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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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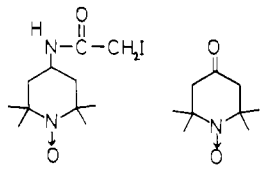

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FIGURE 1: (I) Ketone spin label. (II) Iodoacetamide spin label.

preceding paper (Andree, 1978a). This paper describes approaches to obtain information about the relations between the different sites by the introduction of paramagnetic spin-label probes in the enzyme.

Experimental Section

Bovine liver glutamate dehydrogenase was obtained as a suspension in ammonium sulfate from Boehringer (Mannheim). Purification and the determination of enzymatic concentrations and activities as well as the source of all chemicals are described in the preceding paper (Andree, 1978a).

Unless otherwise stated, all the experiments including the modifications were carried out in Tris-HCl buffer containing 0.05 M Tris, 0.1 M NaCl, and 0.1 mM EDTA at pH 7.4. In most of the NMR experiments 17–18% dioxane was added to the same buffer, but now containing 0.2 M NaCl. The dioxane was always distilled from KOH pellets immediately before use. In NMR experiments with α-KG, perdeuteriodioxane was used to make both methylene resonances detectable. When the NMR spectra of coenzymes or nucleotides were recorded, the EDTA concentration was raised from 0.1 to 0.5 mM in order to prevent line broadening effects from paramagnetic impurities.

NMR spectra were recorded on a Varian XL-100 spectrometer at a temperature between 15 and 20 °C in D_2O buffer. H_2O was exchanged for D_2O by dialyzing concentrated enzyme solution three times against a five- to tenfold volume of D_2O buffer. The determination of α-KG line broadenings is described in the preceding paper (Andree, 1978a). Line widths of GTP and NADP protons were measured at half height and corrected for field inhomogeneity effects by subtracting the width of the HDO line.

ESR spectra were recorded at room temperature on a Varian E-4 spectrometer equipped with a variable temperature unit in a Varian aqueous solution sample cell. The number of spins was determined by digitizing the recorded spectrum and double integration using a CDC Cyber 74-16 computer. A procedure was developed to correct for appreciable baseline shifts often present after the first integration (Andree, 1975). As an absolute reference the high-field resonance of free spin-label solution was used. The concentration of this solution was calculated by weight. The reference spectrum was recorded after exchanging the solution of labeled enzyme for free label employing a syringe and without removing the sample cell from the cavity. In this way quite reproducible values for the numbers of spin labels incorporated in the protein were obtained. The results compared well with calculations of these numbers from optical spectra.

N-(1-Oxyl-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide was prepared as described by McConnell et al. (1969). 1-Oxyl-2,2,6,6-tetramethyl-4-oxypiperidine was synthesized by oxidation as described by Rozantsev (1970) of 2,2,6,6-tetramethyl-4-oxypiperidine (triacetonamine) prepared according to Weiner (1969). The melting points for the two compounds were 117–120 °C (lit. 118–121 °C) and 35–38 °C (lit. 38 °C). These two compounds will be referred to as iodoacetaminde spin label and ketone spin label (see Figure 1).

Modification with the iodoacetamide spin label was performed by dissolving 15 mM spin label in a 10 mg/mL protein solution. The reaction was allowed to proceed for several hours at room temperature and subsequently the labeled enzyme was purified by thorough dialysis. In the case of the time-dependent labeling experiments, the purification was achieved by Sephadex-G 25 column chromatography. The enzyme used in the reported NMR experiments had reacted for 14 h at pH 8 with and without the addition of 3 mM NADH and 16 mM α-KG in the reaction mixture. The unprotected enzyme (without additions) contained 2.5 spin label molecules per protomer and was 18% active. The protected enzyme had reacted with 0.92 label molecules per protomer and was 100% active.

