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Covalent Flavinylation of L-Aspartate Oxidase from *Escherichia coli* Using N^6-(6-Carboxyhexyl)-FAD Succinimidoester

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L-Aspartate oxidase is a flavoprotein catalyzing the first step in the *de novo* biosynthesis of pyridine nucleotides in *E. coli*. Binding of FAD to L-aspartate oxidase is relatively weak (K_d 6.7 × 10^{-7} M), resulting in partial loss of the coenzyme under many experimental conditions. Only the three-dimensional structure of the apo-enzyme has been obtained so far. In order to probe the flavin-binding site of the enzyme, apo-L-aspartate oxidase has been reacted with N^6-(6-carboxyhexyl)-FAD succinimidoester. The structural characterization of the resulting N^6-(6-carbamoylxyhexyl)-FAD-L-aspartate oxidase shows the covalent incorporation of 1 FAD-analog/ monomer. Residue Lys38 was identified as the target of the covalent modification. N^6-(6-carbamoylxyhexyl)-FAD-L-aspartate oxidase shows only 2% catalytic activity as compared to the native enzyme. Comparison of some properties of the flavinylated and native enzymes suggests that, although the flavin is covalently bound to the former in the region predicted from molecular modeling studies, the microenvironment around the isoallossazine is different in the two forms.

KEY WORDS: L-Aspartate oxidase; nadB; flavoproteins; N^6-(6-carbamoylxyhexyl)-FAD-L-aspartate oxidase; NAD biosynthesis.

1. INTRODUCTION

The first step in the *de novo* biosynthesis of pyridine nucleotides in *E. coli* is catalyzed by L-aspartate oxidase (EC 1.4.3.16), which oxidizes L-aspartate to iminoaspartate using either O_2, fumarate, or quinones (Griffith *et al.*, 1995; Tedeschi *et al.*, 1996, 1997). Iminoaspartate is then condensed with dihydroxyacetonephosphate by quinolinate synthase to give quinolinate, which is subsequentally converted to NAD through a common pathway in all organisms (Tritz, 1987).

L-Aspartate oxidase is a 60-kDa flavoprotein containing 1 mol of noncovalently bound FAD per monomer. The three-dimensional structure of the apo-enzyme has recently been determined to 2.2.-Å resolution, showing a novel fold for a FAD-dependent protein (Mattevi *et al.*, 1999). Despite the fact that the protein preparation used for crystallization contained fully active hole-enzyme and that excess FAD was added to the crystallizazion medium, it was not possible to obtain crystals of the holo form. This result confirms those obtained during the biochemical characterization of the enzyme overexpressed in *E. coli* which suggested that binding of FAD is relatively weak (K_d 6.7 × 10^{-7}M) (Mortarino *et al.*, 1996) compared to other flavoproteins (Muller and Van Berkel, 1991), resulting in partial loss of the flavinic coenzyme under many experimental conditions. As a consequence no accurate description of the FAD–protein interactions could be inferred from the crystallographic data despite the fact

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that the FAD-binding site in the three-dimensional structure of the enzyme could be identified by comparison with other flavoproteins displaying structural homology with L-aspartate oxidase (Mattevi *et al.*, 1999).

Recently, a new FAD derivative N^6-(6-carboxyhexyl)-FAD succinimidoester (Fig. 1) has been synthetized and successfully used to prepare a dimeric, fully active flavinylated D-amino acid oxidase (pig kidney), with one N^6-(6-carboxyhexyl)-FAD moiety/subunit, bound to Lys163 (Stocker *et al.*, 1996). The three-dimensional structure of holo-D-amino acid oxidase confirmed that the adenylic portion of FAD is indeed suitably positioned to allow covalent flavinylation at Lys163 (Mattevi *et al.*, 1996). In order to gain some insight in the interaction between the flavin coenzyme and L-aspartate oxidase and to strengthen the hypothesis that the three-dimensional structure of the apo-enzyme does in fact represent a good starting model to study the properties of the active holo form, the new flavin derivative has been used to obtain a covalent conjugate N^6-(6-carbamoylhexyl)-FAD-L-aspartate oxidase. The properties of the flavinylated enzyme, including the identification of the amino acid residue of the primary structure to which N^6-(6-carboxyhexyl)-FAD is covalently coupled, are reported.

