Coenzyme Analog Inhibitors of Apoglutamate Decarboxylase*

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ABSTRACT: Bacterial glutamate decarboxylase apoenzyme reacts slowly with 5-nitrosalicylaldehyde to form a Schiff base which is devoid of catalytic activity. The inhibition constant is 1.1 mM at 0°, pH 5.4. Binding of this aldehyde prevents the recovery of catalytic activity on addition of pyridoxal phosphate. The binding reaction is accelerated by inorganic phosphate and inhibited by acetate. The apoenzyme is also inhibited by 3-nitrosalicylaldehyde (inhibition constant 2 mM), 3,5-dinitrosalicylaldehyde (6 mM), and 2-hydroxy-5-nitroacetophenone (8 mM). Results of kinetic and equilibrium binding studies on 5-nitrosalicylaldehyde indicate that this is not a simple one-step binding process, but that more than one kinetically significant step is involved.

Bacterial L-glutamate decarboxylase (EC 4.1.1.15) was first isolated and studied by Gale (1946). Other early work included that of Najjar and Fisher (1954) and Koppelman *et al.* (1958). Shukuya and Schwert (1960a) purified the enzyme extensively and showed that it contains pyridoxal 5′-phosphate as a prosthetic group. Their suggestion of a Schiff-base linkage between coenzyme and protein was confirmed by the borohydride reduction studies of Anderson and Chang (1965). Absorption spectra (Shukuya and Schwert, 1960b) and circular dichroism (Huntley and Metzler, 1967a; Sukhareva and Torchinsky, 1966) have been reported. Although the enzyme is most active toward glutamic acid, γ-methylene glutamic acid and *threo*-β-hydroxyglutamic acid are also decarboxylated (Homola and Dekker, 1967). The holoenzyme can be resolved by treatment with α-methylglutamic acid, followed by gel filtration or dialysis (Huntley and Metzler, 1967b).

It is likely that this enzyme follows the general mechanism of pyridoxal 5′-phosphate catalyzed reactions proposed by Metzler *et al.* (1954), by Braunstein and Shemyakin (1952), and (in this case) by Westheimer (Mandeles *et al.*, 1954). However, the enzyme is most active at pH 4.0–5.0 (Shukuya and Schwert, 1960a; M. H. O'Leary and D. T. Richards, 1968, unpublished data) and it appears that the state of ionization of the coenzyme in this case is different from that in aspartate aminotransferase (Guirard and Snell, 1964). The transition from the active form of the enzyme to the inactive form with increasing pH seems to occur with the simultaneous dissociation of more than one proton (M. H. O'Leary and D. T. Richards, 1968, unpublished data).

Although much is known about the catalytic function of pyridoxal 5′-phosphate in enzymes and in model systems (Bruice and Benkovic, 1966), little is known about the nature of the interactions between this coenzyme and various pyridoxal 5′-phosphate dependent enzymes (Snell, 1958; Fasella, 1967). The existence of a Schiff base between the aldehyde group and a lysine residue of the enzyme has been established in a number of cases (Fisher, 1964; Fasella, 1967). However, since pyridoxamine phosphate also binds firmly to many pyridoxal 5′-phosphate dependent enzymes, other factors must also be important. Support for the role of the phosphate group comes from the finding that pyridoxamine phosphate can be displaced from aspartate aminotransferase by a high concentration of P_i (Braunstein, 1964). Pyridoxine phosphate and deoxypyridoxine phosphate have also been found to bind to these enzymes (Meister *et al.*, 1954; Evangelopoulos and Sizer, 1965).

Analogs of pyridoxal 5′-phosphate in which various alkyl groups are placed in the 2 and 6 positions of the pyridine ring activate a variety of pyridoxal phosphate dependent enzymes about as well as does pyridoxal 5′-phosphate itself (Morino and Snell, 1967; Bocharov *et al.*, 1968). Pyridoxal (Evangelopoulos and Sizer, 1965) and 5-deoxypyridoxal (Olivard and Snell, 1955) also bind to apoenzymes of this group. The last authors also reported that 4-nitrosalicylaldehyde is a potent inhibitor of apoalanine racemase.

Most of the studies which have been reported up to the present time have dealt with coenzymes analogs which are quite similar in structure to pyridoxal 5′-phosphate. It seemed appropriate to investigate a diverse series of coenzyme analog inhibitors in order to obtain more information about the nature of the coenzyme binding. Recent interest in irreversible active-site-directed enzyme inhibitors (Baker, 1967) also indicates the importance of such a study.