The reaction with the ketone spin label was performed in 0.15 M sodium phosphate buffer (pH 8). A solution was prepared which contained 24 mg/mL spin label and 15 mg/mL protein. After 30 min the solution was cooled in an ice bath and $NaBH_4$ was added in a slight molar excess over the spin label under continuous stirring. Care was taken to keep the pH of the solution below 8.5. Subsequently the reaction mixture was allowed to reach room temperature and after some hours it was transferred to a dialysis bag and the reagents were removed by exhaustive dialysis against Tris buffer at 4 °C. The enzyme used in the NMR experiments contained as an average 1.0 spin label per protomer (from the ESR spectrum) and was 10% active.

The nitroxide group of the enzyme labeled with this ketone analogue was reduced by addition of a twofold molar excess of ascorbic acid to the enzyme followed by one dialysis started after 30 min of reaction. The enzyme used in these experiments was about 20% active before and after the reduction while the number of paramagnetic groups per protomer dropped from 0.95 to 0.05.

Pyridoxal 5′-phosphate was irreversibly coupled to the enzyme as described by Brown et al. (1973), but using 0.15 M sodium phosphate buffer and employing dialysis for the purification. Modification of the essential lysine residue was 80–85% complete as deduced from the remaining activity.

The number of free SH groups of native and modified GluDH was determined by a slight modification of the procedure described by Rosen et al. (1973). A small amount of enzyme solution was mixed with 2 mL 0.25% sodium dodecyl sulfate and 0.2 mL of 0.01 M 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) in standard Tris buffer at pH 8. The final enzyme concentration was 0.15 mg/mL. The number of free SH groups was calculated from the measured extinction at 412 nm, using an extinction coefficient of 1.36×10^{-4} cm^{-1} M^{-1}. Regeneration experiments were performed by incubating the enzyme at room temperature and pH 7.4 with 0.08 M dithiothreitol. The regenerated activity was determined after a constant value was reached (this took usually some hours).

Sedimentation velocity experiments were carried out at 20 °C in a Beckman Spinco E analytical ultracentrifuge equipped with Schlieren optics at 50 740 or 59 789 rev/min. Sedimentation coefficients were determined from photographs obtained at different time intervals by the use of a Nikon Model 6C optical comparator.

Results

1. *Modification with the Ketone Spin Label.* After addition of the ketone spin label to the enzyme no reversible inhibition

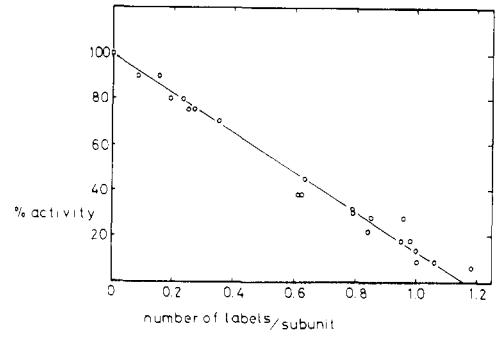

FIGURE 2: The residual activity of GluDH dependent on the number of ketone spin-label molecules incorporated per enzyme protomer.

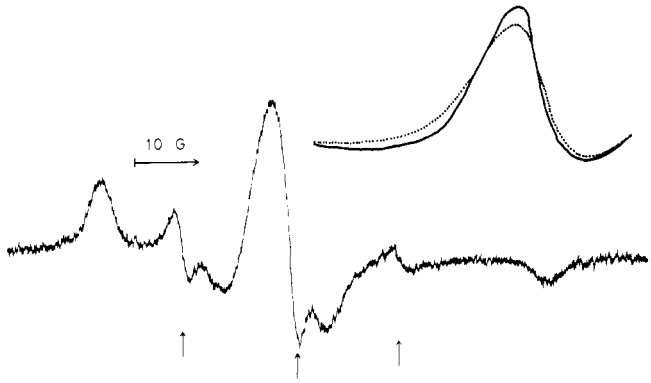

FIGURE 3: The ESR spectrum of GluDH after reaction with the ketone spin label. Conditions: pH 7.4 in 0.05 M Tris/HCl buffer with 0.1 M NaCl; room temperature. The insert shows a comparison of the low-field peak before (—) and after (• • •) the addition of 3 mM NADPH.

