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Nuclear Magnetic Resonance Studies on the Binding of Substrate, Coenzymes, and Effectors to Glutamate Dehydrogenase†

Piet Jan Andree‡

ABSTRACT: The binding of substrate, coenzymes, and effectors to the enzyme glutamate dehydrogenase was studied by ligand nuclear magnetic resonance (NMR) line broadening. Binary complexes, in fast exchange with the free ligand, of the enzyme with the substrate α-ketoglutarate and oxidized and reduced coenzyme were detected by this method. The binding of the inhibitor succinate is comparable to that of the oxidized substrate indicating that neither the precise length of the carbon chain nor the carbonyl group is crucial for the binding. The affinity of the enzyme for the reduced substrate, L-glutamic acid, is apparently much weaker. The formation of ternary complexes with enzyme, substrate, and coenzyme results in a drastic decrease of the observed NMR line broadenings. Quantitative studies show that substrate and coenzyme enhance each others binding by one or more orders of magnitude. Temperature-dependent measurements lead to the proposition that the binding of α-ketoglutarate and oxidized coenzyme in the ternary complex occurs via a first fast step, which is followed by a slow isomerization of the complex. The same two-step binding mechanism seems to occur on formation of the binary complex with reduced coenzyme, but not with oxidized coenzyme. The effects of the allosteric effectors GTP and ADP on the ternary complex formation indicate that inhibition is related to stronger binding and slower release of the bound ligands from the enzyme.

The enzyme glutamate dehydrogenase (L-glutamate: $NAD(P)^+$ oxidoreductase (deaminating), EC 1.4.1.3) from bovine liver catalyzes the reversible oxidation of L-glutamate by NAD(P) to α-ketoglutarate, ammonia, and NAP(P)H. The enzymatic activity is subject to regulatory effects of a large number of compounds of which the inhibitor GTP and the activator ADP are the most studied ones. Despite the large number of studies concerning the enzyme many important questions about the relationship of catalytic and regulatory sites and the role of the subunit structure are still awaiting their final answers.

We have undertaken NMR[1] studies of ligand binding to GluDH which were directed mainly to α-KG and oxidized coenzyme since these compounds are difficult to study by optical techniques. A preliminary report of part of our studies has been published (Andree & Zantema, 1974).

Theory

The theory for nuclear magnetic relaxation of small ligands bound to macromolecules has been well developed in the past years (Swift & Connick, 1962; Navon et al., 1970; Sykes et al., 1970; Beard & Schmidt, 1973). We will briefly review and expand the present theory as far as it is used to interpret our experimental data.

For the equilibrium

$$E + L \rightleftharpoons EL \qquad (1)$$

the following relation between the NMR line width and the total concentrations of ligand (L_0) and enzyme (E_0) holds, provided that the ligand is in large excess over the enzyme:

$$E_0/B = \pi(K_d + L_0)T_2^i \qquad (2)$$

K_d is the dissociation constant for equilibrium 1. B denotes the line broadening of the ligand and specifically means the dif-

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[1] Abbreviations used: NMR, nuclear magnetic resonance; GluDH, glutamate dehydrogenase; α-KG, α-ketoglutarate.

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ference in NMR line width of the observed resonance before and after enzyme addition.

We have introduced the term T_2^i, which we call the apparent intrinsic relaxation time of the bound ligand. An expression for T_2^i is given by Swift & Connick (1962). In cases where the difference in resonance frequencies of the ligand in its free and bound state does not contribute to the relaxation, the Swift & Connick (1962) expression reduces to

$$T_2^i = T_{2b} + \tau_b \tag{3}$$

As is apparent from expressions 2 and 3, the broadening can be determined either by T_{2b} or by τ_b. When T_{2b} is the largest term, the complex is in fast exchange on the NMR time scale and the line width will in general decrease on raising the temperature. If τ_b is larger the complex is in slow exchange and, in this limit, the broadening increases at higher temperature. This was discussed for example by Sykes et al. (1970).

For protons the relaxation is caused largely by dipolar coupling with other protons and the relaxation time is given by (Solomon, 1955):

$$\frac{1}{T_{2b}} = \frac{3}{40}\sum_{ij}\frac{h^2\gamma^4}{r_{ij}^6} \times \left(6\tau_R + \frac{10\tau_R}{1+\omega^2\tau_R^2} + \frac{4\tau_R}{1+4\omega^2\tau_R^2}\right) \tag{4}$$

where τ_R is the rotational correlation time of the bound ligand, ω the used resonance frequency, and r_{ij} the interproton distances.