We report here a quantitative study of the interaction of a number of aromatic aldehydes and one aromatic ketone with apoglutamate decarboxylase. Both the rates of binding and the binding equilibria have been studied.

Experimental Section

Ultraviolet spectra were taken with a Cary 15 spectrophotometer. Infrared spectra were taken with a Beckman IR-8. Melting points were taken with a Thomas-Hoover

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apparatus and are uncorrected. All pH measurements were made with a Radiometer Model 26 pH meter.

Materials. Pyridoxal 5′-phosphate was obtained from Sigma. Eastman 5-nitrosalicylaldehyde was recrystallized from ethanol–water before use. 3,5-Dinitrosalicylaldehyde was synthesized by the method of Gilliand *et al.* (1941). 2-Hydroxy-5-nitroacetophenone was synthesized by the method of Furka and Szell (1960). Ammonium sulfate was Mann Enzyme Grade. Other reagents were commercial materials of high quality. Doubly distilled deionized water was used for making all buffers. Pyridine hydrochloride buffers, 0.1 M in total pyridine, were used except where otherwise noted.

Enzyme Assay. A Gilson differential respirometer was used for all kinetic studies. Assays were conducted at 37.0° in pH 4.9 pyridine hydrochloride buffer containing 25 mM L-glutamic acid (Sigma). In the main chamber of the Warburg flask was placed 3.0 ml of the glutamic acid solution. An appropriate amount of the enzyme, usually 25–50 μl, was put in the side arm and the flask was equilibrated at 37° for 15 min. The contents of the flask were then mixed and the volume of gas evolved was recorded at 100-sec intervals for at least 10 min.

Enzyme Isolation. Acetone powder of *E. coli* (ATCC 11246) was obtained from Worthington Biochemical Corp. The isolation procedure used was as described by Shukuya and Schwert (1960a), with the following exceptions. (1) All buffers contained 10^{-4} M dithiothreitol and 10^{-4} M pyridoxal 5′-phosphate. (2) The cell walls were broken with lysozyme (20 mg/g of acetone powder). (3) After the protamine sulfate step the enzyme was precipitated with ammonium sulfate in the fraction between 50 and 70% of saturation. (4) Only one DEAE-cellulose chromatography was used. The specific activity of the final enzyme was similar to that reported by Shukuya and Schwert (1960a).

Resolution of Holoenzyme. To a solution of holoenzyme at 0° in pH 4.9 buffer free of excess pyridoxal 5′-phosphate was added an equal volume of a solution of α-methylglutamic acid (0.06–0.3 M). After 15 min a second, like portion of α-methylglutamic acid was added. After 1 hr the solution was desalted on a small Sephadex G-25 column at 4°. The resulting enzyme retained less than 10% of its original activity, but full activity could be restored on addition of pyridoxal 5′-phosphate.

Binding of Aldehydes to the Apoenzyme. A slight variation of the normal assay method was used. Instead of the usual chimney on the side arm of the Warburg flask, a serum cap was used. The side arm contained 50 μl of 4 mM pyridoxal 5′-phosphate and the main chamber contained 3.0 ml of 0.025 M glutamic acid (pH 4.9). The flask (containing no enzyme) was equilibrated at 37° and then an aliquot of the enzyme solution was added to the side arm by use of a hypodermic syringe. The assay was started 1 min later. The recovered activity of the apoenzyme was the same whether this procedure was used or the apoenzyme–pyridoxal 5′-phosphate incubation was done at 0°.

In a typical binding experiment, 250 μl of apoenzyme solution and 250 μl of a solution of the compound under study (0.5–4.0 mM) were mixed in an ice bath. Aliquots were withdrawn at intervals with a hypodermic syringe and assayed as described above.

The binding experiments in the presence of other ions were conducted using the usual pH 5.4 buffer to which had been added K_2HPO_4, NaOAc, or glutaric acid.

5-Nitrosalicylaldehyde–Butylamine Schiff Base. About 0.5 g of 5-nitrosalicylaldehyde was dissolved in 25 ml of methanol and 1 g of *n*-butylamine was added. The solution was heated on a steam bath for 15 min and then cooled in a Dry Ice bath. The crystals of the yellow Schiff base precipitated and were collected by filtration. The Schiff base melted at 106°: infrared spectra ($CHCl_3$), 6.1, 6.2, 6.5, and 7.5 μ; nuclear magnetic resonance spectra, δ 1.0 (triplet, 3 H), 1.6 (multiplet, 4 H), 3.7 doublet, 1 H), and 8.2 (multiplet, 3 H); ultraviolet spectrum (0.1 M HCl) 310 mμ.