2. MATERIALS AND METHODS

2.1. Materials

L-Aspartate oxidase was overexpressed and purified according to Mortarino *et al.* (1996). N^6-(6-Carboxyhexyl)-FAD and N^6-(6-carboxyhexyl)-FAD succinimidoester were prepared according to Stocker *et al.* (1996).

RP-HPLC purified FAD was prepared from commercial FAD according to Negri *et al.*, (1997). 5-Deazaflavin-3-sulfonate was a generous gift of Dr. Vincent Massey, University of Michigan. All other reagents were of the highest purity commercially available. All experiments were conducted in 50 mM Hepes, pH 8.0, at 25°C, unless otherwise specified.

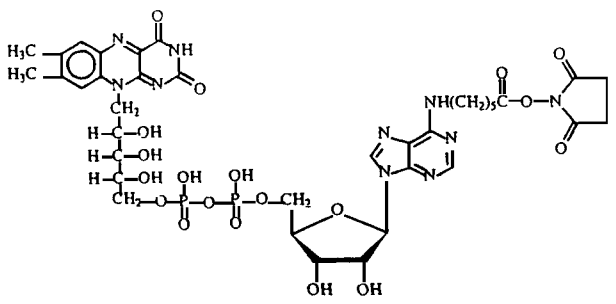

Fig. 1. N^6-(6-Carboxyhexyl)-FAD succinimidoester.

2.2. Apo-Protein Preparation and Flavinylation

Apo-L-aspartate oxidase was freshly prepared by the procedure described in Tedeschi *et al.* (1999). In summary, L-aspartate oxidase was incubated with 0.5% activated charcoal (Sigma) in 50 mM Hepes, pH 8.0, at room temperature until no visible absorption was detected following centrifugation to separate the charcoal.

Flavinylation was carried out essentially as described in Stocker *et al.* (1996): freshly prepared apoprotein (2–3 mg/ml) in 50 mM Hepes, pH 8.0, was mixed with eight-fold molar excess of freshly prepared N^6-(6-carboxyhexyl)-FAD succinimidoester, the pH adjusted to 6.6 with 0.5 N sodium acetate, pH 3.5, and the material incubated for 20 hr at room temperature in the dark. Removal of excess reagents was accomplished repeating the procedure described above for apo-protein preparation until the ratio between the absorbances at 280 and 450 nm remained constant, followed by gel filtration on PD10 (Pharmacia, Sweden) equilibrated in 50 mM Hepes, pH 8.0.

2.3. Identification of the Amino Acid Residue Covalently Modified by N^6-(6-Carboxyhexyl)-FAD Succinimidoester

Ten nmol N^6-(6-carbamoylhexyl)-FAD-L-aspartate oxidase was concentrated by precipitation with 10% trichloroacetic acid. The yellow pellet was washed with cold acetone:HCl 1 N (39:1), resuspended in 2 M urea, 0.1% NH_4HCO_3, pH 8.0, and digested with TPCK-trypsin (Boheringer Mannheim sequence grade) using a protease:substrate ratio 1:20, at 37°C for 16 hr. The digested material was fractionated by RP-HPLC using an Aquapore RP300 C8 column (4.6 × 250 mm) (Perkin-Elmer) on a Jasco (Japan Spectroscopy, Japan) HPLC. Conditions: buffer A, 0.1% trifluoroacetic acid in water; buffer B, 0.075% trifluoroacetic acid in CH_3CN; gradient, from 0% to 50% B in 70 min; flow rate, 0.7 ml/min.

Repurification of the fraction with retention time of 33 min was accomplished under the same experimental conditions except that buffers A and B were 5 mM NaPi, pH 6.0, and CH_3CN, respectively.

The N-terminal amino acid sequence of the flavinylated peptide was determined using an Applied BioSystems Model 477/A automated protein sequencer.

