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# CONFORMATIONAL CHANGES OF ASPARTATE AMINOTRANSFERASES IN THE REGION OF Cys-45 RESIDUE OBSERVED BY MEANS OF SPIN LABEL

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SUMMARY

Two thiol groups located at the surface of the aspartate aminotransferase molecule (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) can be alkylated by a paramagnetic label, *viz.* 2,2,6,6-tetramethylpiperidine-1-oxyl-4-iodoacetate. The modified enzyme restores the activity almost completely. Under the conditions used the spin label is not bound by any other functional groups of the aspartate aminotransferase molecule. Binding of ligands (substrates and quasisubstrates) at the enzyme active site induces changes of the EPR spectrum, which are reversible: removal of ligands by gel filtration through a Sephadex G-25 column results in the disappearance of these changes. One of the accessible thiol groups (Group I) was selectively alkylated by maleic acid whereas the other one (Group II) was labeled with the spin label. In this case addition of ligands did not affect the mobility of the spin label. It can be concluded, therefore, that it is only the spin label bound to the Group I that is sensitive to the interaction of a ligand with the active site. This group in the native enzyme was labeled with [$^{14}$C]maleic acid. Chromatography of the chymotrypsin digest of the treated protein yielded radioactive peptide; thereafter its partial structure was identified. The amino acid sequence of the peptide corresponds to that of the enzyme region from 41 to 48 (Trp residue in the peptide under study was destroyed during acid hydrolysis).

Previously it has been shown that the residue Tyr-40 is located in the region of the enzyme active site. The localization of the cysteine under study (Cys-45) makes it possible to think that this amino acid residue is rather close to the active site of the aspartate aminotransferase. However, the distance (>17 Å) between this residue and the active site as seen from the data obtained is not sufficient to provide spacial overlapping between the spin label and the Schiff's base of coenzyme with the substrate molecule. Therefore, the effect of substrate upon the mobility of the spin label is mediated by conformational changes in a certain region of the active site.

Abbreviation: PCMB, *p*-chloromercuribenzoate.

## INTRODUCTION

Previously it has been shown[1,2] that the exposed thiol groups of the aspartate aminotransferase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) can be alkylated by labels containing iminoxyl radical. Thiol groups which were able to bind these labels were shown to be located at a distance not less than 17–19 Å from the enzyme active site and not to be essential for enzymatic activity. Michaelis complexes between the substrates or quasisubstrates and the spin-labeled aminotransferases being formed, changes were observed in the EPR spectrum of the labels bound to the enzyme. These changes were induced with local conformational transitions in the enzyme region proximate to the label. In this work the reversibility of these changes has been shown and the localization of the cysteine residue involved in the region subject to the rearrangement has been identified.

## EXPERIMENTAL PROCEDURE

### *Materials*

Cytosol aspartate aminotransferase (EC 2.6.1.1) was isolated from pig heart as had been described previously[3]. The enzyme preparation contained a mixture of $\alpha$ and $\beta$ forms. Aminotransferase concentration was evaluated spectrophotometrically $D_{280} = 1.4$ (0.1%). For all the calculations the molecular weight of one aminotransferase subunit was taken as equal to 46 700 that was deduced from the primary structure of the enzyme[4]. Enzymatic activity was determined by the direct spectrophotometric method[5]. Chymotrypsin treated with the trypsin inhibitor was purchased from Worthington.

L-Aspartate was purchased from Reanal; $\alpha$-ketoglutaric acid from Calbiochem; *p*-chloromercuribenzoate (PCMB) from Serva; L-erythro-$\beta$-hydroxyaspartic acid was kindly presented by Dr V. L. Florentiev; [$^{14}$C]maleic anhydride from Amersham. Iminoxyl spin label, *viz.* 2,2,6,6-tetramethylpiperidine-1-oxyl-4-iodoacetate was synthesized as described previously[6].