Binding in Two Steps. Swift & Connick (1962) described a situation in which the bound ligand can exist in two states only one of which can dissociate into the bulk solution. In this case there are two equilibria that have to be taken into consideration:

$$\underset{a}{E} + L \underset{}{\overset{K_1}{\rightleftharpoons}} \underset{b}{EL} \overset{K_2}{\rightleftharpoons} \underset{c}{EL'} \tag{5}$$

Certain limiting cases of this situation are discussed earlier by Sykes et al. (1970) and Beard & Schmidt (1973).

The line width of the ligand resonance is given by eq 17 in the paper of Swift & Connick. We have proceeded from this equation to derive an expression for the concentration dependence of the line width similar to eq 2. The following assumptions and substitutions have been made. All chemical shifts have again been neglected. This is probably a reasonable assumption for high molecular weight proteins for which high values for the relaxation rates of the bound ligand are expected. Relationships between rate constants and equilibrium constants have been used. The equilibrium constants are defined as

$$K_2 = \frac{[EL']}{[EL]} \tag{6}$$

and

$$K_d = \frac{[E][L]}{[EL] + [EL']} \tag{7}$$

The resulting equation is identical with eq 2 with the following expression for the apparent intrinsic relaxation time T_2^i:

$$T_2^i = (K_2 + 1)\left(\tau_{ba} + \frac{1}{1/T_{2b} + K_2/(T_{2c} + \tau_{cb})}\right) \tag{8}$$

It is of interest to note that in the two-step binding mechanism a slow exchange situation can exist that does not lead to the typical slow-exchange temperature-dependent behavior (i.e., increasing relaxation at higher temperatures). To illustrate this we consider an extreme case in which the exchange in the second step is very slow ($\tau_{cb} \gg K_2T_{2b}$). Formula 8 then reduces to:

$$T_2^i = (K_2 + 1)(\tau_{ba} + T_{2b}) \tag{9}$$

Comparison with eq 3 shows that the slow exchange indeed reduces the relaxation (by a factor of $K_2 + 1$), while on the other hand the temperature dependency might indicate fast exchange because it is determined by the first step in the binding process.

In general, this result means that, if fast exchange is indicated by temperature-dependent measurements while other evidence shows that the relaxation is exchange limited, one can conclude that a two-step or more complicated binding mechanism is operative.

Binding of a Second Ligand. The observed line broadening of a ligand can be changed if a second ligand binds to the complex. This will be the case if a ternary complex is formed in which the relaxation of the observed ligand differs from that in the binary complex. Formulas will be presented to express the line broadening of the observed ligand (L) as a function of the concentration of the second ligand (C).

Consider the random binding of two ligands L and C:

$$K_b^L = \frac{[E][L]}{[EL]} \qquad K_t^L = \frac{[EC][L]}{[ELC]}$$
$$K_b^C = \frac{[E][C]}{[EC]} \qquad K_t^C = \frac{[EL][C]}{[ELC]} \tag{10}$$

The four dissociation constants are related by $K_b^L K_t^C = K_b^C K_t^L$. The total line broadening is obtained from the contributions of the binary and ternary complexes. The intrinsic relaxation times of the ligand in the binary and the ternary complex will be designated as T_{2b}^i and T_{2t}^i. The broadening of L is then given by

$$B = \left(\frac{[EL]}{T_{2b}^i} + \frac{[ELC]}{T_{2t}^i}\right)\frac{1}{\pi L_0}$$

In a practical case we can neglect binary complex with C and write for the observed ligand $[L]_f \approx L_0$. We find from the equilibrium expressions and the mass conservation laws:

$$[ELC] = C_0 - [C] = \frac{E_0 L_0 [C]}{K_b^L K_t^C + L_0(K_t^C + [C])} \tag{11}$$

from which [C] can be calculated

$$[C] = \tfrac{1}{2}(p - \sqrt{p^2 - 4q}) \tag{12}$$

where $p = (K_t^C/L_0)(L_0 + K_b^L) - C_0 + E_0$ and

$$q = -C_0\frac{K_t^C}{K_b^L}(L_0 + K_b^L)$$

The line broadening now can be given as a function of all known, total, concentrations by expressing [ELC] and [EL] as a function of [C]:

$$B = \frac{1}{\pi}\frac{E_0}{(1 + L_0/K_b^L + L_0[C]/K_b^L K_t^C} \times \left(\frac{1}{K_b^L T_{2b}^i} + \frac{[C]}{K_b^L K_t^C T_{2t}^i}\right) \tag{13}$$

Experimental Section

Bovine liver glutamate dehydrogenase was obtained as a suspension in ammonium sulfate from Boehringer (Mannheim). The Boehringer enzyme was reported to have