2.4. Miscellaneous Methods

L-Aspartate oxidase activity was determined as described in (Mortarino *et al.* (1996). The standard assay was conducted at 25°C in a total volume of 1 ml of 50 mM Hepes, pH 8.0, containing 10 μmol of L-aspartate, 64 μg of horseradish peroxidase, 90 μg of *o*-dianisidine-2 HCI ($\varepsilon_{436} = 11.6 \times 10^3\ M^{-1}\ cm^{-1}$ for oxidized *o*-dianisidine) ± 20 nmol FAD. Absorption spectra were measured using an HP8452 diode array spectrophotometer (Hewlett Packard). Protein content was determined using $\varepsilon_{452} = 11.6 \times 10^3\ M^{-1}\ cm^{-1}$ for holo-L-aspartate oxidase and N^6-(6-carbamoylhexyl)-FAD-L-aspartate oxidase, $A_{280} = 1.3$ for a 1 mg/ml solution of apo-L-aspartate oxidase (Mortarino *et al.*, 1996). Flavin fluorescence was determined using a JASCO FP750 spectrofluorimeter. Experiments under anaerobic conditions were conducted as described in Tedeschi *et al.* (1996).

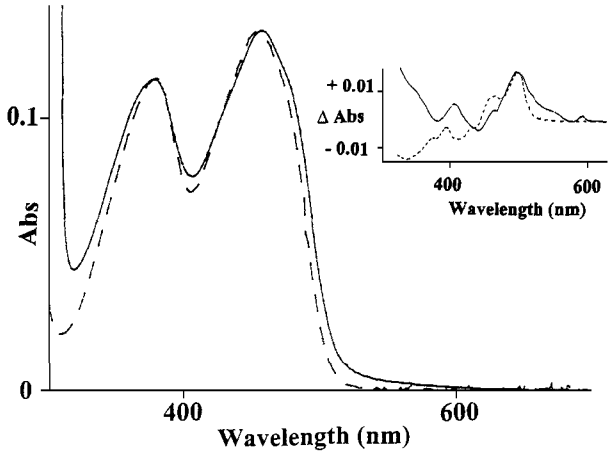

Fig. 2. Visible absorption spectrum of N^6-(6-carboxyhexyl)-FAD free (- - -) and bound to L-aspartate oxidase (—) in 50 mM Hepes, pH 8.0. Inset: Differential spectrum between L-aspartate oxidase-bound and free N^6-(6-carboxyhexyl)-FAD (—) and FAD (- - -).

3. RESULTS

3.1. Synthesis and Characterization of N^6-(6-carbamoylhexyl)-FAD-L-Aspartate Oxidase

Flavinylation of the apo-enzyme with N^6-(6-carboxyhexyl)-FAD succinimidoester at pH 6.6 resulted in a mixture of 53 ± 8% (three preparations) N^6-(6-carbamoylhexyl)-FAD-L-aspartate oxidase and 47% residual apo-L-aspartate oxidase, as deduced from the visible and UV absorption spectrum of the material.

The differential spectrum between N^6-(6-carbamoylhexyl)-FAD-L-aspartate oxidase and the free FAD derivative resembles that of FAD free and bound to native L-aspartate oxidase (Fig. 2). In accordance, the visible absorption maximum of N^6-(6-carbamoylhexyl)-FAD-L-aspartate oxidase is 452 nm, as observed for the native enzyme.

The visible absorption spectrum of N^6-(6-carbamoylhexyl)-FAD-L-aspartate oxidase is not perturbed in the presence of succinate, fumarate, oxaloacetate, or iminoaspartate, which are known to cause spectral perturbation in the case of the native enzyme (Tedeschi *et al.*, 1996; Mortarino *et al.*, 1996). In keeping with this result, no semiquinone was observed during anaerobic photoreduction of N^6-(6-carbamoylhexyl)-FAD-L-aspartate oxidase in the presence of succinate and no oxidation was observed upon mixing the photoreduced enzyme with fumarate, again in contrast with the results observed with native L-aspartate oxidase (Tedeschi *et. al.*, 1996). Finally, as observed for the native enzyme, N^6-(6-carbamoylhexyl)-FAD-L-aspartate oxidase photoreduced in strictly anaerobic conditions is readily oxidized by O_2 upon admission of air in the anaerobic cell.

