzyme B_{12} requiring enzyme which catalyzes the conversion of ethanolamine to acetaldehyde and ammonia. This enzyme, first described by Bradbeer in 1965 (Bradbeer, 1965a,b), was purified to homogeneity by Kaplan and Stadtman (1968a). These workers studied a number of the physical properties of the enzyme and carried out preliminary experiments on the interaction of the enzyme with various cobamides, including 5′-deoxyadenosylcobalamin, hydroxocobalamin, cyanocobalamin, and methylcobalamin (Kaplan and Stadtman, 1968b).

In this communication we report further investigations on the interaction of cobamides with ethanolamine deaminase. We have observed that upon binding to ethanolamine deaminase, both 5′-deoxyadenosylcobalamin, a cofactor for the reaction, and hydroxocobalamin, a powerful inhibitor, undergo alterations in their circular dichroism spectra. Such changes arising from the interaction of cobamides with enzymes have not hitherto been reported. Titrations based on these spectral alterations indicate that a molecule of enzyme binds two molecules of cobamide. Kinetic studies confirm this result and show, in addition, that each of the two cobamide binding sites represents a separate and independent active site.

Materials and Methods

Ethanolamine deaminase was purified and resolved of bound cobamides by the method of Kaplan and Stadtman (Kaplan and Stadtman, 1968a). 5′-Deoxyadenosylcobalamin was the generous gift of Professor R. H. Abeles. Hydroxocobalamin was prepared from 5′-deoxyadenosylcobalamin by photolysis. Ethanolamine-1,2-^{14}C (New England Nuclear) was purified by chromatography on Dowex 50-X8 (H^+) (Weissbach and Sprinson, 1953). After purification, the compound was 98% radiochemically pure as judged by its ability to serve as substrate in the ethanolamine deaminase reaction. The substrate analog, 1-amino-2-propanol (Aldrich, practical), was purified by fractional distillation (bp 70° (15 mm)). Other chemicals were reagent grade, and were used without further purification.

Circular dichroism spectra were obtained at 23° with the circular dichroism attachment of a Cary Model 60 spectropolarimeter. The slit widths were programmed to yield constant energy over the range of wavelengths examined. The instrument was calibrated using a standard optically active compound, camphorsulfonic acid, and an optically inactive compound, sodium dichromate. The latter assured that the instrument was free from absorbance artifacts at absorbances of 2.0 or less. A quartz cell with a path length of 2 cm was used for the spectra taken between 300 and 600 mμ, while a cell with a 1-cm path length was used for spectra at lower wavelengths. The volumes of the cells were 3.1 and 3.0 ml, respectively. The data are expressed in terms of molar ellipticity ($[\theta]$, (deg cm^2)/dmole), or observed ellipticity (θ_{obsd}, deg). For the kinetic studies, incubations were carried out in the dark at 23° under conditions given in the legends of Figures 4 and 5. The reactions were started with enzyme, and were terminated by the addition of 0.1 ml of 6% trichloroacetic acid. Each solution was then diluted to 1 ml with water and immediately passed over a column of Dowex 50-X8 (H^+) (packed resin volume, 0.2 ml). The column was then washed with 1 ml of water. The pooled effluents constituted the first fraction, which contained the product acetaldehyde. The unreacted ethanolamine was recovered from the column by elution with 2 ml of 2 N NH_4OH. Without further delay, aliquots of the fractions were added to Bray's solution and the radioactivity was determined in a Nuclear-Chicago liquid-scintillation counter. Under these con-

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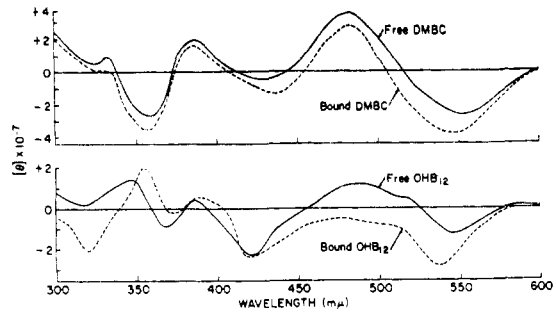

FIGURE 1: Circular dichroism of free and bound cobamides. Above: 5′-deoxyadenosylcobalamin. The samples contained 17 μM 5′-deoxyadenosylcobalamin in 10^{-2} M potassium phosphate buffer (pH 7.4). The volume of each sample was 3.1 ml. For the spectrum of enzyme-bound 5′-deoxyadenosylcobalamin, the concentration of ethanolamine deaminase was 8.2 μM. Below: hydroxocobalamin. The concentration of hydroxocobalamin was 14 μM. The enzyme and buffer concentrations and the sample volumes were as described for 5′-deoxyadenosylcobalamin. Spectra were obtained as described in the text. Operations involving 5′-deoxyadenosylcobalamin were conducted in dim light.

ditions, losses of acetaldehyde through evaporation were negligible.