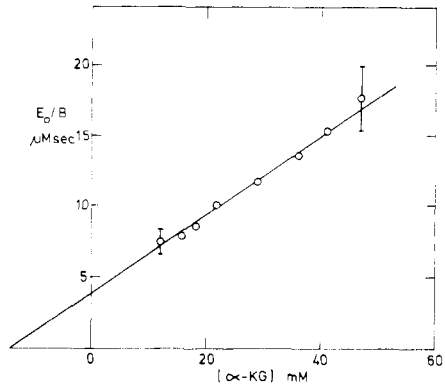

FIGURE 1: The reciprocal NMR line broadening of α-KG as a function of its concentration. Temperature was 18 ± 1 °C. [GluDH] was 2.3 mg/mL.

maximum activity and to give no evidence for heterogeneity by recrystallization, chromatography, or ultracentrifugation (Engel & Dalziel, 1969; Frieden, 1963; Sund, 1961). Unless stated otherwise, the buffer used was 0.05 M Tris-HCl, 0.1 M NaCl, and 10^{-4} M EDTA at pH 7.4. Before use the enzyme was dialyzed against 0.05 M EDTA solution at pH 8 to remove possible metal contaminations followed by exhaustive dialysis against the buffer indicated above. Enzyme concentrations were calculated from the extinction at 280 nm using an extinction coefficient of 0.93 mL cm^{-1} mg^{-1} (Egan & Dalziel, 1970). Molar concentrations in protomers were calculated by assuming a molecular weight of 56 100 (Smith et al., 1970).

Enzyme activity was measured spectrophotometrically at pH 8 by following the oxidation of NADH by α-KG and ammonia. The concentrations in the assay were: 9 mM α-KG, 140 mM NH_4Cl, and 100 μM NADH. The specific activity usually was 65 ± 10 units (mol min^{-1} mg^{-1}). No loss in activity was observed after performing the NMR measurements.

Coenzymes, nucleotides and α-KG were obtained from Sigma and other chemicals from Merck. All solutions were prepared immediately before use. Concentrations were calculated by weight except for NAD(P)H which concentration was calculated from the extinction at 340 nm using a specific absorbance of 6.2 mM^{-1} cm^{-1}.

NMR spectra were recorded on a Varian XL-100 spectrometer at a temperature between 15 and 20 °C in D_2O buffer. A small amount of denatured material was removed by centrifugation at 3000*g* prior to the experiments. α-KG stock solutions were prepared with a concentration of 0.05 M in Tris buffer (pH 7.4) without NaCl which is necessary to minimize effects of ionic strength differences. The line widths of the α-KG spectra were determined by comparison with a series of spectra with different Lorentzian width, simulated with Varian 620 f computer. The line broadening caused by the enzyme was obtained from this width by subtracting (a) the width of the HDO peak to correct for field inhomogeneity; (b) the intrinsic line width of free α-KG under the actual experimental conditions.

Results

1. *Binary Complex with α-KG.* The formation of a binary complex of GluDH with the substrate α-KG is demonstrated by the large increase in the width of the 100-MHz NMR spectrum of α-KG if enzyme is added to the solution. The widths of the two methylene resonances were always found to be exactly equal. The broadening effect results from specific interaction of α-KG with the enzyme as appears from the fact that many other compounds were shown to be not affected.

The substrate dependency of the line broadening is given in Figure 1. In the interpretation of these results, the possible contribution of chemical shift terms was considered (cf. theoretical section). The measured line widths at 60 and 360 MHz were equal to those obtained at 100 MHz within experimental error. Thus chemical shift difference between the bound and the free state does not contribute to the relaxation. Formula 2 was used in evaluating the results. From the linear regression analysis: $K_d = 13 \pm 4$ mM; $T_2^i = T_{2b} + \tau_b = 0.105 \pm 0.01$ ms at 18 °C (average for several experiments, not all shown in Figure 1).

Temperature Dependence. The results of temperature-dependent experiments (Figure 4) show decreasing broadenings at higher temperature from which the conclusion follows that T_{2b} is dominant over τ_b. The similarity of the results at two highly different α-KG concentrations shows that the decrease in the broadening at higher temperature not merely reflects the temperature dependence of K_d. The deviation from linear behavior at lower temperature, which was shown to be reproducible, indicates that the fast exchange condition ($T_{2b} \gg \tau_b$) begins to break down and that τ_b will contribute to the sum $\tau_b + T_{2b}$ at low temperature. From the results, a rough estimate for τ_b (3×10^{-5} s at 10 °C) was obtained by assuming a 40% contribution of τ_b in the sum $\tau_b + T_{2b}$.