### *Determination of the enzyme thiol groups*

Thiol groups were determined by the method of Boyer[7] in 1% solution of sodium dodecyl sulfate in 0.05 M acetate buffer, pH 5.4. The protein solution (volume = 1 ml) of a precisely evaluated concentration (in the range from 0.3 to 0.5 mg/ml) was placed in the cell of the double-beam spectrophotometer and the aliquots of PCMB solution were added by means of a micrometric pipette. The same amount of PCMB was added to the blank cell by using an identical pipette. The solutions in both cells were made 1% in respect to sodium dodecyl sulfate. The final volume of the PCMB solution added was about 0.3–0.5 ml (dilution of the enzyme sample was taken into account for calculations). At this kind of determination the error was not cumulated in the course of titration and was $\pm$0.05 equiv. mercaptide. The slope of the titration curve did not depend on the concentration of PCMB or protein since the abscissae axis corresponded to the number of $\mu$moles PCMB. The exact concentration of PCMB in the initial solution was measured by using the value of its molar extinction equal to $1.69 \cdot 10^4$ at 232 nm in 0.1 M phosphate buffer, pH 7.

*Labeling with iminoxyl radical*

The preparation of aspartate aminotransferase in the sodium acetate buffer, pH 5.5, was incubated in the presence of 0.3% 2,2,6,6-tetramethylpiperidine-1-oxyl-4-iodoacetate for 24 h. The excess of the reagent was removed by gel filtration through a Sephadex G-25 column. The enzyme was concentrated to yield a 15–20 mg/ml solution.

*Labeling of cysteine thiol groups with maleic acid (with formation of a dicarboxyethyl derivative)*

[$^{14}$C]Maleic anhydride was hydrolysed for 30 min in the alkaline medium, pH 8.5, added to the enzyme solution containing 0.25 M Tris–HCl buffer, pH 7.5, (molar ratio 2:1) and incubated for 24 h. Thereafter non-radioactive maleic acid was added to a final concentration of 0.05 M and the incubation was continued for 24 h. Free maleic acid was removed by gel filtration through a Sephadex G-25 column. The protein solution was treated by an excess of $\beta$-mercaptoethanol and EDTA. Free thiol groups of the enzyme were alkylated with non-radioactive iodoacetate in 8 M urea under nitrogen. Thereafter the mixture was passed through a Sephadex column equilibrated with 50% acetic acid. The eluate was evaporated to give the volume of 25 ml and dialysed for 2 days.

*Separation and purification of chymotrypsin digest peptides*

Dialysed enzyme solution was adjusted with 0.1 M NaOH up to pH 8.6. Chymotrypsin (3% of the enzyme weight) was added stepwise for 18 h at 37 °C; the whole procedure was carried out in a vessel of titrimetre (Radiometer TTA3). The digest was lyophilised, dissolved in 2 ml 0.2 M pyridine–acetate buffer, pH 2.3, and centrifuged. The supernatant was applied to the column containing Aminex resin Q-150S (0.9 cm × 16 cm) and eluted with a two-step gradient of pyridine–acetate buffer (see Results). Chromatography was carried out at 37 °C, the flow rate was 40 ml/h. The content of peptides in the eluate was measured by means of the flow automatic analyser Technicon using alkaline hydrolysis followed by ninhydrin reaction (the authors are thankful to Dr C. A. Egorov for valuable advice and help in manipulating the apparatus). The fraction containing radioactivity was chromatographed on the paper in the system pyridine–buthanol–acetic acid–water (10:15:3:12, by vol.). To determine radioactivity, 0.05 ml of the sample (evaporated to give the volume of 2–3 ml) was dissolved in 10 ml of scintillation liquid (0.2 g/l POPOP, 4 g/l PPO, 100 g/l naphthalene in dioxane). The radioactivity was measured in the scintillation liquid spectrophotometre SL-30 (Intertechnique, France).

*Amino acid analysis*

Amino acid analysis was carried out using the analyser Biocal (Munich) BC 201 after hydrolysis under standard conditions (5.7 M HCl, 105 °C, 24 h).

