MAGNETIC RESONANCE STUDIES ON GLUTAMATE DEHYDROGENASE

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1. Introduction

Among the class of pyridine nucleotide dependent dehydrogenases glutamate dehydrogenase (GluDH) is a very interesting one because of its complex allosteric behaviour. Important questions concerning the binding of substrates and effectors and the relationships between catalytic and regulatory sites have not yet been clarified.

Optical techniques have been widely used to study the interaction of the enzyme with its ligands [1–5]. Enzyme modification is used to clarify the role of specific amino acid residues in the enzymatic function [6–8].

In this paper we report the use of magnetic resonance techniques to get both dynamical and structural information. The broadening of the NMR spectrum of a ligand caused by the enzyme turns out to be a useful parameter to study the interaction of this ligand with this enzyme in binary and higher complexes. It was found possible to attach a spin label to a group in the protein that is necessary for the activity and to study the interaction of this paramagnetic label with bound α-ketoglutarate (α-KG).

2. Materials and methods

Glutamate dehydrogenase was obtained as ammonium sulphate suspension from Boehringer (Mannheim). Before use the enzyme was dialyzed against a solution containing 0.05 M EDTA at pH 8 and subsequently dialyzed exhaustively against a buffer containing 0.05 M Tris and 0.1 M NaCl at pH 7.4. The same buffer was used in all further experiments.

Enzyme concentrations were calculated from the extinction at 280 nm, using an extinction coefficient of 0.93 cm^{-1} ml mg^{-1} [9]. To calculate molar concentrations a subunit molecular weight of 56 000 was assumed [10]. Enzyme activity was measured by following the oxidation of NADH by α-KG and ammonia at pH 8. Coenzymes, nucleotides and α-KG were obtained from Sigma and other chemicals from Merck with the highest purity available.

N-(1-Oxyl-2,2,6,6-tetramethyl-4-piperidinyl) iodoacetamide was prepared as described by McConnell [11]. ESR spectra were recorded on a Varian E-4 spectrometer in an aqueous solution sample cell.

NMR spectra were recorded on a Varian XL-100 spectrometer at a temperature between 15 and 20°C in D_2O buffer at pH 7.4 (meter reading). H_2O was exchanged for D_2O by dialyzing the enzyme solution three times against a 5- to 10-fold volume of the D_2O buffer. The linewidth of the α-KG spectra was determined by comparison with a series of spectra with different Lorentzian width, simulated with a Varian 620 f computer. The line broadening caused by the enzyme was calculated from this width by applying two corrections:

a) The width at half height of the HDO peak was subtracted as a correction for the field inhomogeneity.
b) A value of 0.1 Hz was subtracted for the line width of free α-KG.

3. Results and discussion

3.1. *Spin labeling experiments*

In fig. 1 the two types of ESR spectra are presented

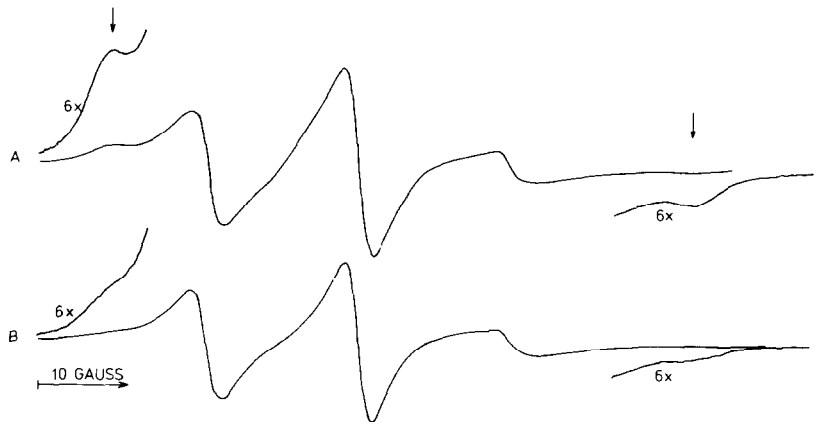

Fig. 1. ESR spectra of spin labeled GluDH. Conditions: Protein 13 mg/ml. Buffer 0.05 M Tris–0.1 M NaCl pH 7.4. The labeling reaction was carried out without additions (A) and in the presence of 3.1 mM NADH and 1.8 mM GTP (B). The arrows indicate the positions of the outer peaks of the immobilized spectrum. The spectra were recorded using a power of 20 mW and a modulation amplitude of 0.8 G.