Association. GluDH shows a concentration dependent association (Olson & Anfinsen, 1952; Eisenberg & Tomkins, 1968). This is a complicating factor when one wants to use the NMR line broadening as a parameter for further investigations because the width is in principle determined by the molecular motion of the complex which will depend on the association state of the enzyme. Therefore, the α-KG line broadening was measured over an enzyme concentration range that should cause the average molecular weight of the enzyme to change a factor 2 or 3 (Eisenberg & Tomkins, 1968). No significant change in T_{2b} was apparent. This shows that small ligand induced changes in enzyme association need not be considered in interpreting the line broadening measurements.

Other Binary Complexes. At conditions similar to those used in experiments with α-KG, no comparable enzyme caused line broadenings were detected for the reduced substrate L-glutamic acid. With the competitive inhibitor succinic acid a binary complex is formed which appears to be very similar to that with α-KG. The parameters derived from a plot as shown in Figure 1 are:

$$K_d = 8 \pm 5 \text{ mM}; \tau_b + T_{2b} = 0.14 \pm 0.02 \text{ ms at } 19\ ^\circ\text{C}$$

2. *Coenzyme Binding.* Effects of the enzyme on NMR line widths were observed for the coenzymes in our laboratory and by other workers (Jallon & Roux, 1974; Markau, 1974). NMR studies of coenzyme binding are complicated by the existence of more than one binding site per protomer. This is well known for NADH, but recently Krause et al. (1974) showed that this second site also binds NADPH, certainly at the high coenzyme concentrations necessary for NMR experiments. No detailed concentration dependent measurements were performed. Transverse relaxation times were directly calculated from measured line broadenings, assuming dissociation constants smaller than the concentrations used (Krause et al., 1974; Dalziel & Egan, 1972) and fast exchange conditions as justified by temperature dependent measurements. T_{2b} values calculated in this way are 0.31 and 0.22 ms for the nicotinamide and adenine protons of NAD. NADH is significantly less broadened by the enzyme: $T_{2b} = 1.2$ ms was calculated for the adenine C-2 and C-8 protons.

3. *Formation of Ternary Complexes.* Addition of small

TABLE I: Effects of Ternary Complex Formation on NMR Line Broadenings.

Resonance obsd	Concn (mM)	[GluDH] (mg/mL)	B (Hz)	Addition
α-KG	28	4.0	6.0	
α-KG	28	4.0	1.2	2.8 mM NAD
α-KG	20	2.3	0	1.0 mM NADH
NAD(A-2H)	11	7.3	13	
NAD(A-2H)	11	7.3	4	3 mM α-KG
NADH(A-2H)	14	7.6	2.2	
NADH(A-2H)	14	7.6	0.2	1 mM α-KG

TABLE II: Results Obtained for the Binding of Coenzyme to the Binary GluDH α-KG Complex.[a]

	$T_{b,K}{}^i$ (ms)	$T_{t,K}{}^i$ (ms)	$K_t{}^C$ (μM)
NADP	0.092 ± 0.01	1.23 ± 0.2	84 ± 15
NAD	0.112 ± 0.01	0.594 ± 0.1	33 ± 5
NADPH	0.105 ± 0.01	10	0.9 ± 0.4
NADP in 0.1 M phosphate pH 7.5	0.115 ± 0.01	0.73 ± 0.15	20 ± 4

[a] Conditions: temperature 17 ± 2 °C; [GluDH] = ±2 mg/mL. Values for the binary dissociation constants with substrate were substituted as 13 mM in Tris and 7 mM in phosphate as determined in the binary complex studies. K refers to α-KG and C to coenzyme.

TABLE III: Results for the Binding of α-KG to the GluDH NAD Complex.[a]

	$T_{b,C}{}^i$ (ms)	$T_{t,C}{}^i$ (ms)	$K_t{}^K$ (mM)
Nicotinamide protons	0.31 ± 0.05	5 ± 1.5	0.45 ± 0.15
Adenine protons	0.22 ± 0.04	1 ± 0.5	0.35 ± 0.15

[a] Conditions: T = 17 ± 2 °C; [GluDH] = 7.3 mg/mL. In the calculation a binary dissociation constant for coenzyme binding of 1 mM was used. This value is not of large influence on the results obtained.

amounts of coenzyme to enzyme and α-KG strongly effects the substrate line width (Table I). Oxidized coenzyme causes only partial decrease in the broadening while in the presence of reduced coenzyme almost no broadening is detectable. A trivial explanation of these observations would be that the coenzyme displaces the substrate from its binding site. This explanation, however, cannot be valid. Not only the substrate relaxation is decreased by coenzyme, but the same is observed for coenzyme if α-KG is added. In both experiments the concentration of the second ligand necessary to exert its effect is low compared with the first one. This implies that the second added ligand binds stronger than the first one, but this cannot be true for both ligands. Because of this inconsistency we conclude that the observed effects reflect the formation of a ternary complex. The decrease in relaxation is probably a consequence of slower dissociation from the bound state.