N^6-(6-Carbamoylhexyl)-FAD-L-asparte oxidase shows typical flavin fluorescence (75% of free FAD, excitation and emission maxima at 450 and 520 nm, respectively). It should be pointed out, however, that native L-aspartate oxidase shows an increase in flavin fluorescence (120%) compared to free FAD, suggesting that the microenvironment around the isoallosazinic subnucleus is different in native and N^6-(6-carbamoylhexyl)-FAD-L-aspartate oxidase.

N^6-(6-Carbamoylhexyl)-FAD-L-aspartate oxidase shows a specific activity of 0.0127 μmol substrate oxidized $min^{-1}\ mg^{-1}$ when assayed under the usual assay conditions but without excess FAD in the reaction mixture. Such a value corresponds to about 2% L-aspartate oxidase activity as compared to the native holo-enzyme under the same experimental conditions. Since the apoprotein before incubation with N^6-(6-carboxyhexyl)-FAD succinimidoester showed no activity in the absence of excess FAD in the reaction mixture, it can be concluded that the activity reported above has to be attributed to N^6-(6-carbamoylhexyl)-FAD-L-aspartate oxidase. In agreement with this observation, overnight incubation of N^6-(6-carbamoylhexyl)-FAD-L-aspartate oxidase with L-aspartate under anaerobic conditions resulted in a very slow and largely incomplete flavin reduction (data not shown). No kinetic parameters for the flavinylated enzyme could be determined due to the very low reaction rates observed.

3.2. Identification of the Amino Acid Residue Modified by N^6-(6-Carboxyhexyl)-FAD Succinimidoester

Figure 3 shows the RP-HPLC separation of the tryptic digest of 10 nmol N^6-(6-carbamoylhexyl)-FAD-L-aspartate oxidase monitored at 220 and 360 nm. The latter wavelength was used to detect the presence of the flavin derivative covalently bound to peptide(s) (Stocker *et al.*, 1996). Most of the material with 360 nm absorbance was associated with a fraction with retention time 33 min, which, however, was shown by N-terminal sequence analysis to contain more than one peptide and was rechromatographed as shown in Fig. 4.

The N-terminal amino acid sequencing of the peptide contained in the fraction with retention time of 33 min from the second RP-HPLC chromatography resulted in the following sequence: LeuAlaAspGlnHis-

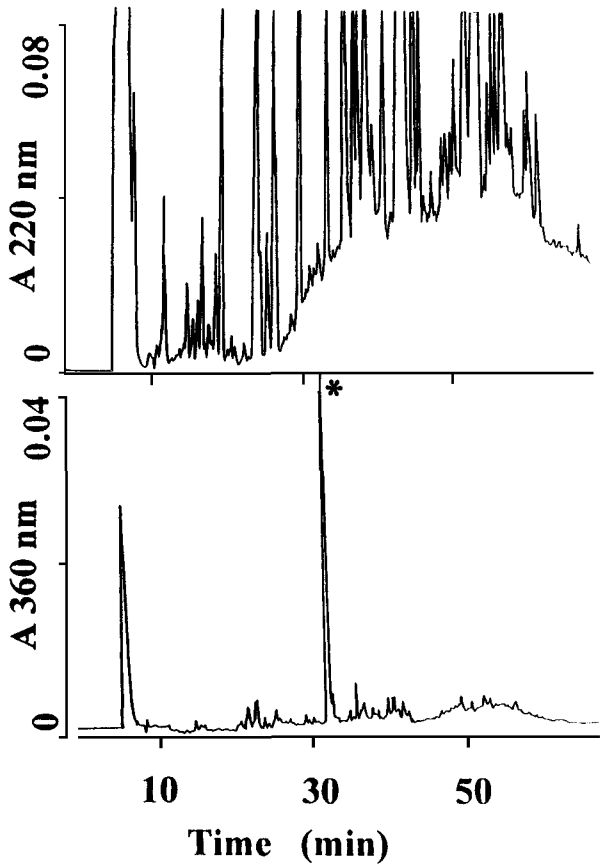

Fig. 3. RP-HPLC cromatography of the peptides obtained following tryptic digestion of N^6-(6-carbamoylhexyl)-FAD-L-aspartate oxidase under the conditions described in Materials and Methods. The flavin-containing fraction (marked by the asterisk, retention time of 33 min) was rechromatographed as described in Fig. 4.