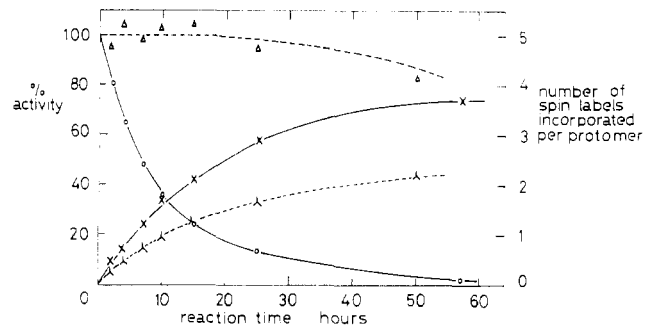

FIGURE 4: The reaction of GluDH with the iodoacetamide spin label. Plotted are the activity (O and Δ), and the number of spin labels incorporated per protomer (X and λ). The modification was carried out with (- - -) and without (—) the presence of 16 mM α-KG and 2.5 mM NADH.

was observed, but irreversible inhibition could be achieved by addition of $NaBH_4$. Figure 2 shows how the activity of the enzyme changes with the amount of spin label incorporated in the enzyme. It appears that (almost) complete activity loss results from the reaction of one amino acid residue.

The ESR spectrum of spin-labeled GluDH (Figure 3) shows a signal from strongly immobilized nitroxide radicals with an outer peak separation of 69 G. The peaks indicated by the arrows represent rather mobile label molecules. By double integration we found that they only amount to 5–10% of the total intensity. We ascribe this mobile signal to aspecific labeling which might also account for the small excess of label incorporated in fully inactivated enzyme.

The broadening of the outer lines is partly inhomogeneous and increases when the temperature is decreased or glycerol is added to the solution to increase the viscosity. The rotational correlation time was obtained from temperature dependent spectra (Mason & Freed, 1974), and from a comparison of the outer peak distance with the maximum distance at infinite immobilization by extrapolation to infinite glycerol concentrations (Goldman et al., 1972). Both methods give an estimate of 1.5×10^{-7} s for τ_R at room temperature. However, because of the low mobility the estimates obtained are rather inaccurate. We consider 5×10^{-8} s as a reasonable lower limit value for τ_R.

We found that addition of NADPH causes an extra broadening of the ESR spectrum as is shown in the insert of Figure 4 for the low-field peak. This extra broadening most likely represents a further immobilization.

2. *Modification with the Iodoacetamide Spin Label.* The inactivation of GluDH with a spin-labeled iodoacetamide analogue (II) was reported earlier by us (Andree & Zantema, 1974). It was found that the ESR spectrum of the spin-labeled enzyme shows a superposition of spectra of immobilized (outer peak distance 65 G) and a larger amount of weakly immobilized labels. The labeling caused inactivation of the enzyme which could be prevented by adding protecting compounds during the reaction. The spectrum component for the immobilized label was nearly absent in protected spin-labeled enzyme. New studies showed that NADH together with the substrate α-KG or the inhibitor GTP were protecting while, if present alone, none of these compounds protected against activity loss and the appearance of an immobilized nitroxide spectrum. The concentrations in these experiments were saturating according to Fisher (1973). The results of quantitative experiments are presented in Figure 4. The data suggest that modification of one residue, accessible only in the absence of protecting compounds, is responsible for the loss of enzymatic activity. Estimates of the amount of strongly immobilized nitroxide, obtained by comparing the height of the low-field resonance with the same peak for ketone spin-labeled enzyme, confirm that the amount of strongly immobilized nitroxide is about one per protomer of completely inactivated enzyme.

Since Rosen et al. (1973) have found that all 6 SH groups of GluDH could react with 4-iodoacetamidosalicylic acid, we determined the number of free SH groups as described by these authors. The number of free sulfhydryls in native enzyme was 6.0 and after reaction with the spin label at room temperature for 14 h it dropped to 5.3, independent of the presence of protecting ligands.