## RESULTS

*Reversible effect of ligands on the spectra EPR of spin label*

Two thiol groups (so named "external" thiols) of the aspartate aminotransferase molecule may be associated with the iminoxyl radical. The alkylated enzyme res-

tores more than 90% of the initial activity. This fact is in agreement with the data obtained earlier[3]. The spin-labeled enzyme has the same absorption and circular dichroism spectra (280–500 nm, pH 5.5) as the unlabeled one.

EPR spectra of the 2,2,6,6-tetramethylpiperidine-1-oxyl-4-iodoacetate iminoxyl radical covalently bound by the aspartate aminotransferase are presented in Fig. 1. Following the addition of substrates or quasisubstrates (mixture of $5 \cdot 10^{-2}$ M glutamic acid and $5 \cdot 10^{-3}$ M α-ketoglutaric acid; $2.5 \cdot 10^{-3}$ M L-erythrohydroxy-


Fig. 1. EPR spectra of the iminoxyl radical 2,2,6,6'-tetramethylpiperidine-1-oxyl-4-iodoacetate covalently bound to two "external" thiol groups of the aspartate aminotransferase. A. Spectrum in the absence of ligands. B. Highfield component of the EPR spectrum. ———, in the absence of substrates; – – –, after addition of erythro-β-hydroxyaspartic acid. C. The same after removal of the ligand by gel filtration. Spectra were read under identical conditions: protein concentration 20 mg/ml, buffer Tris–HCl, pH 8.0. Each sample contained 10 μl of enzyme solution in a polyethylene capillary. Inconsiderable variations in amplitude were eliminated by approximating the intensities of all spectra to the amplitude of the central component.

aspartic acid) some changes occur in the EPR spectra that indicates alterations in the mobility of the bound label. Since all the ligands mentioned have been shown previously[1,2] to affect the mobility of the spin label in a similar way we were able to use only one of these compounds throughout the whole work. L-Erythrohydroxyaspartic acid was chosen since it has the highest affinity for the enzyme. Changes occurring in the EPR spectra after addition of this compound to the spin-labeled transaminase are clearly seen in Fig. 1B. After the ligand had been removed from the

solution by gel filtration through a Sephadex G-25 column, the EPR spectrum was read again. Since this operation resulted in about a 1.5-fold dilution of the enzyme, spectra were compared by juxtaposition of the amplitudes of the central components. Figs 1B and 1C show that the initial spectrum and that of the spin-labeled enzyme after removal of the ligand are almost identical. This means that the conformational changes of a certain enzyme region induced by the ligand are reversible.

*Selective labeling of the SH group II*

Titration of aminotransferase by PCMB in 1% sodium dodecyl sulfate solution prior to and after binding of 2,2,6,6-tetramethylpiperidine-1-oxyl-4-iodoacetate has shown that the both "exposed" thiol groups of the enzyme can be alkylated. Therefore, each of the labels or both may be responsible for the changes in the EPR spectra. If the enzyme containing 4 titratable SH groups is modified by maleic acid, one thiol group disappears (SH group I) (Table I). The enzymatic activity is com-

TABLE I

QUANTITATIVE DETERMINATION OF THE NUMBER OF SPIN LABELS BOUND TO ASPARTATE AMINOTRANSFERASE

| *Enzyme sample* | *Activity (%)* | *Number of SH groups (in sodium dodecyl sulfate)* | *Number of spin labels bound to the enzyme* |
|---|---|---|---|
| 1. Unmodified aspartate transferase | 100 | 4.0 | 0 |
| 2. The same, *plus* 2,2,6,6′-tetramethylpiperidine-1-oxyl-4-iodoacetate | 95 | 2.1 | 1.9 |
| 3. Aspartate aminotransferase treated by maleic acid | 92 | 3.2 | 0 |
| 4. The same, *plus* 2,2,6,6′-tetramethylpiperidine-1-oxyl-4-iodoacetate | 89 | 2.2 | 1.0 |

pletely restored after this kind of treatment. The maleinated enzyme was treated by 2,2,6,6-tetramethylpiperidine-1-oxyl-4-iodoacetate and in this case only one iminoxyl radical was bound by the protein (the spectrum is shown in Fig. 2). The binding of the label is accompanied by the disappearance of one more thiol group. It can be seen from Fig. 2A that the spectrum of the label bound to the SH group II is distinct from that of the two radicals bound to SH Groups I and II. Broadening of the high-field component (Fig. 2B) indicates strong immobilization of the label bound with SH group II.