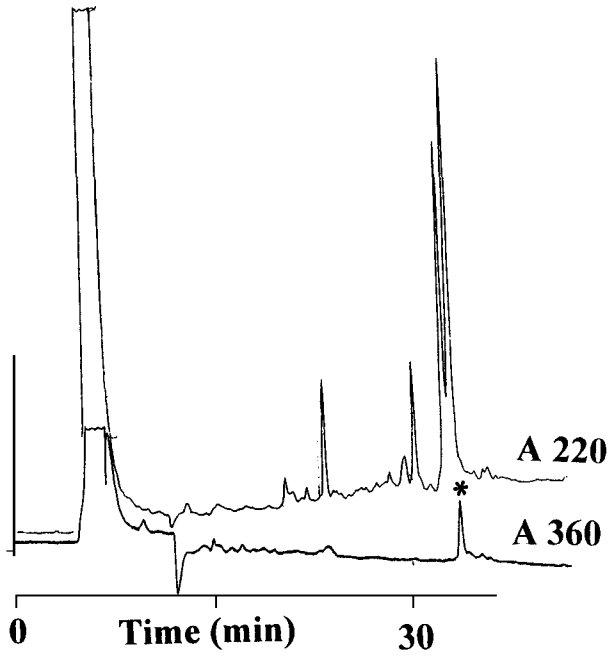

Fig. 4. Repurification of peptide with retention time 33 min from the chromatography shown in Fig. 3 under the conditions described in Materials and Methods. The absorbance unit full scale was 0.02 and 0.04 when monitored at 220 and 360 nm, respectively. The flavin-containing peptide (marked by the asterisk) was submitted to Edman degradation.

GlnValIleValLeuSerXxx. Although the analysis was carried on for an additional five degradation cycles, no further amino acid residues were detected. Since this sequence corresponds to tryptic peptide Leu27–Lys38 in the primary structure of L-aspartate oxidase (Flachmann *et al.*, 1989) (Fig. 5), and the conditions used are such that the flavin derivative reacts only with primary amino groups (Stocker *et al.*, 1996), it is concluded that the site of modification by N^6-(6-carboxyhexyl)-FAD succinimidoester is Lys38. In conclusion, the structural characterization indicates that the conditions for preparing semisynthetic L-aspartate oxidase lead to a conjugate with one FAD-analog/monomer bound to Lys38.

4. DISCUSSION

This paper reports the use of a very peculiar active-site-directed reagent to probe the flavin-binding site of flavoproteins. Reaction of apo-L-aspartate oxidase with the active-site-directed reagent N^6-(6-carboxyhexyl)-FAD succinimidoester leads to the very

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                                                          ↓
Ecol                MNTLPEHSCDVLIIGSGAAGLSLALRLADQ--HQVIVLSKG 39
Psae                MSQHYQH--DVLVIGSGAAGLSLALTLPEHLR--IAVLSKG 37
Mlep               MAVPDWKHAADVVVIGTGVAGLAAALAAHRAGR-NVVVLTKA 41
Mtub               MAGPAWRDAADVVVIGTGVAGLAAALAADRAGR-SVVVLSKA 41
Scoe      MTSTGIRLHAPAPGWAIAADVVVVGSGVAGLTAALRCESAGL-RTVVVTKA 50
Syny                MGANQFDVVVVGSGAAGLYACLCLPGHYR--VALVTKA 36
Aqae      MNYFLRFDTSLLPEEEAKVLICGSGIGGLATAISLKELGIE-PLILTRG 48
Pyho               MMEMRVGIVGGGLAGLTAAIALAEKGFDVSIIGPRS 36
Bsub               MSKKTIAVIGSGAAALSLAAAFPPS--YEVTVITKK 34
Mjan               MKTDILIIGGGGAAARAAIECRDK---NVIIAVKG 32
                        : : * * ::                 :  :