Results

Properties of the Apoenzyme. Apoglutamate decarboxylase is conveniently prepared by treating a solution of the holoenzyme with 0.1–0.2 M α-methylglutamic acid (Huntley and Metzler, 1967b). After dialysis or gel filtration the enzyme retains less than 10% of its original activity and has no ultraviolet absorbance above 300 mμ. Essentially complete activity can be restored by addition of 1 mM pyridoxal 5′-phosphate.

The apoenzyme is considerably less stable than the holoenzyme. No activity can be recovered after heating the apoenzyme at 37° for 5 min at pH 4.9. The apoenzyme is stabilized by the presence of reducing agents such as dithiothreitol and glutathione. In the presence of 10^{-4} M dithiothreitol less than 10% of the recoverable activity of the apoenzyme is lost in 1 week at 0°. In the presence of dithiothreitol at pH 4.9 the enzyme is stable for at least several hours at 25°. At pH 5.9 the apoenzyme is stable for less than 1 day at 0°. The apoenzyme is less stable at low than at high concentration.

Binding of 5-Nitrosalicylaldehyde to the Apoenzyme. Apoglutamate decarboxylase is catalytically inactive, but activity can be restored by brief treatment with 2 mM pyridoxal 5′-phosphate. When the apoenzyme is incubated with 1.0 mM 5-nitrosalicylaldehyde at 0° the amount of activity which can be recovered on brief treatment with pyridoxal 5′-phosphate gradually decreases. Results of a typical experiment are shown in Figure 1. Aliquots of the solution containing 5-nitrosalicylaldehyde and the apoenzyme were removed at various times and added to 50 μl of 5 mM pyridoxal 5′-phosphate in the side arm of a Warburg flask which had previously been equilibrated at 37°. After about 1 min the assay was begun. Quantities of evolved gas were recorded each 100 sec for a total of 10 min. Plots of microliters evolved *vs.* time were linear and showed no evidence of reconversion of the inactive enzyme to an active form during assay.

That the binding of 5-nitrosalicylaldehyde to the apoenzyme is reversible was shown by an experiment in which the apoenzyme was incubated with 2 mM 5-nitrosalicylaldehyde at pH 4.9 for 3 hr at 0°. This solution was then dialyzed for 12 hr at 0° in the presence of 1 mM pyridoxal 5′-phosphate. After this treatment

the enzyme was found to have recovered more than 90% of its original activity.

Rate and equilibrium constants for the binding of 5-nitrosalicylaldehyde to the apoenzyme were computed using data such as those in Figure 1. The equilibrium constant defined by eq 1 can be calculated from eq 2,

$$K = \frac{[\text{5-nitrosalicylaldehyde}][\text{apoenzyme}]}{[\text{complex}]} \quad (1)$$

$$K = \frac{V_\infty[\text{5-nitrosalicylaldehyde}]}{V_0 - V_\infty} \quad (2)$$

where V_0 is the velocity of the decarboxylation in the presence of pyridoxal 5'-phosphate before 5-nitrosalicylaldehyde is added to the solution, V_∞ is the velocity of the decarboxylation in the presence of pyridoxal 5'-phosphate after the 5-nitrosalicylaldehyde binding equilibrium has been reached, and [5-nitrosalicylaldehyde] is the concentration of 5-nitrosalicylaldehyde. When necessary these data were corrected for the presence of small amounts of holoenzyme in the apoenzyme preparation.

Rate and equilibrium data for the binding of 5-nitrosalicylaldehyde to the apoenzyme are summarized in Table I. The reported equilibrium constants are estimated to be accurate to about ±20%.

The rate constant for the binding of 5-nitrosalicylaldehyde to the apoenzyme can also be obtained from these data. Since the concentration of 5-nitrosalicylaldehyde is much greater than that of the apoenzyme we assume that in a given experiment the binding is pseudo first order. Thus a plot of log $(V - V_\infty)$ *vs.* time gives a straight line of slope k_1. The interpretation of data from a series of experiments at different 5-nitrosalicylaldehyde concentrations is more difficult and will be considered later. The rate constants in Table I refer to the rate when the 5-nitrosalicylaldehyde concentration is 1.0 mM.

