BBA 61217

Mobility of the paramagnetic label bound by aspartate aminotransferase

According to KOSHLAND's theory, the formation of an enzyme–substrate complex involves transconformation of the enzyme both at and outside the active site[1,2]. During the last few years, electron paramagnetic resonance (EPR) of the stable iminoxyl radicals bound by proteins has been successfully used in the investigation of these conformational changes. The EPR spectrum affords information concerning the mobility of radicals.

In this work aspartate aminotransferase (E.C.2.6.1.1) was studied. Contemporary ideas concerning the transamination mechanism suggest that conformational changes occur in the active site of aspartate aminotransferase during the formation of its complexes with substrates[3].

It has already been shown that the EPR spectrum of the iminoxyl radical covalently bound by the protein molecule allows the local conformational changes of the protein in the neighbourhood of the paramagnetic label to be studied[4–6]. We have introduced radicals outside the active site of aspartate aminotransferase.

Highly purified cytoplasmic aspartate aminotransferase was obtained from pig heart by the method described in a previous paper[7]. The labeling radicals used were substances which alkylate the thiol groups: iodacetamide- (2,2,6,6-tetramethylpiperidine-1-*N*-oxyl) and maleimide- (2,2,6,6-tetramethylpiperidine-1-*N*-oxyl), designated as Ac-R· and M-R·, respectively.

The enzyme solution (15–20 mg/ml) was incubated at 2–4° in 0.05 M acetate buffer (pH 5.5) with excess Ac-R· or M-R· for 5 h and 20 h, respectively. The nonreacted radicals were removed using a Sephadex G-50 column. The number of radicals covalently bound by the transaminase molecule was estimated by comparing the EPR signal intensity of the labeled protein solution with that of a standard solution, both frozen at 77°K. The standard solution contained $5 \cdot 10^{-4}$ M of the iminoxyl radical in a water–ethylene glycol mixture. In a series of experiments, the number of free SH-groups before and after binding of a radical was determined by means of parallel titration by the method of BOYER[8]. The results of determinations of the number of blocked SH-groups and of SH-groups remaining free were in good agreement. The experiments were performed with transferase containing 1–2 equivalents of M-R·, or 4–5 equivalents of M-R·, per dimer (mol. wt. 90 000). The labeled enzyme maintained no less than 85–90% of its original activity, showing that the label is bound by thiol groups which are not important for enzymatic activity. The absorption spectra and circular dichroism spectra of the labeled enzyme in the region 280–500 nm do not differ from those of the native enzyme at pH 5.5 and pH 8.0. A sample (0.01 ml) was put into a polyethylene capillary with a diameter of 0.5 mm, and the EPR spectra were obtained using the apparatus X-band EPR-spectrometer. The spectrum was measured using the following parameters: a modulation amplitude of 1 gauss with a time constant of 0.5 sec and a 4-min scanning time of the magnetic field. The measurements were made at room temperature.

Figs. 1 and 2 shows the EPR spectra of the radicals covalently bound to the thiol groups of aspartate aminotransferase, which contained pyridoxal 5'-phosphate as coenzyme, and also the spectra of the free radical in water and in a water–ethylene