Rosen et al. (1973) further demonstrated that, when the reaction was carried out at low pH, an essential methionine could react faster than lysine while at high pH a lysine was the main modified essential residue. When the activity loss resulted from methionine modification, regeneration of the activity could be achieved by addition of dithiothreitol. We obtained similar results for the reaction with the spin label. Enzyme was allowed to react at pH 8 for 14 h and after addition of dithiothreitol 15% of the activity loss was generated. The activity of enzyme incubated for 14 h at pH 6 was regenerated for 65%.

3. *NMR Experiments.* To avoid uncertainties due to enzyme association we added 17.5% of dioxane to the solutions. Both spin-labeled and native enzyme in a mixture of H_2O buffer with 10% dioxane showed one single sharp peak in the ultracentrifugation pattern, with an s value of 10.3 S (uncorrected for D_2O viscosity and density) which corresponds to the single hexamer in agreement with the results published by Churchich & Wold (1963). The enzyme was active under the conditions used.

NMR Study of α-Ketoglutarate Binding. The line width

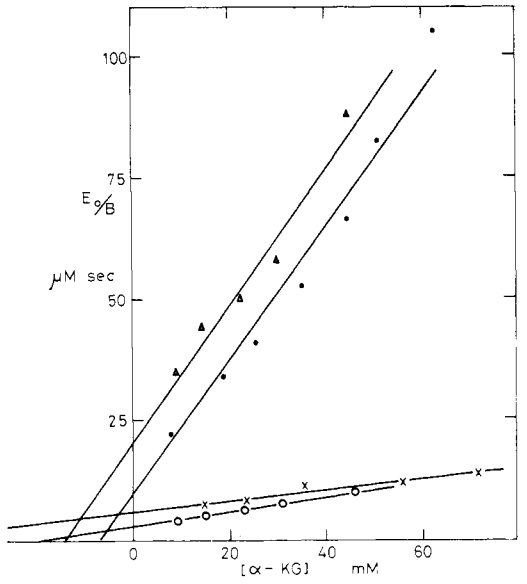

FIGURE 5: The reciprocal line broadening of α-KG dependent on its concentration. Generally, the two methylene resonances had the same width. A slight difference observed with the ketone spin-labeled enzyme was averaged. Buffer conditions: 82.5% 0.05 M Tris-HCl (pH 7.4) with 0.2 M NaCl and 17.5% deuteriodioxane. (•) Native enzyme, (Δ) iodoacetamide spin-labeled enzyme with protection (0.92 label per protomer, 100% active). (O) Iodoacetamide spin-labeled enzyme without protection (2.5 labels per protomer, 18% active). (X) Ketone spin-labeled enzyme (1.18 labels per protomer, 7% active). The enzyme concentration was about 2 mg/mL in all experiments (18 °C).

TABLE I: Results of NMR Study of α-KG Binding to Native and Modified GluDH at 17–18 °C.

Enzyme modification	K_d (mM)	$\tau_b + T_{2b}$ (ms)
Native	7 ± 5	0.46 ± 0.10
Protected IAL[a]	14 ± 5	0.45 ± 0.11
Unprotected IAL	18 ± 7	0.050 ± 0.008
KL	57 ± 25	0.035 ± 0.014
Pyr 5′-phosphate	10 ± 5	1.14 ± 0.20

[a] IAL = iodoacetamide spin label. KL = ketone spin label.

of the two methylene resonances in the NMR spectrum of α-KG is strongly increased after addition of native or spin-labeled GluDH. Figure 5 gives the results of NMR line broadening measurements in the presence of native enzyme and the two spin-labeled enzymes. The data are plotted according to

$$E_0/B = \pi(K_d + [\alpha\text{-KG}])(\tau_b + T_{2b}) \quad (1)$$

where E_0 is the total enzyme concentration, B the observed broadening, K_d the dissociation constant for the observed ligand, and T_{2b} and τ_b are the relaxation time and the average lifetime of the ligand in the bound state, respectively. A decrease in T_{2b} (enhanced relaxation) can result from interaction with a paramagnetic group as is observed for both the spin-labeled and inactivated enzymes. No paramagnetic relaxation is observed for the active enzyme which was modified with the iodoacetamide spin label in the presence of protecting ligands. The inactivation obviously decreases the affinity of the enzyme for the substrate. The values for K_d and $\tau_b + T_{2b}$ obtained by regression analysis are summarized in Table I.