No dissociation of the 5-nitrosalicylaldehyde–enzyme complex to give apoenzyme occurs during the assay. Formation of apoenzyme would be followed rapidly by formation of holoenzyme, since reaction of the apoenzyme with pyridoxal 5'-phosphate is rapid under these conditions. Holoenzyme formation would have resulted in curvature of the plots of gas evolved *vs.* time. The 5-nitrosalicylaldehyde–apoenzyme complex has no detectable decarboxylase activity.

The 5-nitrosalicylaldehyde–apoenzyme complex does not dissociate readily into 5-nitrosalicylaldehyde and apoenzyme. The apoenzyme was incubated at pH 4.9, 0° with 2.0 mM 5-nitrosalicylaldehyde for 2 hr (sufficient time for the binding equilibrium to be established), after which an equal volume of 2 mM pyridoxal 5'-phosphate was added. No increase in the activity of this solution was noted in 3 hr. Dissociation of the 5-nitrosalicylaldehyde–apoenzyme complex would have resulted in formation of holoenzyme, since the reaction of pyridoxal 5'-phosphate with the apoenzyme under these conditions is much more rapid than the reaction with 5-nitrosalicylaldehyde. Assuming that less than 10% reversion to holoenzyme has occurred, this places an upper limit of 10^{-5} sec^{-1} on the rate of dissociation of the apoenzyme–5-nitrosalicylaldehyde complex.

The 5-nitrosalicylaldehyde–apoenzyme complex can be freed of excess 5-nitrosalicylaldehyde by gel filtration at 0°. Ultraviolet absorption maxima of this complex at various pH values are summarized in Table II. Spectra of 5-nitrosalicylaldehyde and the Schiff base of 5-nitrosalicylaldehyde with *n*-butylamine are also given. The apoenzyme has no absorption above 300 mμ.

Treatment of the complex with 0.1 M NaOH for 10 min at 80° followed by centrifugation leads to the recovery of 5-nitrosalicylaldehyde, as shown by its ultraviolet spectra in 0.1 M NaOH and in 0.1 M HCl.

The holoenzyme is not inactivated by incubation with 5-nitrosalicylaldehyde. A sample of holoenzyme free of excess pyridoxal 5'-phosphate was incubated with 2 mM 5-nitrosalicylaldehyde for 2 hr at 0°, pH 4.9, and then excess 5-nitrosalicylaldehyde was removed by gel filtration. The activity of the enzyme was not changed by this treatment. However, the ultraviolet spectrum of the enzyme was slightly changed. An increase of approximately 50% in the absorbance at 320 and 360 mμ

TABLE I: Binding of 5-Nitrosalicylaldehyde to Apoglutamate Decarboxylase at 0°.

pH	Added Ion (10 mM)	K_{equil} (mM)	Binding Rate[a] (10^4 sec^{-1})
5.4	None	1.1	5
4.9	None	1.0	6
4.4	None	1.4	
5.4	PO_4^{3-}	1.0	9
5.4	$CH_3CO_2^-$	0.9	3
5.4	$^-O_2C(CH_2)_3CO_2^-$	1.1	6

[a] 5-Nitrosalicylaldehyde concentration 1.0 mM.

TABLE II: Long-Wavelength Ultraviolet Absorption Maxima.

Compound	pH 3.5	pH 4.9	pH 5.9
Apoenzyme 5-nitrosalicylaldehyde complex	353, 388 (sh)	353, 390	354, 388 (sh)
5-Nitrosalicylaldehyde	308	314, 380 (sh)	354, 388
n-Butylamine–5-nitrosalicylaldehyde Schiff base	360, 387	360, 386	363, 384

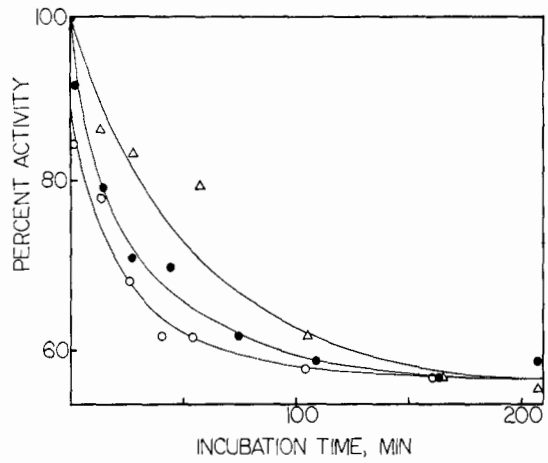

FIGURE 1: Loss in recoverable activity on incubation of apoglutamate decarboxylase with 1.0 mM 5-nitrosalicylaldehyde for various times (●). Same experiment in the presence of 0.01 M phosphate (○) and 0.01 M acetate (△).