Results

Circular Dichroism Spectra. Like all cobamides so far studied, 5′-deoxyadenosylcobalamin and hydroxocobalamin have distinctive circular dichroism spectra between 300 and 600 mμ (Firth *et al.*, 1967). The binding of these two compounds to ethanolamine deaminase leads to significant alterations in these spectra (Figure 1).

Free 5′-deoxyadenosylcobalamin exhibits negative extrema at 555, 430, and 365 mμ and positive extrema at about 482, 385, and 332 mμ. Upon addition to ethanolamine deaminase, the negative extrema at 555 and 430 mμ shift to 545 and 438 mμ, respectively. There is also an increase in the intensity of all the negative extrema while that of the positive extrema decrease. Addition of 1-amino-2-propanol, an inhibitor competitive with ethanolamine ($K_i = 2 \times 10^{-6}$ M), to a final concentration of 10^{-3} M produces no further change in the circular dichroism spectrum of the enzyme–5′-deoxyadenosylcobalamin complex.

Binding of hydroxocobalamin to ethanolamine deaminase induces similarly distinctive alterations in circular dichroism spectra. Free hydroxocobalamin has negative circular dichroism extrema at about 545, 422, and 368 mμ and positive extrema at about 480, 385, and 346 mμ. Upon binding to ethanolamine deaminase, the negative extrema at 545 and 368 mμ shift to 537 and 373 mμ, respectively, and the positive extremum at 346 mμ shifts to 355 mμ. Furthermore, the broad positive extremum at about 480 mμ is abolished and a new negative extremum appears at 320 mμ.

The free enzyme shows no circular dichroism between 300 and 600 mμ.

Stoichiometry of Binding of 5′-Deoxyadenosylcobalamin and Hydroxocobalamin. The intensities of the bands

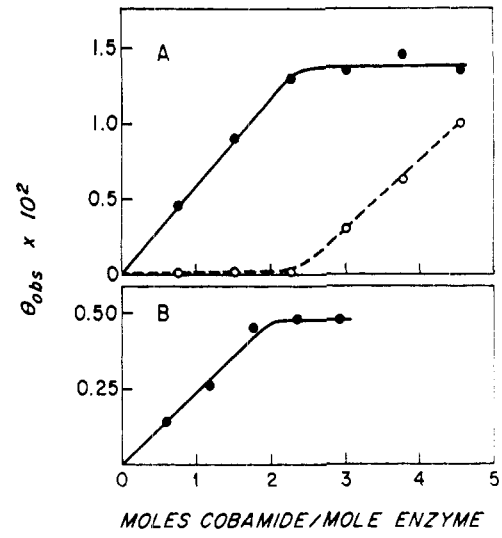

FIGURE 2: Titrations of ethanolamine deaminase with 5′-deoxyadenosylcobalamin and hydroxocobalamin. (A) Titration with 5′-deoxyadenosylcobalamin. The initial sample consisted of 3.1 ml of 6.2 μM ethanolamine deaminase in 10^{-2} M potassium phosphate buffer (pH 7.4). The titration was conducted as described in the text, using aliquots of 1.8 mM 5′-deoxyadenosylcobalamin. Additions of 5′-deoxyadenosylcobalamin were made in dim light. (B) Titration with hydroxocobalamin. The initial sample consisted of 1.0 ml of 9.2 μM ethanolamine deaminase in 10^{-2} M potassium phosphate buffer (pH 7.4). The titration was performed with aliquots of 1.8 mM hydroxocobalamin. A cell with a 1-cm path length was used.

in the circular dichroism spectra of the enzyme–cobamide complexes were found to be directly proportional to the amount of the cobamide bound. This property permitted the measurement of the stoichiometry of interaction in a manner analogous to spectral and optical rotatory dispersion titrations (Li *et al.*, 1962). To perform the titration, small aliquots of concentrated solutions of the cobamides were added to the enzyme, and the changes in the circular dichroism spectra were measured at several wavelengths (Figure 2).