Enzyme labeled with pyridoxal 5′-phosphate also caused broadening of the α-KG NMR spectrum. Some preliminary experiments were carried out to study the effect of coenzyme on the binary complex of α-KG with several modified enzymes.

TABLE II: Effects of Coenzymes on the α-KG NMR Line Broadening in the Presence of Ketone Spin-Labeled and Pyridoxal 5′-Phosphate Modified Enzyme at 17–18 °C.

Enzyme	[GluDH] (mg/mL)	[α-KG] (mM)	Additions	Line broadening (Hz)
Ketone spin labeled	2.0	30		5.1[a,c]
	2.0	30	1 mM NADPH	0.5[a,c]
	2.3	25		3.0[b]
	2.3	25	1.2 mM NAD	1.6[b]
	2.3	25	1.2 mM NAD	
			1 mM GTP	1.7[b]
Pyridoxal phosphate	2.1	22		1.2[b]
Modified (20% active)	2.1	22	1 mM NADH	0.0[b]

[a] In buffer with 11% dioxane. [b] No dioxane added. [c] Temperature was 15 °C.

TABLE III: NMR Results for the Binding of NADP and GTP to Native and Spin-Labeled GluDH.

Enzyme	Proton obsd	K_d (mM)	$T_{2b} + \tau_b$ (ms)
Native	NADP C2-H	0 ± 2	2.40
	NADP C8-H	0 ± 2	2.24
	GTP C8-H	2.5 ± 2	1.09
Ketone spin labeled	NADP C2-H	1.5 ± 2	1.02
	NADP C8-H	1.5 ± 2	0.87
	GTP C8-H	4.5 ± 2	0.42

In general, a decrease or disappearance of the line broadening was observed on addition of coenzyme (see Table II). This effect is also observed with native enzyme and reflects the formation of a ternary complex (see Andree, 1978a).

Binding of NADP and GTP to Ketone Spin-Labeled Enzyme. The NMR line broadenings of the adenine ring C-2 and C-8 protons of NADP in the presence of native and spin-labeled enzyme were measured. A concentration-dependent plot similar to Figure 5 shows that both lines are additionally broadened after the spin labeling, indicating that NADP binds close to the spin label. The plot gives no indication of a decrease in the affinity. Nuclear relaxation enhancement due to the spin label is observed for GTP as well, but the disssociation constant is increased after the labeling. The values obtained for the dissociation constant K_d and the sum $T_{2b} + \tau_b$ are summarized in Table III.

To rule out the possibility that the observed changes are due to a conformational change caused by the bulky spin label or some other, not paramagnetic, effect, we reduced the paramagnetic group with ascorbic acid. This caused a large decrease in the line broadenings which became comparable with those measured for native enzyme. Thus, the relaxation enhancement after spin labeling must be ascribed to the interaction of the observed protons with the paramagnetic group.

Temperature Dependence. From the concentration-dependent plots, the quantity $T_{2b} + \tau_b$ for the bound ligand is obtained and temperature dependent measurements can show whether T_{2b} or τ_b is the largest term in the sum (Dwek, 1973). In all experiments the broadening was measured as a function of the temperature at the highest ligand concentration used.

The results (Andree, 1975) show that for active enzyme the fast exchange condition, where T_{2b} is dominant, holds for all complexes studied. Fast exchange was also observed for the complex of ketone spin-labeled enzyme with GTP. The only exceptions are the complexes of spin-labeled, inactivated enzyme with NADP and α-KG where no change of the line broadenings with temperature was observed at all. Probably τ_b begins to contribute to the sum because T_{2b} is lowered so much by the paramagnetic effect. Because of this possibility the obtained values for $T_{2b} + \tau_b$ have to be considered as maximum possible values for T_{2b} with the paramagnetic enzyme.