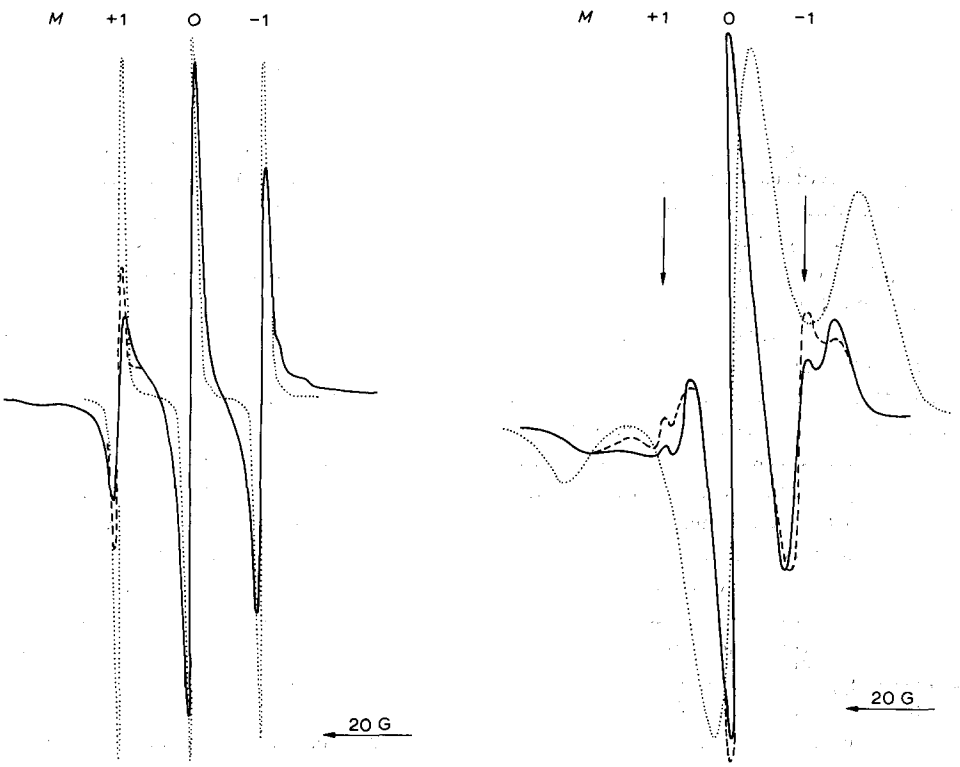

Fig. 1. EPR Spectra* of Ac-R· covalently bound by the pyridoxal 5′-phosphate form of aspartate aminotransferase (———); with holoaspartate aminotransferase in the presence of ligands (— — —). EPR spectrum of the iminoxyl radical ($5 \cdot 10^{-4}$ M; 0.01 ml) in water at room temperature (· · · ·). Protein concentration was 20 mg/ml.

Fig. 2. EPR spectra* of M-R· covalently bound by the pyridoxal 5′-phosphate form of aspartate aminotransferase, (———); with holoaspartate aminotransferase in the presence of ligands (— — —), EPR spectrum of the iminoxyl radical ($5 \cdot 10^{-4}$ M; 0.1 ml) in a water–ethylene glycol mixture at 77° K (· · · · ·). Protein concentration was 30 mg/ml.

glycol mixture at 77°K. The forms of the lines are similar to those previously established[4–6,9]. According to the theory suggested by McCONNELL[10] and KIVELSON[11], the symmetrical form of the line, containing three components with equal intensity for $M = 0, \pm 1$ (M is the nitrogen nuclear spin quantum number) observed for the free rotating radical in water, is disturbed, and this disturbance must increase with correlation time τ. Both radicals bound by aspartate aminotransferase are noticeably immobilized; this is shown by changes in the intensities of the components and by their broadening. M-R· is immobilized to a greater extent than Ac-R·. This fact can be explained by the difference in structure of the alkylating groups[12,13].

The mobility of the radical bound by protein may depend on the conformational changes of the protein moiety induced by nonspecific effects, of pH or temperature.

* The spectra of radicals bound by proteins were measured under identical conditions. Small variations in amplitude were eliminated by approximating the intensities of all spectra to the amplitude of the central component ($M = 0$).

for example[4–6,14]. It was established that the mobility of Ac-R· and M-R· bound by aspartate aminotransferase does not change in the pH range 5–8. In the pH range 8–10 the mobility gradually increases (the amplitudes of the extreme components increase). Further addition of $Na_3PO_4 \cdot 12H_2O$ rapidly increases the mobility of the labels due to denaturation of the enzyme.

Long storage of labeled aspartate aminotransferase at 2–4° produces an increase in radical mobility, the enzymatic activity remaining stable. Addition of urea (6 M) immediately increases the mobility of the radical.