When enzyme was titrated with 5′-deoxyadenosylcobalamin the changes in circular dichroism intensity were followed at 515 mμ, where the signal is due solely to the enzyme–5′-deoxyadenosylcobalamin complex, and at 505 mμ, where only the free coenzyme gives a signal. At 515 mμ, a plateau of maximum intensity is reached when 2 moles of 5′-deoxyadenosylcobalamin is added per mole of enzyme, indicating saturation of binding, whereas at 505 mμ, an increase in intensity is observed only after 2 moles of 5′-deoxyadenosylcobalamin has been added per mole of enzyme. Thus, ethanolamine deaminase appears to bind 2 moles of 5′-deoxyadenosylcobalamin/mole of enzyme (Figure 2a).

The titration of enzyme with hydroxocobalamin was performed at 360 mμ, where only the enzyme–hydroxocobalamin complex exhibits a circular dichroism signal. A plateau is again reached at 2 moles of hydroxocobalamin/mole of enzyme, indicating that as with 5′-deoxyadenosylcobalamin, each mole of ethanolamine deaminase binds 2 moles of hydroxocobalamin (Figure 2b).

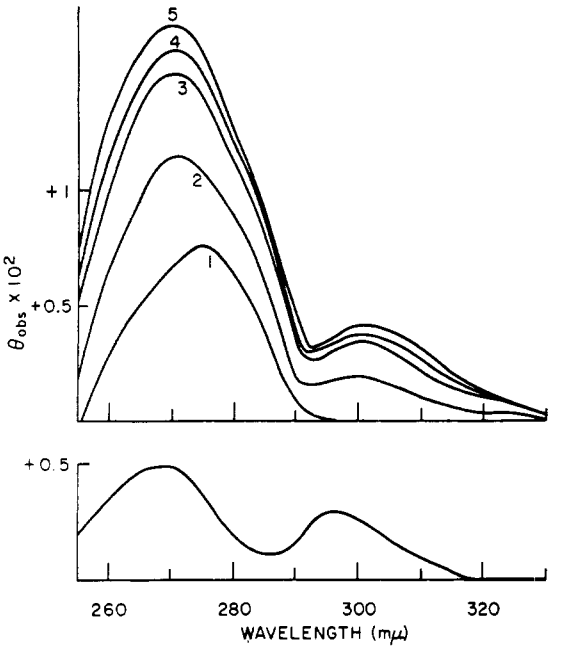

FIGURE 3: Circular dichroism of ethanolamine deaminase, 5′-deoxyadenosylcobalamin, and the enzyme–5′-deoxyadenosylcobalamin complex at low wavelengths. The sample consisted of 3.5 μM ethanolamine deaminase in 10^{-2} M potassium phosphate buffer (pH 7.4). Aliquots of 1.8 mM 5′-deoxyadenosylcobalamin were added to the sample as described for the titrations. The final concentrations of 5′-deoxyadenosylcobalamin in the sample cell were as follows: curve 1, 0; curve 2, 3.5 μM; curve 3, 7 μM; curve 4, 10.5 μM; curve 5, 14 μM. Below is shown the spectrum of 17.5 μM 5′-deoxyadenosylcobalamin in 10^{-2} M potassium phosphate buffer (pH 7.4).

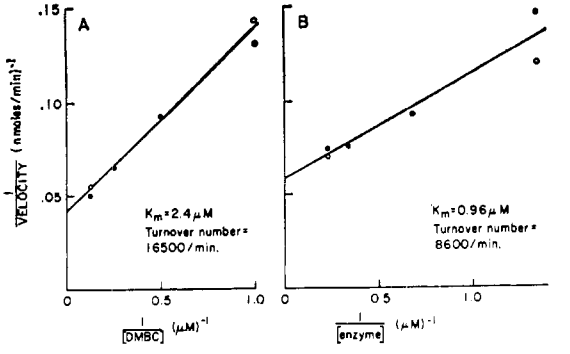

FIGURE 4: Lineweaver–Burk plots of reaction velocity *vs.* the concentration of one component of the enzyme–5′-deoxyadenosylcobalamin couple at low fixed concentrations of the other component. (A) Velocity *vs.* 5′-deoxyadenosylcobalamin concentration. The reaction mixtures contained 1.5×10^{-3} nmole of ethanolamine deaminase, 0.1 μmole (11,000 dpm) of [^{14}C]ethanolamine, 5 μmoles of potassium phosphate buffer (pH 7.4), and 5′-deoxyadenosylcobalamin as indicated in a total volume of 0.1 ml. (B) Velocity *vs.* ethanolamine deaminase concentration. The reaction mixtures contained 2×10^{-3} nmole of 5′-deoxyadenosylcobalamin, 0.1 μmole (11,000 dpm) of [^{14}C]ethanolamine, 5 μmoles of potassium phosphate buffer (pH 7.4), and ethanolamine deaminase as indicated, in a total volume of 0.1 ml. The incubations were conducted as described in the text. (O--O) Reactions which were stopped at 1 min; (●--●) reactions which were stopped at 2 min.