Ternary Complex Titrations. The dependency of the α-KG NMR line broadening on the coenzyme concentration is shown in Figures 2 and 3 for NADP and NADPH, respectively. Assuming different apparent intrinsic relaxation times for α-KG in the binary and ternary complex ($T_{b,K}{}^i$ and $T_{t,K}{}^i$), the line broadening is expressed in eq 12 and 13 as a function of the added concentration of coenzyme C. A least-squares computer program was used to fit the experimental data assuming 13

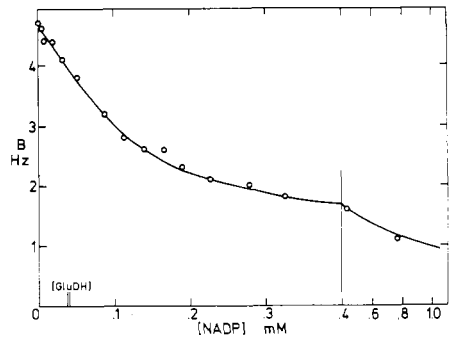

FIGURE 2: NMR line broadening of α-KG (17 mM) in the presence of 2.3 mg/mL GluDH dependent on the NADP concentration at 17 ± 1 °C. The drawn curve is the result of a computer fitting as described in the text.

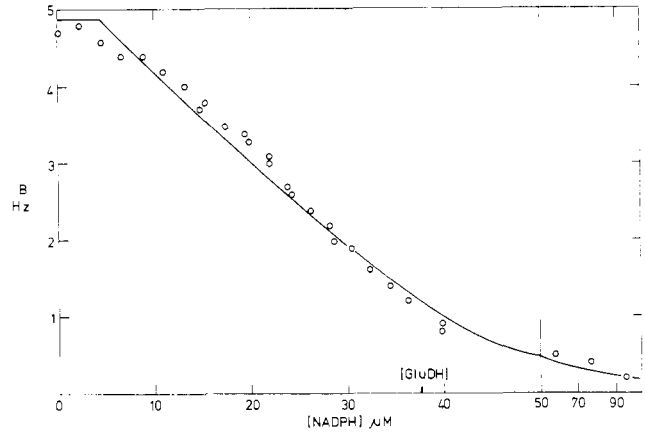

FIGURE 3: NMR line broadening of 20 mM α-KG in the presence of 2.2 mg/mL GluDH, dependent on the concentration of added coenzyme NADPH. The drawn curve gives the computer fit with the parameters given in Table I. A correction was applied for $NH_4{}^+$ ions initially present and causing the oxidation of the coenzyme. The correction was applied for 4.7 μM of $NH_4{}^+$, although only 3.3 μM was determined optically.

mM for $K_b{}^K$, the dissociation constant for the binary complex with α-KG.

The results obtained with reduced coenzyme were corrected for a small amount of unremovable ammonium ions which cause the enzymatic reaction to occur, until they are exhausted. The correct coenzyme concentration was derived from the absorbance at 340 nm. The influence of the small amount (3–6 μM) of oxidized coenzyme formed at the start of the experiment was neglected as justified by the results of Figure 2.

The relaxation times and equilibrium constants obtained are summarized in Table II. Table III shows the results of a titration of the binary NAD complex with α-KG. These results are derived from considerably less data points than those in Table II.

4. *Temperature Dependence in the Ternary Complex.* A decrease in the relaxation rates of the ligands is observed upon the formation of a ternary complex. As pointed out in the theoretical section, a decreased dissociation rate would give rise to less effective broadening in the slow exchange case. However, the temperature dependent data in Figure 4 show the line broadening of α-KG in the ternary complex with oxidized coenzyme to decrease at higher temperatures. This implies that T_{2b}, not τ_b, dominates as in the binary complex (i.e., the complex is still in fast exchange). As shown in the section theory, a two-step binding mechanism can account for decreased relaxation together with fast exchange behavior:

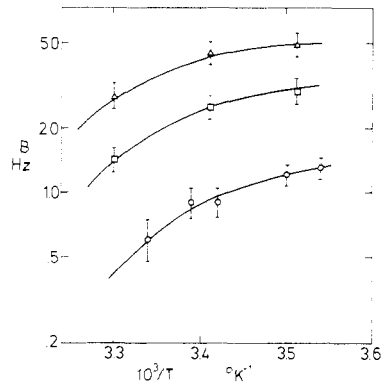

FIGURE 4: NMR line broadening of α-KG caused by GluDH plotted logarithmically against the inverse temperature. (Δ and □) With 13 and 37 mM α-KG, respectively, both in the presence of 1.8 mg/mL GluDH; (○) 29 mM α-KG in the presence of 6 mg/mL GluDH and 6.5 mM NADP.