The addition of specific ligands to aminotransferase with labeled SH Group II did not result in any changes in the EPR spectrum (Fig. 2B). Juxtaposition of the spectrum shown in the Fig. 2B with that presented in Fig. 1C and indicating changes under the effect of specific ligands revealed the identity of both spectra. Hence, the alterations in the spectrum under the action of ligands are probably be more intensive than shown in Fig. 1C, but they are masked by the spectrum of the second label, the form of the latter being unchanged in the presence of ligands.

It was also noticed that in the course of prolonged storage of the enzyme in the absence of mercaptoethanol, EDTA and other SH-protecting agents the number of thiol groups titratable in sodium dodecyl sulfate solution is decreased up to 3 (with

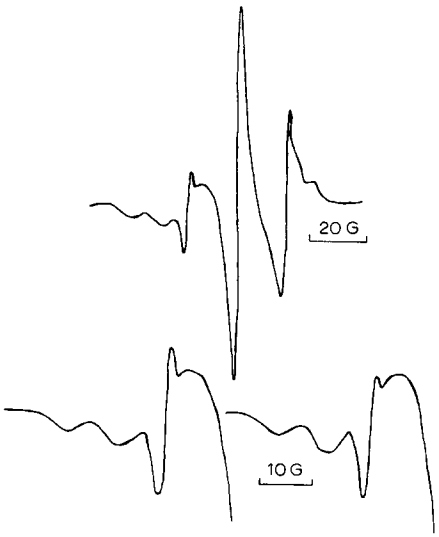


Fig. 2. EPR spectra of the iminoxyl radical 2,2,6,6′-tetramethylpiperidine-1-oxyl-4-iodoacetate covalently bound to the thiol Group II of aspartate aminotransferase. A. Spectrum in the absence of ligands. B. The same for the highfield component. C. Highfield component of the spectrum after addition of L-erythro-β-hydroxy aspartic acid. For experimental conditions see Fig. 1; protein concentration 15 mg/ml.

complete restoration of activity). Results obtained in experiments when such a preparation possessing only one accessible thiol group was labeled were quite identical to the data available from analysis of the dicarboxythylated enzyme. These results are summarized in the Table I; it is clear there is only one label which bound to the thiol Group I, that is sensitive to the interaction of aminotransferase with specific ligands.

One more distinctive feature of the label bound to the thiol Group I consists in its greater ability for hydrolysis. This is observed by the appearance of a sharp component in the course of storage. The hydrolysis rate of the label bound to the thiol Group II under similar conditions is one order less. This feature of the label bound to the Group I was taken into account in experiments. The protein was passed through a Sephadex column just before the spectral measurements were made; the sharp component in the spectrum inherent to the free radical admixture does not prevent the interpretation of the results obtained in experiments with ligands.

*Localization of the thiol Group I in the primary structure*

The thiol Group I of the aspartate aminotransferase was modified by maleic acid and the enzyme was cleaved by chymotrypsin (see under Materials and Methods). The peptide mixture was separated on an Aminex G-150S column (Fig. 3). Most radioactivity (>80%) was found in a single fraction. To purify the radioactive peptide, chromatography on Whatman 3MM paper was carried out. After drying, the $^{14}C$-labeled peptide was localized. In addition to this peptide, only one band was revealed on the chromatogram which was ninhydrin positive but did not contain radioactivity. The amino acid composition of the peptide was determined. A very acidic amino acid was found in the hydrolysate which could be identified as dicar-