Distance Calculations. The contribution of a paramagnetic interaction to the relaxation rate is given by the formula

$$\frac{1}{T_{2p}} = \frac{1}{15}\frac{\gamma_I^2 g^2 \mu_B^2}{r^6}\left(4\tau_c + \frac{3\tau_c}{1+\omega_I^2\tau_c^2}\right) \quad (2)$$

in an approximation suitable for our case (Dwek, 1973). γ_I is the gyromagnetic ratio for the nucleus, g the electron Lande factor, ω_I the nuclear Larmor frequency, μ_B^2 the Bohr magneton, and τ_c the characteristic time with which the proton–spin-label interaction is fluctuating. This correlation time has contributions from three processes: the rotational tumbling of the complex in solution, the electron spin relaxation, and the dissociation of the complex. In formula this is given by:

$$1/\tau_c = 1/\tau_R + 1/\tau_s + 1/\tau_b \quad (3)$$

where τ_R, τ_s, and τ_b are the characteristic times for the three processes mentioned, respectively. A reasonable estimate for τ_R is obtained from the ESR spectrum (1.5×10^{-7} s). In general, τ_b is too high to contribute to τ_c as also indicated by the temperature dependent results. In the approximation used τ_s equals $T_{1,e}$ (Reuben et al., 1970) which has been reported to be in the order of 10^{-5} s and to depend only weakly on the mobility (Hyde et al., 1975). Therefore, τ_s will not contribute either and the distances can be calculated from eq 2 using τ_R obtained from the ESR spectrum. The results are 15.5 and 14.8 Å for the adenine C-2 and C-8 protons of NADP, respectively, 12.8 Å for the C-8 proton of GTP, and 7.8 Å for the methylene protons of α-KG.

Discussion

Distance Calculations. There are several uncertainties in the calculated distances. In the first place, the T-1 values of the bound ligands appear to be determined by other effects than usually are considered (Andree, 1978b). Therefore, only the ESR studies could be used to obtain an estimate for the correlation time. The result (1.5×10^{-7} s) is very close to the estimate of 1.4×10^{-7} s obtained from the Stokes–Einstein relationship. Further, the (remote) possibility exists that τ_s or τ_b contributes to τ_c. For these reasons, the value we have used cannot be significantly too high. τ_R was determined under conditions where the enzyme was more strongly associated than in the NMR experiments, but this is probably not of much importance because the ESR spectrum did not change after the addition of 15% dioxane, causing complete dissociation. The values substituted for T_{2p} might be too high because the relaxation with the paramagnetic enzyme could be partially exchange limited. However, all these possible sources of error work in the same direction. Therefore, the calculated distances, if not correct, can at least be considered as upper limit values. The conclusion that the studied ligands all bind close to the spin label cannot be effected.

The Site of Labeling. A variety of aldehydes are able to inactivate GluDH by formation of a Schiff's base (Anderson et al., 1966). The inactivation can be made irreversible by reducing the Schiff's base with $NaBH_4$. It has been shown that pyridoxal 5′-phosphate is specifically linked to Lys-126 in the amino acid sequence (Piszkiewicz et al., 1970; Moon et al., 1972; Moon & Smith, 1973). With the ketone spin label irreversible inhibition was only obtained after addition of $NaBH_4$, suggesting the formation of a Schiff's base. In view of the similarity with other carbonyl compounds we postulate that the ketone spin label also is linked to Lys-126 in the active center. The similarity in the NMR results for pyridoxal 5′-phosphate and spin label modified enzyme lends further support to the assumption that the same amino acid residue has been labeled in both cases.