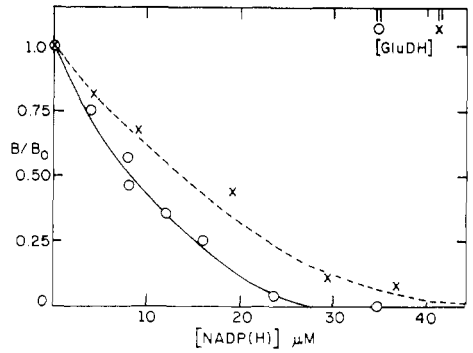

FIGURE 5: Relative NMR line broadening of α-KG in the presence of GTP as a function of coenzyme concentration. (○) With 23 mM α-KG, 1.95 mg/mL GluDH and 0.55 mM GTP (19 °C) titrated with NADPH; (X) 17 mM α-KG, 2.4 mg/mL GluDH, and 0.7 mM GTP titrated with NADP at 17 °C. In this case the drawn curves for NADPH (—) and NADP (- - -) do not represent a computer fit. The enzyme concentrations in the two experiments are indicated in the upper right hand corner.

$$E \cdot NADP + \alpha\text{-KG} \rightleftharpoons E \cdot NADP \cdot \alpha\text{-KG} \rightleftharpoons (E \cdot NADP \cdot \alpha\text{-KG})'$$

The equilibrium constant K_2 for the isomerization of the ternary complex can be calculated assuming that the relaxation time of α-KG is the same in the binary and the ternary complex, and that the final complex does not contribute to the relaxation at all (i.e., the second equilibrium is very slow). Then the ratio of $T_{b,K}{}^{i}$ and $T_{t,K}{}^{i}$ equals $K_2 + 1$ (cf. eq 9) and the values of K_2 are calculated to be 4.4 for NAD and 12.4 for NADP. Because of the assumptions made, these values are considered as lower limits.

5. *Influence of Allosteric Effectors.* The inhibitor GTP and the activator ADP did not show any effect on the binary complex of enzyme and α-KG. Titrations of the enzyme substrate complex with coenzyme in the presence of GTP are shown in Figure 5. The binding is enhanced by the inhibitor which is particularly clear for oxidized coenzyme NADP. Further, the α-KG line broadening is apparently eliminated before the enzyme is saturated, which effect is the most pronounced with reduced enzyme NADPH. No quantitative analysis of these results was attempted because in the course of the titration the enzyme dissociates completely into the hexameric units (Frieden, 1963; Iwatsubo et al., 1973). This dissociation might very well account for a part of the observed decrease in line broadening.

The effects of ADP on ternary complex formation were not studied in detail. Addition of ADP to a solution that contained enzyme, GTP, α-KG, and NADP completely reversed the α-KG line narrowing caused by GTP.

Discussion

1. *Binary Complex with Substrate α-Ketoglutarate.* Our value for the dissociation constant of the binary GluDH-α-KG complex (13 mM) is higher than the values of 1.5 mM obtained from kinetic studies (Engel & Dalziel, 1970) and 2.8 mM from spectrophotometric measurements (Cross et al., 1972). The difference might be the result of different experimental conditions used. We found that the binding was stronger (7 ± 4 mM) in phosphate buffer as was used in the studies cited above. Furthermore, part of the difference might result from the substitution of H_2O by D_2O, as effects of D_2O on enzymatic activity have been reported (Henderson & Henderson, 1969). In conclusion it can be said that the NMR results are consistent with those obtained by other techniques.

A lower limit for the dissociation rate constant $k_{off} = 3 \times 10^4\ s^{-1}$ can be estimated from the temperature dependence of the NMR line broadenings. This gives an upper limit for the rate constant for formation: $k_{on} = 2.5 \times 10^6\ M^{-1}\ s^{-1}$. The leveling off of the line broadening at lower temperatures suggests that the actual values might be close to these limits.