that could be obtained for protein bound spin label. The upper spectrum was recorded after incubating 13 mg/ml enzyme with buffer solution 10 mM in spin label at room temperature for 10 hr, followed by dialysis at 4°C to remove the free label. The presence in the reaction mixture of millimolar concentrations NADH did not change this result, but when the coenzyme was combined with GTP or α-KG the lower spectrum resulted. The upper type spectrum can be explained as a superposition of signals from spin label molecules with different mobilities [11]. The distance between the outer peaks in this spectrum is 65 G, indicating almost complete immobilization. This strongly immobilized signal is only for a small fraction present in the second type of spectrum. While normally the activity decreased during the incubation with the spin label, no such activity loss was observed when the reaction was carried out in the presence of NADH together with α-KG or GTP. No activity loss was observed when the enzyme was stored for a few days at room temperature without adding spin label.

These experiments give evidence for the presence of at least one spin label reactive group in the protein that is necessary for the full activity and can be protected against reaction with the spin label by coenzyme together with substrate or inhibitor. It is possible that the protection is not due to simple steric exclusion of the reactive group by the bound ligands, but to a conformational change that has been suggested [12]. An ESR spectrum with a low amount of immobilized signal was also obtained by modifying the enzyme with pyridoxal-5′-phosphate using a slight modification of the method described by Goldin [8], before performing the reaction with spin label. The modified enzyme was obtained by incubating 8 mg/ml enzyme at room temperature for 30 min in Tris buffer at pH 7.4 with 5 mM pyridoxal-5′-phosphate, followed by reduction with $NaBH_4$ and dialysis at 4°C. Since this reagent reacts specifically [7] with Lys 126 in the sequence as determined by Moon et al. [13] this provides an indication that this particular group is this specific lysine.

3.2. *NMR on binary complexes*

The existence of binary complexes of glutamate dehydrogenase with substrate α-KG and coenzyme NADH is demonstrated by the broadening of the lines in the NMR spectrum of these substances when enzyme is added to a solution of one of them. Under conditions similar to those used for α-KG, L-glutamic acid does not show a broadening, indicating no or a much weaker binding.

In fig. 2 some typical NMR spectra are shown. The interpretation of NMR line broadenings has been discussed at many places in the literature [14–16] and we shall only mention the final results here.

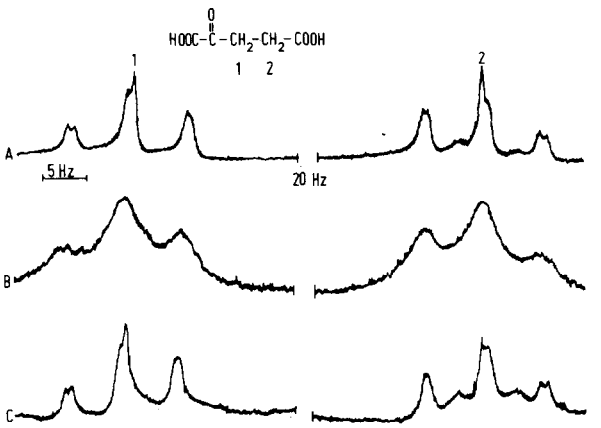

Fig. 2. NMR spectra of α-keto glutarate. Buffer 0.05 M Tris–0.1 M NaCl in D_2O. pH = 7.4 (meter reading). (A) 30 mM α-KG, (B) 30 mM α-KG + 2 mg/ml GluDH, (C) 50 mM α-KG + 6 mg/ml GluDH + 4 mM NADH. The small central lines in spectrum C are due ro partial deuteration of the low field methylene group.