Circular Dichroism Spectra of 5′-Deoxyadenosylcobalamin and the Enzyme–5′-deoxyadenosylcobalamin Complex between 260 and 320 mμ. Since 5′-deoxyadenosylcobalamin differs from hydroxocobalamin in containing an adenyl function which absorbs strongly at 260 mμ, the circular dichroism spectra of the free and the enzyme-bound 5′-deoxyadenosylcobalamin were also examined between 260 and 320 mμ. Free 5′-deoxyadenosylcobalamin exhibits two positive circular dichroism extrema in this region, one at 300 mμ and a more prominent one at 270 mμ. Ethanolamine deaminase shows a single positive circular dichroism band at 275 mμ, presumably due to the aromatic side-chain chromophores of the protein. The enzyme–5′-deoxyadenosylcobalamin complex exhibits strong positive circular dichroism bands at 270 and 300 mμ whose intensities are considerably greater than the algebraic sums of the band intensities of the free enzyme and the free coenzyme, suggesting that the circular dichroism spectrum of the bound coenzyme also differs from that of the free coenzyme in this region. When ethanolamine deaminase is titrated with increments of 5′-deoxyadenosylcobalamin it becomes apparent that the intensities of the circular dichroism extrema at 270 and 300 mμ for bound 5′-deoxyadenosylcobalamin are two to three times greater than those of the free coenzyme (Figure 3). After saturation of binding at 2 moles of 5′-deoxyadenosylcobalamin/mole of enzyme, the increase in intensity upon further addition of 5′-deoxyadenosylcobalamin becomes comparable with that of equivalent concentrations of the free coenzyme.

Reaction Rate as a Function of Concentration of Catalyst. In the ordinary substrate saturation experiment, the velocity of an enzyme-catalyzed reaction is measured as a function of substrate (or cofactor) concentration under conditions in which the amount of enzyme in the reaction mixture is small compared with the amounts of the other constituents. The maximum velocity obtained from such an experiment is expressed as the rate per unit of enzyme, and the Michaelis constant is given in terms of substrate (or cofactor) concentration. Because large quantities of ethanolamine deaminase were available, it was possible to conduct this experiment in reverse, that is, with a large excess of enzyme and limiting amounts of cofactor, as well as in the usual manner. In this way maximum velocities and Michaelis constants for both of the catalytic species were obtained. Since the molecular weight of the enzyme is known (Kaplan and Stadtman, 1968b), these parameters could be expressed in molar terms for the enzyme as well as for the cofactor.

Figure 4 shows the reciprocal plots of the data from such experiments. When either enzyme or cofactor concentrations are varied the data fall close to a straight line, with no apparent deviation from linearity at low concentrations of the varied component. The turnover number for the enzyme is approximately twice the turnover number for the 5′-deoxyadenosylcobalamin, indicating that for full activity two molecules of cofactor are required per molecule of enzyme. (In considering these Lineweaver–Burk plots, it must be borne in mind that the maximum velocity (here expressed as the turnover

number) is given with respect to the catalyst whose concentration is fixed. Thus, in the reciprocal plot of rate *vs.* 5′-deoxyadenosylcobalamin concentration, the turnover refers to moles of substrate per minute per mole of *enzyme*, and *vice versa*.) The Michaelis constants obtained for the enzyme and the coenzyme are also consistent with this interpretation: the K_m for the enzyme is about half the K_m for 5′-deoxyadenosylcobalamin, the result expected if each molecule of enzyme possessed two coenzyme binding sites, each of which had the same affinity for 5′-deoxyadenosylcobalamin.

Relationship between Reaction Rate and Cofactor Concentration in the Presence of Large Amounts of Enzyme. To examine whether or not *both* molecules of cofactor must be bound to the enzyme before it becomes catalytically active, the reaction rate was determined as a function of coenzyme concentration under conditions in which the enzyme was in great excess. If both molecules of cofactor were required for activity, it would be anticipated that the reaction rate would vary as the square of the concentration of 5′-deoxyadenosylcobalamin. However, as shown in Figure 5, the reaction rate is directly proportional to the concentration of 5′-deoxyadenosylcobalamin present in the incubation mixture. This suggests that only one coenzyme molecule is required per active site. It implies further that such an active site–coenzyme complex is catalytically active regardless of whether or not a second molecule of cofactor is bound to the enzyme.