The main aim of this work was to study the mobility of the radical bound by the enzyme during its interaction with specific ligands: substrates and inhibitors. The ligands were added immediately after labeling of the enzyme and the EPR spectra were recorded.

The EPR spectra of both radicals undergo pronounced changes during the formation of complexes between the enzyme and substrates and quasisubstrates: with a mixture of glutamic ($5 \cdot 10^{-2}$ M) and α-ketoglutaric ($5 \cdot 10^{-3}$ M) acid; α-ketoglutaric acid ($5 \cdot 10^{-2}$ M), glutaric acid ($5 \cdot 10^{-2}$ M), β-erythrooxyaspartic acid ($2.5 \cdot 10^{-3}$ M) and alanine (1 M). The concentration of the added ligand was always more than 10 times its affinity constant for aspartate aminotransferase. The experiments were performed in the pH range 8.5–8.6, *i. e.* in the region of optimal enzyme activity. Formation of the complexes was established from the absorption and circular dichroism spectra[3]. The changes in the EPR spectra of Ac-R· are shown by the broken line in Fig. 1. The components corresponding to $M = 0, +1$, retain their forms in the presence of substrate and quasisubstrates; only the component in the high field ($M = -1$) becomes more intense. The EPR spectra of M-R· change much more (*cf.* the broken line in Fig. 2) if the labeled protein interacts with substrates and quasisubstrates. The changes both in low and high fields are quite pronounced. Such changes can be explained by an increase in radical mobility, *i.e.* by a decrease in correlation time. The effective time values can be evaluated with the help of McConnell's theory[5]. The value for Ac-R· calculated from the linear term in M (*cf.* Eqn. (5) ref. 5) is, in the case of the holoenzyme without ligand, $(8.0 \pm 0.4) \cdot 10^{-10}$ sec, and with ligand is $(5.1 \pm 0.4) \cdot 10^{-10}$ sec. The form of the EPR spectrum of M-R· does not allow such an evaluation to be made. However, the correlation time for this radical was established indirectly, by luminescence polarization, to be $3.6 \cdot 10^{-8}$ sec[9]. It is difficult to estimate the change of this value resulting from substrate binding. Evidently it is diminished, since the spectrum intensity (*cf.* the broken line in Fig. 2) increases in the regions shown by the arrows corresponding to the components of the EPR spectrum of the radical rotating free in water. This shows that hindrance of the radical M-R· bound by aspartate amino transferase decreases, *i.e.* the correlation time becomes smaller.

The qualitative changes in the EPR spectra of both radicals produced by interaction of the pyridoxal 5′-phosphate form of aspartate aminotransferase with real substrates (mixture of glutamic and α-ketoglutaric acid) or quasisubstrates, are the same, as seen in Figs. 1 and 2 (broken lines), the mobility of the radical increasing. Addition of substrates produces a complicated equilibrium mixture of enzyme–substrate complexes[3]. The quasisubstrates imitate the single stages of transamination. Glutaric and ketoglutaric acid form Michaelis complexes with the pyridoxal 5′-phosphate form of aspartate aminotransferase under these conditions[15]. β-Erythro-

oxyaspartic acid forms a stabilized quinoid structure arising during the transformation of Schiff's base I into base II during transamination of the real substrate. Alanine is not a good substrate for aspartate aminotransferase, but at high concentrations it transaminates with ketoglutarate and forms an enzyme-substrate complex.

Despite the differences in binding of these ligands by the active site of aspartate aminotransferase, they influence the mobility of the radicals bound by protein in a similar way, suggesting that the local conformational changes in the region of radical binding are induced by the same functional group common to all ligands that interact with aspartate aminotransferase. It can be suggested that this group is α-carboxyl.

As has already been said, the radicals are bound by those thiol groups that are "nonessential" for activity. They are not involved in the active site and are located not less than 15 Å from the pyridoxal 5'-phosphate bound to the active site of aspartate aminotransferase[16]. The behavior of the label bound by "essential" groups may be quite different, both in the holoenzyme and in its derivatives.

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Received July 8th, 1970

Biochim. Biophys. Acta, 220 (1970) 357–360