Table 1
Some results of NMR studies of the binding of α-ketoglutarate (α-KG) by glutamate dehydrogenase

[Enzyme] (mg/ml)	Condition	[α-KG] (mM)	NMR line broadening (Hz)	τ_b+T_{2b} (msec)
1.0	Native enzyme	*	*	0.14
1.0	Spin labeled enzyme	*	*	0.05
1.7	Protected spin labeled enzyme	*	*	0.13
6.0	Native enzyme + 4 mM NADH	50	0.0	> 20
2.0	Native enzyme + 2 mM NAD^+	30	0.7	0.5
1.8	Native enzyme + 0.03 mM NAD^+ +1 mM GTP	23	0.0	> 10

* Value for τ_b+T_{2b} obtained from a series of measurements with varying α-KG concentrations.

If the concentration of the monitored species is high compared to its dissociation constant, the sum $\tau_b + T_{2b}$ is directly proportional to the reciprocal line-broadening and can be calculated from it. In this sum T_{2b} is the transverse magnetic relaxation time of the bound species and τ_b is its mean residence time on the enzyme. When the linewidth decreases with higher temperature T_{2b} is the dominant term in this sum, while the reverse effect means that τ_b is large compared to T_{2b}.

The results of all the measurements including those on ternary and higher complexes are summarized in table 1. Line broadenings of both methylene groups appeared to be the same in all cases.

It is clear that the relaxation of bound α-KG by the protein is enhanced by putting the spin labels on it. However, protein that was spin labeled under protecting conditions did not show this enhancement effect. Tmeperature dependent measurements indicated that the linewidth was determined by T_{2b} in these cases. So this extra effect can be ascribed to the interaction of the substrate molecule with the paramagnetic label on the special group that can be protected against reaction. This means that this group is in the neighbourhood of the α-KG binding site. A rough calculation using some parameters found for other immobilized spin labels [17] yields 5 to 8 Å for the distance between the α-KG protons and the nitroxide moiety.

Work is in progress now to define the labeling sites better and more quantitatively. Furthermore the binding of coenzymes to the spin labeled enzyme will be investigated.

3.3. *NMR on ternary and quaternary complexes*

As is shwon in fig. 2 addition of NADH to the α-KG-GluDH system results in the reappearance of the unbound α-KG spectrum. We explain this effect by a very strong binding of α-KG in the ternary complex. This stronger binding is reflected by an increase of τ_b; now τ_b is no longer small compared to T_{2b} and hence the linewidth will decrease because of an increase of $\tau_b + T_{2b}$. It is interesting to note that also the line broadening of the NADH NMR spectrum disappears after addition of a small quantity of α-KG. Addition of NAD^+ also reduces the α-KG broadening but not to an unobservable value (see table 1). This indicates that the substrate dissociates faster from the complex with coenzyme when the latter is in the oxidized form.

The interactions of inhibitors and activators with the complexes can be studied also by NMR. We found that GTP alone had no observable effect on the α-KG

broadening. In the presence of 1 mM GTP, however the line narrowing caused by NAD^+ became visible at much lower NAD^+ concentrations and contrary to the results obtained without GTP, the narrowing was now complete. Addition of ADP reverses the GTP effect. These observations suggest as a possible explanation for the inhibitory effect of GTP the much tighter binding in the complex E–NAD^+-α-KG when GTP is present. Work is in progress now in order to give a more quantitative description of the processes that occur upon formation of the higher complexes.

4. Conclusion

Both structural and dynamic information GluDH can be obtained by magnetic resonance techniques. The first qualitative findings are:

1) The possibility of putting a spin label on a group that is in the vicinity of the α-KG binding site but does not prevent this binding.

2) There is a very drastic influence of coenzyme and substrate on each other during binding. The residence times on the enzyme of each of these ligands are increased by one or more orders of magnitude when the other is bound simultaneously.

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