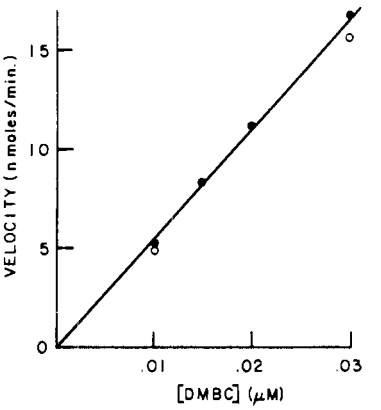

FIGURE 5: Reaction velocity *vs.* 5′-deoxyadenosylcobalamin concentration at high concentrations of ethanolamine deaminase. The reaction mixtures contained 0.15 nmole of ethanolamine deaminase, 0.1 μmole (11,000 dpm) of [^{14}C]-ethanolamine, 5 μmoles of potassium phosphate buffer (pH 7.4), and 5′-deoxyadenosylcobalamin as indicated, in a total volume of 0.1 ml. The incubations were conducted as described in the text. (○--○) Reactions which were stopped at 1 min; (●--●) reactions which were stopped at 2 min.

Discussion

The interactions of enzymes with chromophoric molecules of low molecular weight such as nucleotides or coenzymes frequently lead to changes in the spectroscopic properties of the interacting species (Ulmer and Vallee, 1965). These changes may be detected by measurements of absorption spectra, optical rotatory dispersion, or circular dichroism. For example, the binding of NADH to alcohol dehydrogenase (Ulmer *et al.*, 1961), nucleotides to creatine kinase (Kagi and Li, 1965), and pyridoxal phosphate to aspartic aminotransferase (Fasella and Hammes, 1964) and aspartate decarboxylase (Wilson and Meister, 1966), have all been shown to be accompanied by changes in the optical rotatory dispersion or circular dichroism spectra.

Recently, Firth *et al.* published an extensive study of the absorption and circular dichroism spectra of cobamides (Firth *et al.*, 1967). In this study they showed that these spectra varied more or less continuously from "normal" (*e.g.*, dicyanocobalamin) to "extreme anomalous" (*e.g.*, B_{12r}), depending upon the nature of the ligands in the fifth and sixth positions of the cobalt coordination sphere. They suggested that correlation of these results with the spectra of enzyme–cobalamin complexes could provide information concerning the nature of the interactions between the enzyme and the cobamide.

In the present study, distinct differences have been found between the circular dichroism spectra of free and enzyme-bound cobamides. These differences clearly reflect alterations in the environment of the cobamides that take place upon binding. However, the spectra[1] "anomalousness" is essentially the same whether the compounds are bound or free, suggesting that the binding of a cobamide to ethanolamine deaminase does not lead to major changes in the coordination sphere of the cobalt atom. (These results do not exclude minor modifications, such as the substitution of the dimethylbenzimidazole group by an imidazole residue of histidine.)[1]

The difference between the spectra of bound and free 5′-deoxyadenosylcobalamin consists primarily of a negative displacement of the entire spectrum of the bound cobamide, with relatively little change in the amplitudes and spectral positions of the individual peaks. In contrast, the binding of hydroxocobalamin gives rise to a rather pronounced change in the shape of the circular dichroism spectrum, which comes to resemble closely the spectrum of aquocobalamin. This finding suggests that upon binding to the enzyme, the hydroxyl group in the cobalt coordination sphere is protonated. This protonation probably does not represent an increase in the basicity of the hydroxyl group[2] transmitted through the cobalt, since a significant change in the "anomalousness" of the spectrum would be expected if this were the case (Firth *et al.* 1967). It may be due to an effective increase in the pK of the hydroxyl group arising from negatively charged residues in the vicinity of the bound cobamide, or to the transfer of a proton from a weakly acidic group lying nearby.

The augmentation of the peak at 270 mμ in the spectrum of the 5′-deoxyadenosylcobalamin-enzyme complex may represent an interaction of the adenyl moiety of the coenzyme with the protein, particularly since this augmentation was not observed in the spectrum of the

[1] H. Hogenkamp, personal communication.

[2] The pK_a of aquocobalamin is 7.1 (Adler *et al.*, 1966).

hydroxocobalamin–enzyme complex.[3] However, one cannot exclude the possibility that alterations in the environment of the chromophoric groups of the protein upon binding of 5′-deoxyadenosylcobalamin are responsible in whole or in part for these changes.