It was found that the enzyme modified with the iodoacetamide spin label was inactivated and caused paramagnetic relaxation of bound substrates only if protecting ligands were absent during the reaction. Earlier evidence led us to propose that the particular group that could be protected was Lys-126 (Andree & Zantema, 1974). This is now further supported by the sulfhydryl determinations which confirm that this particular group is not cysteine. That it is a lysine is in accordance with the results reported by Rosen et al. (1973) for 4-iodoacetamidosalicylic acid and our dithiothreitol regeneration experiments.

In conclusion it can be said that all evidence supports (although not strictly proves) that one essential amino residue, lysine-126, is the main target of the two different spin labels. The label attached at this position causes paramagnetic relaxation of the bound substrate. The modification does not completely prevent the binding of α-KG but the binding is considerably weaker in particular for the ketone spin label.

Comparison of Native and Ketone Spin-Labeled Enzyme. It was established in the preceding paper (Andree, 1978a) that the NMR spectra of substrate α-KG and oxidized and reduced coenzyme are broadened by the enzyme. These broadenings decrease upon formation of the ternary complex with α-KG and coenzyme. The observed effects very likely originate from binding of the ligands at their catalytic sites. With the ketone spin-labeled enzyme only very weak binding was observed. Nevertheless, it is unlikely that the observed binding represents aspecific effects because of the strong paramagnetic relaxation by the spin label and the fact that other compounds than the ones studied in general did not show any NMR line broadening. More important, the characteristic decrease in the α-KG line width on coenzyme addition (NADPH and NAD were tested) is still observed showing the formation of a ternary complex. The formation of binary complexes with coenzyme is shown by the NMR studies with NADP and the effect of NADPH on the ESR spectrum of the enzyme.

Although studied in less detail, the pyridoxal 5′-phosphate modified enzyme apparently also binds α-KG and forms a ternary complex, at least with NADH. This is in contrast with the studies reported by Brown et al. (1973) who conclude that the modified enzyme is inactive because of the inability to bind α-KG. A possible explanation of the discrepancy is that the binding could be considerably weaker which might have prevented the detection of the binding by optical methods. It is also possible that, although binding occurs, the characteristic optical effects are absent or much smaller. This would correlate well with the general idea (see Andree, 1978a) that NMR is sensitive to more initial steps in the complex formation than optical techniques. The inability of the complex to assume a conformation that is detected by optical methods might also explain why the modified enzyme is inactive, although it can

bind substrate and coenzyme. Of course, another straightforward explanation for the inactivation would be that the modified lysine is involved chemically in the catalytic reaction.

Ligand Exclusion Theory. Fisher (1973) has proposed that the inhibitory effect of GTP results from direct steric interactions at the active site. Other theories of enzyme regulation, however, imply that the inhibition is due to a conformational change, triggered by binding at a distant site. We observed binding sites of GTP and NADP close to the spin label. For NADP our NMR data are in good agreement with published work (see Andree, 1978a). This suggests that binding at the active site is observed and this in turn is consistent with the fact that the spin label probably reacted with Lys-126 in the active site. Then, GTP must also bind close to the active site. However, the dissociation constant is in the millimolar region while the binding of GTP to the inhibitory site is much stronger (18.6 μM) as reported by Colman & Frieden (1966). Therefore, it seems likely that the site observed by NMR is not the same site. Indeed, an additional kinetically inhibitory site at high GTP concentrations has been detected by Koberstein et al. (1977) who also suggest that these weaker sites for the nucleotide effectors (including ADP) are the ones that are detected by NMR. Therefore, our original question concerning the relation of the strong GTP site and the active site must remain unanswered.

In the course of our work on GluDH some indications for the occurrence of conformational changes were found. First, in order to explain the NMR data on the binary and ternary complexes with oxidized substrate and coenzyme we assumed a slow step in the binding process for the ternary complex. This surely can be defined as a conformational change, but it is not proven that indeed atoms of the enzyme itself are involved. However, some change in the conformation of the enzyme is required to explain the change in ESR spectrum if NADPH is added to the ketone spin-labeled enzyme. Such changes would not agree with the exclusion theory as far as it excludes specific changes in the enzyme.

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