Ecol      PVTE--GSTFYAQGGIAAVFDETD-SIDSHVEDTLIAGAGICDRHAVEFVA 87
Psae      ELSQ--GSTYWAQGGVAAVLDDTD-TVESHVEDTLVAGGGLCREDAVRFTV 85
Mlep      DQRRGETATHYAQGGIAVVLPGSDDSVDAHASDTLAAGAGMCNLDTVYSIV 92
Mtub      AQTH-VTATHYAQGGIAVVLPDNDDSVDAHVADTLAAGAGLCDPDAVYSIV 91
Scoe      RLDD--GSTRWAQGGVAAALGEGD-TPEQHLDDTLVAGAGLCDQDAVRILV 98
Syny      ELKT--GASDWAQGGIAAAIAPTD-SPQSHYEDTLAAGAGLCDGEAVDFLV 84
Aqae      -----IGNTYYSQGGIAAAVLPDD-SPYLHYCDTLRAGRYLNHELHTKLLT 93
Pyho      -TD---SNSYLAQAGIALPLLEGD-SIRIHVLDTIKAGKYINDEEIVWNVI 82
Bsub      SVKN--SNSVYAQGGIAAAYAKDD-SIEAHLEDTLYAGCGHNNLAIVADVL 82
Mjan      LFGK-SGCTVMAEGGYNAVFNPKD-SFKKHFYDTVKGGGFINNPKLVEILV 81
                :   ::*        * :   *  **  :*
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Fig. 5. Alignment of the N-terminal amino acid Sequence of L-aspartate oxidase from *Escherichia coli* (Ecol), *Pseudomonas aeruginosa* (Psae), *Mycobacterium leprae* (Mlep), *Mycobacterium tuberculosis* (Mtub), *Streptomyces coelicolor* (Scoe), *Synechocystis* PCC6803 (Syny), *Aquifex aeolicus* (Aqae), *Pyrococcus horikoshii* (Pyho), *Bacillus subtilis* (Bsub), and *Methanococcus jannashii* (Mjan). Residues identical or conservatively substituted in all sequences are indicated with an asterisk (*) or a colon (:), respectively. The arrow indicates the Lys residue modified by N^6-(6-carboxyhexyl)-FAD succinimidoester.

specific covalent modification of residue Lys38. As shown in Fig. 5, such a residue is highly conserved in all known L-aspartate oxidase sequences except from *A. aeolicus* and *P. horikoshii,* where it is substituted by an Arg. In particular, this Lys is one of the only two basic residues conserved or conservatively subsituted in the first 100 N-terminal amino acids of L-aspartate oxidase, the other being His66. These results allow us to identify Lys38 as one of the residues involved in the binding site of the adenine moiety of FAD, a conclusion which is also strengthened by the comparison studies reported below.

As detailed in Mattevi *et al.* (1999), *E. coli* thioredoxin reductase is the top-scoring protein in a search for structural homologues of L-aspartate oxidase. Thus, it is possible to fit the FAD conformer present in thioredoxin reductase to the experimentally determined three- dimensional structure of apo-L-aspartate oxidase to obtain a model for the holo-enzyme. In order to use such a computer-created model as a starting point to study protein–coenzyme interactions in L-asparatate oxidase, however, it is necessary to obtain independent experimental data to confirm the proposed location of FAD in holo-L-aspartate oxidase. As shown in Fig. 6, the experimental results presented in this report in fact do agree with the modeling studies reported in Mattevi *et al.* (1999) since Lys38 is indeed positioned at or close to the putative adenine binding site of L-aspartate oxidase. In addition, comparison between L-aspartate oxidase and D-amino acid oxidase shows that Lys38 of L-aspartate oxidase is in a position similar to the one occupied by Lys163, the flavinylated residue in D-amino acid oxidase (Stocker *et al.*, 1996; Mattevi *et al.*, 1996) (Fig. 6). In fact, although the two enzymes do not share significant overall primary structure