Titration of the enzyme with 5′-deoxyadenosylcobalamin and hydroxocobalamin shows that a molecule of enzyme will bind two molecules of either of the two cobamides. With 5′-deoxyadenosylcobalamin this result was confirmed by the kinetic studies. Furthermore, the kinetic studies indicate that each of the two enzyme-bound cofactor molecules is associated with an independent active site, and that the K_m for cofactor is the same at each of these sites.

The conclusion that the enzyme possesses independent active sites is based primarily on the observation that when the enzyme is in great excess, the reaction rate is a linear function of 5′-deoxyadenosylcobalamin concentration. This result, however, would also be consistent with a mechanism in which the two active sites are interdependent, or in which the enzyme possesses a single active site requiring two molecules of coenzyme per molecule of enzyme for activity, provided there were sufficient cooperativity between the two coenzyme binding sites. If the cooperativity were great enough, the only species in solution would be the free enzyme and the fully saturated enzyme, and the reaction rate would be proportional to the 5′-deoxyadenosylcobalamin concentration despite the fact that the enzyme required two molecules of cofactor for activity.

If one assumes that the cooperative mechanism is valid in the present case, a constraint is placed on the relationship between the apparent binding constants for the two sites, because of the observation that with enzyme in excess, the reaction rate is directly proportional to the concentration of 5′-deoxyadenosylcobalamin (see Appendix). It is possible to obtain independent estimates of these binding constants from the data in Figure 4. These estimates show that the observed binding constants do not obey the constraint imposed by the cooperative mechanism, thus excluding this mechanism and providing further evidence that ethanolamine deaminase possesses two independent active sites, each of which is capable of binding one molecule of 5′-deoxyadenosylcobalamin.

In their studies on the binding of cobamides to ethanolamine deaminase, Kaplan and Stadtman found that while 1 mole of enzyme could be titrated to inactivity with 2 moles of hydroxocobalamin, the enzyme was capable of binding much larger quantities of hydroxocobalamin (Kaplan and Stadtman, 1968b). Furthermore, these large quantities of cobamide remained bound to the enzyme even after extensive dialysis. Nevertheless, the fact that there appear to be two active sites, each of which binds one molecule of cobamide, together with the observation that the changes in the circular dichroism spectra which accompany the binding of cobamide to ethanolamine deaminase are complete upon the addition of 2 moles of cobamide/mole of enzyme, suggests that the excess of hydroxocobalamin which is taken up by the enzyme is bound nonspecifically.

Appendix

The Constraints Imposed upon the Michaelis Constants by a Cooperative Mechanism in Which Two Molecules of Cofactor per Molecule of Enzyme Are Required for Catalytic Activity. This situation can be expressed in terms of the following model

$$E + C \underset{}{\overset{K_1}{\rightleftharpoons}} E_1$$

$$E_1 + C \underset{}{\overset{K_2}{\rightleftharpoons}} E_2$$

where E_2 is the catalytically active species.

The concentrations of the various species are given by the equations

$$K_1EC = E_1$$

$$K_1K_2EC^2 = E_2$$

$$C_t = C + E_1 + 2E_2$$

$$E_t = E + E_1 + E_2$$

where E = free enzyme, E_t = total enzyme, C = free cofactor, C_t = total cofactor, and E_1 and E_2 are the complexes of the enzyme with one and two molecules of cofactor, respectively.

Since the only catalytically active species is E_2, the reaction rate is expressed by the equation $V = kE_2 = kK_1K_2EC^2$.

1. THE CONDITIONS UNDER WHICH THE REACTION RATE WOULD BE DIRECTLY PROPORTIONAL TO THE CONCENTRATION OF COFACTOR, WHEN THE ENZYME IS IN GREAT EXCESS (SEE FIGURE 5). With the enzyme in great excess, $E \sim E_t$, where E_t is the total enzyme concentration. Under these conditions

$$C_t = C(1 + K_1E_t) + 2K_1K_2E_tC^2 \qquad (1)$$

Solving for C in terms of C_t

$$C = \frac{b\left(\sqrt{1 + \frac{8aC_t}{b^2}} - 1\right)}{4a} \qquad (2)$$

where $a = K_1K_2E_t$, $b = 1 + K_1E_t$.