The value obtained for the relaxation time T_{2b} for α-KG when bound at the enzyme allows us to calculate the rotational correlation time of the bound substrate. Taking

$$\sum_{ij} r_{ij}^{-6} = 4.3 \times 10^{46}\ cm^{-6}$$

for two adjacent methylene groups (Sykes et al., 1970), the value calculated from eq 4 of the theoretical section for τ_R is 8.8×10^{-7} s which is rather high compared with the value of 1.4×10^{-7} s estimated from the Stokes-Einstein equation for the rotational tumbling time of a sphere with the mass of the hexameric unit of GluDH. Since enzyme association showed little or no effect on the line broadening, we postulate that the high calculated value for τ_R results from additional dipolar interaction between protein and substrate protons. This is also suggested by the results of T-1 experiments (Andree, 1978). We conclude that the α-KG molecule, although it binds rather weakly, must be (almost) completely immobilized at the enzyme surface and is probably close to protons of the protein molecule.

Comparing the different substrates it appears that the much smaller line broadening of L-Glu, compared with α-KG, can result from a low degree of saturation of the enzyme in accordance with the studies of Prough et al. (1972) who measured a dissociation constant of 47 mM.

We found that succinate behaved very similar to α-KG in forming a binary complex. This seems to disagree with results of Caughey et al. (1957) which indicate that succinate is too short to be an effective competitive inhibitor. However, preliminary NMR data suggest that binding in the ternary complex is much weaker with succinate which would be in accordance with the kinetic studies. The results indicate that neither the carbonyl group nor the precise length of the carbon chain is important for the binding of the substrate in a binary complex with GluDH. The structural requirements for strong binding in a ternary complex appear to be more severe than for the binary one.

2. *Binary Complex with Coenzyme.* NAD bound in the binary complex with the enzyme was found to have a low re-

laxation time (0.22 ms) which is difficult to explain by intramolecular dipolar coupling because the protons are several angstroms apart. It seems likely that the NAD molecule is in close contact with the protein and that intermolecular-dipolar coupling occurs.

Although NADH is known to bind stronger to the enzyme than NAD and furthermore possesses a second binding site (Jallon & Iwatsubo, 1971; Krause et al., 1974), its proton NMR lines were much less broadened (estimated $T_{2b} = 1.2$ ms). Temperature-dependent experiments showed fast exchange behavior with a dissociation rate constant of 10^3 s^{-1} or faster. This seems not to agree with stopped-flow studies of NADPH dissociation by di Franco & Iwatsubo (1972), who reported a rate constant of 30 s^{-1}.

Both this discrepancy and the small line broadening can be explained if the binding occurs in two steps (cf. section on theory). The NMR line broadening would only result from the first step which is in fast exchange while optically the second step is detected. A very similar interpretation based on the same type of evidence was proposed by Czeisler & Hollis (1973) for their NMR data obtained for NADH binding to liver alcohol dehydrogenase.

3. *Ternary Complexes with α-KG and Coenzyme.* Cross et al. (1972) found a dissociation constant of 9.6 μM for the binding of NADP to the GluDH–α-KG complex (in phosphate buffer). This is much lower than our value in Tris (84 μM), but again we could demonstrate a significant buffer effect. The remaining difference between the value obtained by us in phosphate (20 μM) and the result of Cross can be a result of further differences in the conditions. We find that NAD has a somewhat higher affinity than NADP. This agrees with the findings of Dalziel & Egan (1972) for the binding of NAD and NADP to the enzyme–glutarate complex.

The decreased relaxation of both α-KG and oxidized coenzyme in the ternary complex, together with fast-exchange temperature-dependent behavior of the α-KG relaxation, suggests that the binding in the ternary complex occurs via a slow isomerization step. This explanation depends critically on our assumption that the small α-KG relaxation observed in the presence of NADP originates from the ternary complex. It could, however, also result from remaining binary complex which might be present at high NADP concentrations if there existed negative cooperativity in the binding, as is reported by Dalziel & Egan (1972) for the ternary complex with NADP and glutarate. We could not obtain a satisfactory fit of our data with a model including negative cooperativity and complete elimination of the line broadening in the ternary complex. It was found by Zantema et al. (1977) for a spin-labeled NADP analogue that cooperative effects are much more pronounced in phosphate buffers than in Tris.

The binary dissociation constant for the binding of NAD to GluDH can be calculated directly from the other dissociation constants obtained from the NMR titrations. The result is 1.1 mM which compares well with the result obtained by Dalziel & Engel (1972) by equilibrium dialysis (0.47 mM) and the value calculated by Cross et al. (1972) from their experiments for NADP (2.2 mM).

Reduced Coenzyme. From the NMR data, obtained by titrating the GluDH–α-KG complex with NADPH, we calculated $K_d = 0.9$ μM for the binding of the coenzyme, which is in reasonable agreement with the results of di Franco & Iwatsubo (1972) who found 0.4 μM (in Tris buffer, but at lower temperature) and the value of 0.5 μM obtained by Cross (1972) in phosphate. The slow exchange rates that the first authors determined for the ternary complex agree with our observation that all NMR line broadening disappears.