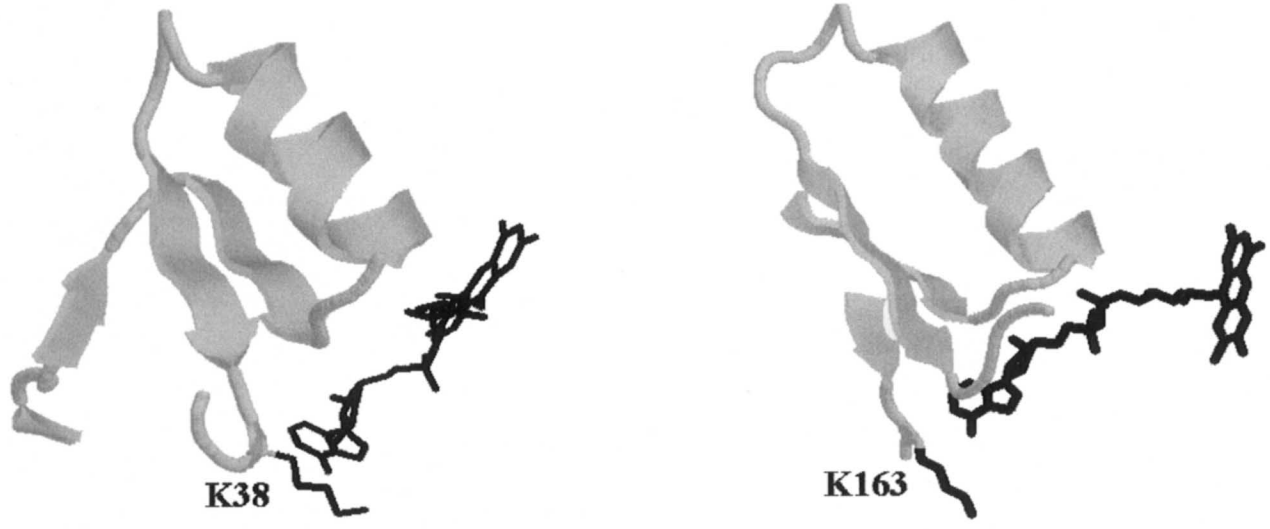

Fig. 6. Cartoon model of the three-dimensional structure of the N-terminal portion of (left) L-aspartate oxidase (residues 2–40) (Mattevi *et al.*, 1999) and (right) D-amino acid oxidase (residues 2–40 and 160–164) (Mattevi *et al.*, 1996), showing the position of Lys38 (L-aspartate oxidase) and Lys163 (D-amino acid oxidase) relative to the typical βαβ-fold common to most FAD-binding flavoproteins and the adenylic portion of FAD. The model of FAD in L-aspartate oxidase is drawn to indicate the putative position of the coenzyme (see text for details).

similarity, both Lys38 (L-aspartate oxidase) and Lys163 (D-amino acid oxidase) are positioned at or close to the C-terminus of a βαβ motif common to both enzymes and to most FAD-binding proteins and known to be involved in the binding of the ADP moiety of FAD (Wierenga *et al.*, 1986).

The biochemical properties of N^6-(6-carbamoylhexyl)-FAD-L-aspartate oxidase indicate that this enzymatic form, even if it presents the same absorption spectrum as the native enzyme, does not possess the same properties since it has a very low L-aspartate oxidase activity, no fumarate reductase activity, and does not show spectral perturbations in the presence of molecules known to have such an effect on native L-aspartate oxidase. This indicates that, although the flavin derivative is bound to the region predicted from sequence similarity and molecular modeling studies, the microenvironment around the isoallossazine is different as compared to the native enzyme. This result agrees with previous studies based on site-directed mutagenesis of residues likely to be involved in the isoallosazinic binding site of L-aspartate oxidase which showed that also substitutions causing minor changes in the polarity of the environment around FAD (such as Ser45His) cause total loss of enzymatic activity (Mortarino *et al.*, 1996). In conclusion, the present results confirm the model proposed for the binding of FAD to apo-L-aspartate oxidase, but the flavinylation procedure reported is not suitable for the preparation of active L-aspartate oxidase with improved catalytic properties.

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