The relationship of interest is the dependence of C^2 upon C_t, since by postulate the reaction velocity is proportional to C^2. Rearranging and squaring eq 2

$$\left(\frac{4aC}{b}\right)^2 = \frac{8aC_t}{b^2} - 2\sqrt{1 + \frac{8aC_t}{b^2}} + 2 \qquad (3)$$

A graph of this function is shown in Figure 6. It is

[3] Unpublished results.

clear that this curve approximates a straight line only for values of $8aC_t/b^2$ exceeding 2. Even then, the line would not appear to pass through the origin unless much larger values of $8aC_t/b^2$ were used, with a corresponding reduction in the scale of the x axis.

For values of $8aC_t/b^2$ large enough so that the graph of eq 3 approximates a straight line passing through the origin

$$\sqrt{1 + \frac{8aC_t}{b^2}} - 1 \cong \sqrt{\frac{8aC_t}{b^2}}$$

Substituting in eq 2

$$C \cong \sqrt{\frac{C_t}{2a}}$$

$$V = kaC^2 = \frac{kC_t}{2} \tag{4}$$

Thus, the plot of the reaction rate *vs.* cofactor concentration under these conditions would be a straight line.

2. THE UPPER LIMIT OF K_1. This can be evaluated from Figure 4b, the reciprocal plot of velocity *vs.* enzyme concentration when the enzyme is in great excess. Here again, since the free enzyme $\sim$ the total enzyme.

$$C_t = C(1 + K_1E_t) + 2K_1K_2E_tC^2 = \left(\frac{b}{C} + 2a\right)C^2 \tag{5}$$

By postulate

$$V = kaC^2 = \frac{kaC_t}{b/C + 2a} = \frac{kaC_t}{b\sqrt{\frac{2a}{C_t}} + 2a}$$

since

$$C \cong \sqrt{\frac{C_t}{2a}}$$

under conditions in which the reaction rate is a linear function of cofactor concentration. Substituting and rearranging

$$\frac{1}{V} = \frac{2}{kC_b\sqrt{2K_1K_2C_t}}\frac{1}{\sqrt{E_t}} + \frac{2K_1}{kC_t\sqrt{2K_1K_2C_t}}\sqrt{E_t} + \frac{2}{kC_t} \tag{6}$$

Inspection of this equation shows that the velocity falls to zero at both extremes of enzyme concentration. The fall in velocity as the enzyme concentration rises is due to competition between the unsaturated enzyme and the half-saturated enzyme for the limited amount of available cofactor. The plot of $1/v$ *vs.* $1/E_t$, therefore, passes through a minimum.

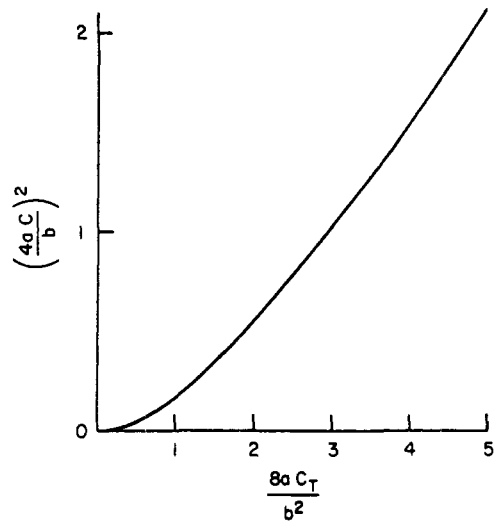

FIGURE 6: Graph of eq 3.

$$\frac{d\left(\frac{1}{v}\right)}{d\left(\frac{1}{E_t}\right)} = E_t^{3/2}\left(\frac{1}{kC_t\sqrt{2K_1K_2C_t}}\frac{1}{E_t} - \frac{K_1}{kC_t\sqrt{2K_1K_2C_t}}\right) \tag{7}$$

$$= 0 \text{ at the minimum}$$

At this point

$$\frac{1}{E_t} = K_1 \tag{8}$$

Since the slope of the curve shown in Figure 4b is always positive, the minimum must lie to the left of the point closest to the ordinate. Therefore, by eq 8, $K_1 < 10^6$. Substitution of this value of K_1 along with the appropriate concentrations into the expression $8aC_t/b^2 > 2$ gives the result $K_2 > 5 \times 10^7$.

3. THE VALUE OF K_2. For the mechanism under consideration, the plot of $1/v$ *vs.* $1/C_t$ when the enzyme is limiting and the cofactor is present in great excess (see Figure 4A) is given by the equation (Reiner, 1959)

$$\frac{1}{V} = \frac{1}{V_m}\left(\frac{1}{K_1K_2C_t^2} + \frac{1}{K_2C_t} + 1\right)$$

When the data fall on a straight line, the quadratic term can be ignored. Under those conditions, then,

$$\frac{1}{V} = \frac{1}{V_m}\left(\frac{1}{K_2C_t} + 1\right)$$

and $K_m = 1/K_2$. Thus, from Figure 4a, $K_2 = 5 \times 10^5$. This observation conflicts with the constraint that $K_2 > 5 \times 10^7$ and indicates, therefore, that the mechanism under consideration is not consistent with the data.