TABLE III: Equilibrium Constants for the Binding of Aldehydes and Ketones to Apoglutamate Decarboxylase.[a]

Compound	K_{equil} (mM)
5-Nitrosalicylaldehyde	1.1
3-Nitrosalicylaldehyde	2
3,5-Dinitrosalicylaldehyde	6
5-Nitro-2-hydroxyacetophenone	8
Salicylaldehyde	*b*
m-Nitrobenzaldehyde	*b*

[a] Determined at pH 5.4, 0°. [b] Binding occurs, but is too slow to be measured accurately.

was observed. The protein was removed by denaturation and centrifugation, and the ultraviolet spectrum of the supernatant solution was recorded. In 0.1 M NaOH a shoulder was noted at 360 mμ. This absorption is not present in pyridoxal 5′-phosphate and is presumably due to the presence of a small amount of 5-nitrosalicylaldehyde.

Binding of 5-Nitrosalicylaldehyde to the Apoenzyme in the Presence of Other Ions. The data in Table I show that the binding of 5-nitrosalicylaldehyde to the apoenzyme is not strongly affected by pH over the pH range from 4.4 to 5.4. The rate of binding at pH 5.4 is strongly affected by the presence of acetate or phosphate (Figure 1). The binding of 5-nitrosalicylaldehyde to the apoenzyme is accelerated about twofold by the presence of 0.01 M phosphate, but the equilibrium constant for the binding is the same in both cases. The binding of 5-nitrosalicylaldehyde to the apoenzyme is inhibited by the presence of acetate. In the presence of 0.01 M acetate the rate of binding decreases by about a factor of 2. Again, the equilibrium constant is not affected. The rate and equilibrium for binding of 5-nitrosalicylaldehyde to the apoenzyme are not affected by the presence of 0.01 M glutarate at pH 5.4.

Binding of Other Aldehydes and Ketones to the Apoenzyme. Several other aromatic aldehydes and one aromatic ketone were found to bind to the apoenzyme. These data are summarized in Table III. None of the other compounds tested is as good an inhibitor as is 5-nitrosalicylaldehyde. The binding constants for the other compounds listed in Table III were not determined as accurately as that for 5-nitrosalicylaldehyde and are only approximate. The binding of salicylaldehyde and of *m*-nitrobenzaldehyde to the apoenzyme are so slow that binding constants were not determined. No decarboxylase activity was observed in the complex formed by 3,5-dinitrosalicylaldehyde and the apoenzyme.

Discussion

A number of aromatic aldehydes, most notably 5-nitrosalicylaldehyde, bind slowly and reversibly to apoglutamate decarboxylase. This binding prevents the activation of the apoenzyme by pyridoxal 5′-phosphate. Ultraviolet spectral data indicate that this binding occurs *via* a Schiff-base linkage. Although our experiments do not conclusively prove that this inhibition occurs because of binding of 5-nitrosalicylaldehyde to the same lysine which ordinarily binds pyridoxal 5′-phosphate, our data are consistent with this possibility and several lines of evidence argue in favor of this: (1) the binding of 5-nitrosalicylaldehyde prevents activation of the apoenzyme by pyridoxal 5′-phosphate; (2) the binding of pyridoxal 5′-phosphate to the active site prevents the inhibition by 5-nitrosalicylaldehyde; (3) the effect of phosphate on the binding of 5-nitrosalicylaldehyde to the apoenzyme is similar to its effect on the binding of pyridoxal 5′-phosphate to a number of other enzymes; and (4) the effect of acetate on the binding of 5-nitrosalicylaldehyde to the apoenzyme is similar to the effect of acetate on the binding of pyridoxal 5′-phosphate (Huntley and Metzler, 1967b).

Some nonspecific binding of 5-nitrosalicylaldehyde to the holoenzyme also occurs, but this binding can easily be differentiated from the active-site binding because of the inhibition produced by 5-nitrosalicylaldehyde when bound to the active site. Nonspecific binding of pyridoxal 5′-phosphate to aspartate aminotransferase also occurs (Banks *et al.*, 1966).

The formation and decomposition of Schiff bases of aromatic aldehydes have been shown to be catalyzed by the presence of an *o*-hydroxyl group (French *et al.*, 1965; Reeves, 1965). A similar catalytic role has been ascribed to the phenolic hydroxyl group of pyridoxal 5′-phosphate in enzymatic processes (Bruice and Benkovic, 1966). Our results show that the formation of the enzyme–5-nitrosalicylaldehyde Schiff base (and therefore the enzyme–pyridoxal 5′-phosphate Schiff base) is subject to similar catalysis. It is not possible on the basis of the present data to assess the effect of the phenolic hydroxyl group on the equilibrium constant for the binding of 5-nitrosalicylaldehyde to the apoenzyme. In model systems the phenolic hydroxyl group increases the strength of the binding (French *et al.*, 1965).