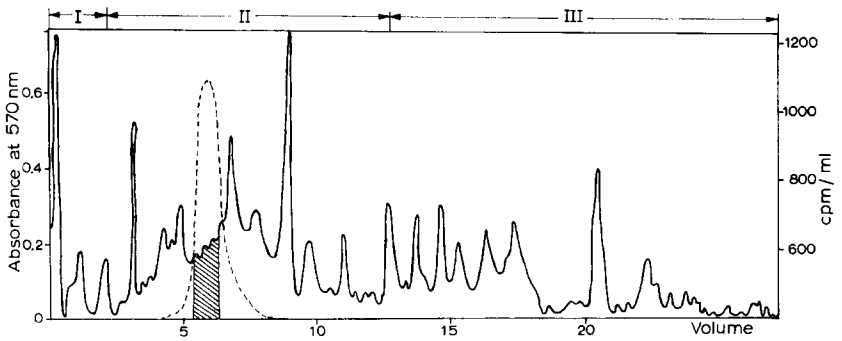


Fig. 3. Elution profile of soluble chymotryptic peptides on 0.9 cm × 150 cm column of Aminex G-150S. The column was developed with a double linear gradient of pyridine–acetate (see below) and monitored automatically by ninhydrin analysis (———) after alkaline hydrolysis. Fractions of 3.0 ml were collected. Radioactivity (– – –) was counted on 100-$\mu$l aliquots taken from alternate fractions. Change of gradient pyridine–acetate buffer: I, 0.2 M, pH 3.1; II, 0.2 M, pH 3.1–0.5 M, pH 5.0; III, 0.5 M, pH 5.0–2 M, pH 5.0.

boxyethyl cysteine. The following amino acid residues were present in the peptide: dicarboxyethyl Cys, 0.7; Asp, 2.2; Thr, 1.3; Glu, 1.3; Pro, 1.0; Ala, 0.9; Arg, 0.8. Admixtures of serine and glycine were also detected (these amino acids are usually formed from tryptophan under conditions of acid hydrolysis; the latter is present in the peptide). The N-terminal sequence was established using another sample by the Edman procedure combined with determination of the N-terminal residue after dansylation (the authors are grateful to Dr M. Yu. Feygina for the help). The N-terminal amino acid was represented by arginine, the following residue was found to be threonine. Analysis of the data show that the labeled peptide represents a part of the sequence of the aspartate aminotransferase polypeptide chain from the 41st to the 48th residue:

Arg–Thr–(Asp,Asp,$\overset{*}{\text{Cys}}$,Glu,Pro)–
Arg–Thr–Asp–Asp–Cys–Glu–Pro–Trp– 41–48 residues

## DISCUSSION

The use of spin labels makes it possible to study local conformational changes in the enzyme molecule. The most important part of such investigations is thought to consist of the determination of the enzyme region involved in the conformational changes and their connection with specific interactions in the enzyme active site. Structural rearrangements in the region of the active site of aspartate aminotransferase were postulated on the basis of some suggestions[8] and were proved in experiments concerning syncatalytic modifications of the enzyme functional group in the presence of substrates and some substrate analogues[9–11]. It was interesting to evaluate the size of the region involved in conformational changes induced by interaction with substrates and the degree of selectivity of these rearrangements. Three functional groups of the aspartate aminotransferase protein have been labeled: two thiol groups and one lysine residue[1,2] of unknown localization. Both thiol groups may be considered accessible for alkylation according to the classification suggested by many authors[3,12].