Acknowledgments

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References

Adler, N., Medwick, T. and Pozananski, T. J. (1966), *J. Am. Chem. Soc. 88*, 5081.
Bradbeer, C. (1965a), *J. Biol. Chem. 240*, 4669.
Bradbeer, C. (1965b), *J. Biol. Chem. 240*, 4675.
Bray, G. A. (1960), *Anal. Biochem. 1*, 279.
Fasella, P., and Hammes, G. G. (1964), *Biochemistry 3*, 530.
Firth, R. A., Hill, H. A. O., Pratt, J. M., Williams, R. J. P. and Jackson, W. R. (1967), *Biochemistry 6*, 2178.
Kagi, J. H. R., and Li, T. K. (1965), *Fed. Proc. 24*, 385.
Kaplan, B. H., and Stadtman, E. R. (1968a), *J. Biol. Chem. 243*, 1787.
Kaplan, B. H., and Stadtman, E. R. (1968b), *J. Biol. Chem. 243*, 1794.
Li, T. K., Ulmer, D. D., and Vallee, B. L. (1962), *Biochemistry 1*, 114.
Reiner, J. M. (1959), Behavior of Enzyme Systems, Minneapolis, Minn., Burgess, p 66.
Ulmer, D. D., Li, T. K., and Vallee, B. L. (1961), *Proc. Natl. Acad. Sci. U. S. 47*, 1155.
Ulmer, D. D., and Vallee, B. L. (1965), *Advan. Enzymol. 27*, 37.
Weissbach, A., and Sprinson, D. B. (1953), *J. Biol. Chem. 203*, 1013.
Wilson, E. M., and Meister, A. (1966), *Biochemistry 5*, 1166.

Hydrophobic Nature of the Active Site of Firefly Luciferase*

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ABSTRACT: Anilinonaphthalenesulfonates and toluidinonaphthalenesulfonates bind to firefly luciferase with an enhancement of fluorescence. Approximately 2 moles of dye is bound per mole of enzyme. The affinity of 2,6-toluidinonaphthalenesulfonate for the enzyme is much greater than 1,5-anilinonaphthalenesulfonate or the corresponding isomer 2,6-anilinonaphthalenesulfonate. Both 2,6-toluidinonaphthalenesulfonate and 1,5-anilinonaphthalenesulfonate are competitive inhibitors of luciferin. The addition of 2 moles of dehydroluciferyl adenylate/mole of enzyme completely removes the bound dye from the enzyme. Inhibition of the enzymatic activity by reaction of the two essential sulfhydry groups with *N*-ethylmaleimide decreases the affinity of the enzyme for 2,6-toluidinonaphthalenesulfonate but does not alter the number of dye molecules bound. The binding of 2,6-toluidinonaphthalenesulfonate is independent of pH between 6 and 9. The increase in fluorescent intensity and the shift in the emission maximum of the bound dye are indicative of hydrophobic binding sites on the enzyme.

The data support the conclusion that the dyes bind at the normal luciferin binding sites, thus inhibiting catalytic activity.

The use of dyes as probes for hydrophobic sites on proteins is now well documented (Weber and Young, 1964; Stryer, 1965; McClure and Edelman, 1966, 1967; Brand *et al.*, 1967). The interaction between dye and protein may be followed by the increase of fluorescence observed when the dye is bound in a hydrophobic environment. In the case of firefly luciferase the dyes used are competitive inhibitors of the enzyme indicating a specific interaction where either the dye or the substrate may be bound at the same hydrophobic site. These studies with luciferase as well as similar findings with other enzymes (Brand *et al.*, 1967; Stryer, 1965; Hymes *et al.*, 1965) suggest the role of such hydrophobic sites may be to dissolve the substrate in a specific region of the protein thus making it available in high concentration at the active site where catalysis takes place.

Methods

Five-times-crystallized firefly luciferase was prepared as described by Green and McElroy (1956). The enzyme was stored at 0° as a concentrated solution (10–20 mg of protein/ml) in 10% $(NH_4)_2SO_4$. This stock solution was diluted into 0.025 M glycylglycine (pH 7.5) just prior to use. Protein concentration was measured by

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