Although numerous aldehydes have been reported to form Schiff bases with pyridoxal 5′-phosphate dependent enzymes, to our knowledge no ketones have previously been shown to bind. The binding of 2-hydroxy-5-nitroacetophenone to apoglutamate decarboxylase is considerably weaker than that of 5-nitrosalicylaldehyde. Although this difference might be due to steric interference by the methyl group of the ketone, it is more likely that this effect is purely an inductive one. Methyl ketones form carbonyl derivatives less easily than do the corresponding aldehydes (Arai, 1961).

P_i has been reported to have an effect on the binding of pyridoxal 5′-phosphate to a number of enzymes. The binding of pyridoxal 5′-phosphate to aspartate aminotransferase (Banks *et al.*, 1963; Braunstein, 1964) and to kynurenine aminotransferase (Mason, 1957) is inhibited by the presence of P_i. Wada and Morino (1964) have shown that mitochondrial aspartate aminotransferase apoenzyme catalyzes the transamination between aspartate and pyridoxal provided that P_i is present. Catalysis of the binding of 5-nitrosalicylaldehyde to apoglutamate decarboxylase by P_i is presumably related to the same phenomenon; binding of P_i to the site normally occupied by the phosphate group of pyridoxal 5′-phosphate.

The effect of acetate on the binding rates of pyridoxal 5′-phosphate and 5-nitrosalicylaldehyde is apparently not due to binding of acetate at the substrate binding site. If it were, then the effect of glutarate on the binding should be greater than that of acetate. Since glutarate has no effect on the binding of 5-nitrosalicylaldehyde, acetate must effect the coenzyme binding site, rather than the substrate binding site.

The binding of 5-nitrosalicylaldehyde to apoglutamate decarboxylase is considerably weaker than that of pyridoxal 5′-phosphate. At pH 5.4 the binding constant for 5-nitrosalicylaldehyde is 1.1×10^{-3} M. Preliminary evidence (M. H. O'Leary, 1968, unpublished) indicates that the binding constant for pyridoxal 5′-phosphate is similar to that found in other cases, *i.e.*, 10^{-6}–10^{-8} M (Snell, 1958).

One important discrepancy exists in the present data. The equilibrium constants based on eq 1 can most simply be related to binding according to eq 3.

$$\text{apoenzyme} + \text{5-nitrosalicylaldehyde} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{complex} \quad (3)$$

The equilibrium constant is then related to the two rate constants k_1 and k_2 by eq 4. The value of K has been

$$K = \frac{k_2}{k_1} \quad (4)$$

obtained in this study and the value of k_1 can be obtained if the assumption is made that binding process is first-order in apoenzyme and first-order in 5-nitrosalicylaldehyde. However, the value of k_2 calculated from our data and eq 4 is greater than the estimated upper limit of k_2 by a factor of 50. Thus the simple scheme given in eq 3 is not adequate to explain these data. A similar discrepancy has been observed in one other case. Meister *et al.* (1954) reported that the equilibrium constant for the binding of pyridoxal 5′-phosphate to apoaspartate aminotransferase at pH 7.5 and 37° is 4.4×10^{-6} M. From their data the binding rate (analogous to k_1 in eq 3) can be estimated to be 10^2 M^{-1} sec^{-1}. Thus the rate of dissociation of the holoenzyme is estimated to be 4×10^{-4} sec^{-1} and the time required for 50% dissociation is about 30 min. However, Meister *et al.* reported that no dissociation of the holoenzyme occurred in 60 hr at 5°. Unless the effect of temperature on the dissociation rate is extraordinarily large, it must be concluded that the simple scheme analogous to that given in eq 3 is not correct.

The discrepancy between predicted and observed rates of dissociation of the holoenzyme or of the apoenzyme–5-nitrosalicylaldehyde complex might be due to any of several factors. Two particularly intriguing explanations which must be considered are: (1) formation and decomposition of a carbinolamine intermediate are kinetically significant; (2) the apoenzyme must undergo a conformational change before binding the coenzyme or inhibitor. Further speculation concerning this question is not profitable until more data are available. Investigation of this phenomenon is continuing.

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