The observation that the substrate line broadening has disappeared when one NADPH molecule is bound strongly suggests that all α-KG NMR line broadening originates from binding at one specific site.

4. *Influence of Effectors.* The NMR results show that the inhibitor GTP actually enhances the binding of coenzyme in the ternary complex. This is in agreement with other studies (Pantaloni & Lecuyer, 1973; Dalziel & Egan, 1972). The antagonistic behavior of ADP is also confirmed by other reports (Frieden, 1963). For the complex with oxidized coenzyme it is demonstrated that the exchange rate of α-KG is decreased in presence of GTP, which agrees with studies that showed GTP inhibition to result from slower release of ligands (di Franco, 1974).

It is interesting to note that in the presence of GTP an apparent half of sites effect is observed for NADPH binding. There are several other reports of either induced or preexisting asymmetry in the hexameric units. Evidence for such effects is obtained in binding (Koberstein et al., 1973; Krause et al., 1974), stopped-flow (di Franco, 1974), and chemical modification (Rasool et al., 1976) studies and it is usually found that GTP enhances such effects.

The results obtained by the NMR technique are in good agreement with other, mainly spectrophotometric studies. It is very likely that the spectrophotometrically observed complexes are directly related with those occurring in the catalytic mechanism (di Franco, 1974). This strongly suggests that the NMR results also reflect binding of substrate and coenzyme at their catalytic sites.

We have observed three cases where the NMR line broadening was (almost) an order of magnitude smaller than that of a similar ligand or that of the same ligand in a different situation. Because the temperature-dependent data indicate fast exchange, we propose that in these cases the relaxation is limited by slow exchange in a second (or higher) step in the binding process. On the basis of formula 4 the difference might also be explained as arising from a lower degree of immobilization of the ligand on the enzyme, or generally increased proton–proton distances. We consider this explanation as very unsatisfactory because the smaller line broadenings are observed when the interaction between ligand and enzyme is much stronger.

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Electron Spin Resonance and Nuclear Relaxation Studies on Spin-Labeled Glutamate Dehydrogenase†

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ABSTRACT: The reaction of glutamate dehydrogenase with two different stable nitroxides (spin labels) is reported. The two compounds contain a carbonyl and an iodoacetamide group as their reactive parts. The carbonyl compound inactivates the enzyme by the formation of a 1:1 covalent complex after $NaBH_4$ reduction of an intermediate Schiff's base. Evidence indicates that the enzyme is modified at lysine-126 in the active site. The electron spin resonance (ESR) spectrum of spin-labeled enzyme indicates a high degree of immobilization of the nitroxide. The binding of reduced coenzyme NADPH is reflected by a change (immobilization) of the ESR spectrum. Nuclear relaxation of bound substrate, oxidized coenzyme, and inhibitor by the paramagnetic group is observed. This shows the existence of a binding site for these compounds close to the active site. The distances of selected protons of the binding ligands to the nitroxide are calculated. The iodoacetamide spin label reacts with several groups, one of which is not a sulfhydryl. The reaction of this particular group causes inactivation of the enzyme. Protection against this inactivation could be achieved with certain ligands. Only enzyme that was spin labeled without such protection caused paramagnetic relaxation of bound substrate and coenzyme.

Glutamate dehydrogenase (GluDH)[1] catalyzes the reversible oxidation of L-glutamic acid by NAD(P) to α-ketoglutarate, NAD(P)H, and ammonia. The catalytic activity is affected by a large number of compounds of which the activator ADP and the inhibitor GTP are studied the most intensively. One important question concerning GluDH is the relation between the catalytic and regulatory sites. It is often assumed that the activity of enzymes can be controlled by allosteric effectors which do not bind close to the active center and exert their effect through a conformational change of the enzyme. Fisher & co-workers (1970) have pointed out that "allosteric" effects can be explained as well by direct steric interactions of substrate, coenzyme, and effectors with each other. This ligand exclusion theory was used as a basis to interpret many of the experimental results published for GluDH (Fisher, 1973). Chemical modification studies also lend some support to the hypothesis that catalytic and regulatory sites are close to each other (e.g., Goldin & Frieden, 1972).

We have undertaken magnetic resonance studies with the objective to elucidate these and other questions. The relevant complexes of the enzyme with substrates, coenzymes, and effectors can be studied by the NMR method as is shown in the

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[1] Abbreviations used: NMR, nuclear magnetic resonance; ESR, electron spin resonance; GluDH, glutamate dehydrogenase; α-KG, α-ketoglutarate; DTNB, 5,5-dithiobis(2-nitrobenzoic acid).

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