The total number of cysteine residues in the polypeptide chain of aspartate aminotransferase is equal to 5; thiol groups may be divided into two types: external and internal ones. Recently, the localization of two accessible (Cys-45 and Cys-82) and three internal (Cys-191, Cys-252 and Cys-390) thiol groups has been identified[13]. The masked thiol groups were selectively labeled with monoiodo[$^{14}$C]acetic acid in 8 M urea solution after alkylation of the external cysteine residues with non-radioactive monoiodoacetic acid. The comparison of the results obtained in these two lines of investigations makes it possible to conclude that in the native enzyme the spin-label 2,2,6,6-tetramethylpiperidine-1-oxyl-4-iodoacetate is bound to Cys-45 as well as to Cys-82. Cys-45 being modified, the label can react with Cys-82 only. In this paper it is shown that it is only the label bound to Cys-45 that undergoes changes in the mobility after addition of substrates. Distance from the spin labels to pyridoxal phosphate in the active site has been evaluated by means of titration of spin-labeled aspartate aminotransferase with $Cu^{2+}$ (ref. 2). It has been shown that after addition of 6 copper equivalents (per 1 enzyme subunit) the incorporation of the $Cu^{2+}$ into the chelate complex involving the pyridoxal phenol moiety and the internal aldimine NH group is completed. Dipole–dipole interaction between enzyme-bound $Cu^{2+}$ and spin labels was not observed. The absence of this kind of interaction is possible only if the distance between the labels and active site exceeds 17–19 Å. No interaction between the labels was revealed by analysis of EPR spectra. Therefore, spin labels bound to the cysteine residues Cys-45 and Cys-82 and coenzyme are localized at the apices of a triangle with sides of length not less than 17–19 Å. This distance is not sufficient to provide spacial overlapping of the spin labels with the Schiff base of a pyridoxal phosphate with substrate. It follows from this fact that the influence of a substrate on the spin-label spectrum is mediated by conformational changes in a certain region of the aminotransferase protein globula namely that surrounding Cys-45. Naturally, a question arises as to which intermediate *ES* complex is responsible for this effect. Previously it has been shown[1,2] that all the substrates and substrate analogues affect the spin label EPR spectrum in the same way if the concentration of the corresponding ligand exceeds $K_m$ by about 10 times. In particular, this effect is induced by glutaric acid ($5 \cdot 10^{-2}$ M) and even by alanine at a saturating concentration of 1 M. It could be suggested, therefore, that formation of the initial Michaelis complex with a substrate is sufficient to provide the appearance of changes in the EPR spectrum of the spin label. With respect to the specificity of ligand action this effect is essentially distinct from the syncatalytic modification of one of aminotransferase tyrosine residues. This type of modification was shown[9] to proceed rapidly and selectively only in the presence of the substrate pair or erythro-$\beta$-hydroxyaspartic acid rather than $\alpha$-methylaspartate, which means that it involves a catalytic step preceding formation of the intermediate ketimine (Schiff II). It has been shown[14] that it is the tyrosine residue Tyr-40 that is syncatalytically nitrated. Selective syncatalytic modification of this residue was accounted for by its high accessibility for a modifying reagent in the presence of substrates[10]; the unmasking was regarded as a consequence of certain conformational rearrangements. Recently it has been shown[15,16] that one more residue exists which undergoes syncatalytic modification, namely that of cysteine. The latter one is modified at the ketimine step only (according to the primary structure[4], Cys-320). Therefore, it is evident that discrete steps of interactions and transformations of substrates in the active site of aminotransferase

TABLE II

SYNCATALYTIC CONFORMATIONAL CHANGES IN THE MOLECULE OF ASPARTATE AMINOTRANSFERASE

| *Ligand* | *Step of enzymatic process* | *Residue* | *Effect* | *Ref.* |
|---|---|---|---|---|
| Dicarboxylic acids, alanine, substrates | Michaelis complex | Cys-45 | Changes in spectra of EPR labels | — |
| Erythro-β-hydroxyaspartic acid*, mixture of substrates (not α-methylaspartate) | Between intermediate aldimine and ketimine | Tyr-40 | Selective and rapid modification | 9,14 |
| Mixture of substrates | Intermediate ketimine | Cys-390 | Selective and rapid modification | 15,16 |

* Unpublished data.

correspond to conformational changes of definite regions of the enzyme molecule proximate to the active site.

The data concerning this subject are summarized in Table II.

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