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Biochimica et Biophysica Acta 1229 (1995) 381–385

BBA Biochimica et Biophysica Acta

Changes in secondary structure and flavin microenvironment between *Azotobacter vinelandii* lipoamide dehydrogenase and several deletion mutants from circular dichroism

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Received 31 October 1994; revised 23 January 1995; accepted 30 January 1995

Abstract

Circular dichroism (CD) has been used to investigate the secondary structure of wild-type lipoamide dehydrogenase from *Azotobacter vinelandii* and two deletion mutants lacking 9 and 14 C-terminal amino acids, respectively (these mutants are referred to as Δ9 and Δ14). From analysis of CD-spectra in the 190–240 nm region it was found that the α-helix content did not change (32%) among the three proteins, but the β-sheet structure is distinctly less in case of the deletion mutants as compared to the wild-type enzyme. Upon dilution of the Δ14 mutant from 30 μM to 2 μM the CD spectrum showed a drastic reduction in α-helix content (from 32% to 17%) which is ascribed to a weakening of the subunit-subunit interaction. The region where the flavin prosthetic group absorbs light (300–500 nm) exhibits characteristic changes between wild-type protein (or Δ9 mutant) and the Δ14 mutant. While wild-type and Δ9 mutant proteins have virtually no optical activity within the lowest energy absorption band, the Δ14 mutant gives a negative Cotton effect in this region. The second absorption band at higher energy shows strong positive optical activity in case of Δ9 mutant and wild-type enzyme and a smaller effect in case of the Δ14 mutant. Since the optical activity of the flavin chromophore in flavoproteins is very sensitive to its microenvironment, the experimental results are indicative of a changed surrounding of the flavin, which is in full agreement with the partial exposure of the flavin to the solvent in the Δ14 mutant (in contrast to the shielded flavin in wild-type enzyme), as found previously with fluorescence relaxation spectroscopy (Bastiaens, P.I.H., Van Hoek, A., Van Berkel, W.J.H., De Kok, A. and Visser, A.J.W.G. (1992) Biochemistry 31, 7061–7068).

Keywords: Secondary structure; Circular dichroism; Cotton effect; Flavin microenvironment; Deletion mutant

1. Introduction

Lipoamide dehydrogenase belongs to the class of disulfide oxidoreductases. This homodimeric flavoprotein catalyses the NAD^+-dependent oxidation of reduced lipoyl groups which are covalently attached to the core component (lipoate acyltransferase) in different multienzyme complexes [1]. The three-dimensional structure of lipoamide dehydrogenase isolated from *Azotobacter vinelandii* has been solved to quite high resolution [2]. In a previous study, the fluorescent FAD prosthetic group has been used as a natural marker to probe the dynamical microenvironment of the flavin fluorophore in the protein [3]. Using a viscous solvent, it was found from fluorescence relaxation spectroscopy that the flavin in the wild-type enzyme is rigid on a nanosecond timescale and is shielded from the solvent. In contrast, a deletion mutant lacking 14 C-terminal amino acids shows rapid dipolar relaxation arising from both protein and solvent dipoles. In this study we describe the use of circular dichroism as an additional technique to investigate the flavin surrounding medium as applied earlier for site-selected mutants of lipoamide dehydrogenase [4]. Circular dichroism (CD) spectra of flavoproteins in the visible and near ultraviolet flavin light-absorbing region are very characteristic and they show good correlation between the type of CD spectrum observed and the particular function of that flavoprotein [5–7]. Rather than conventional spectrophotometry the optical activity of the visible and near ultraviolet absorption bands is a sensitive indicator for any changes occurring in the vicinity of the flavin prosthetic group. CD spectra in the far ultraviolet peptide light-absorbing region of proteins yield information on the secondary structure of

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SSDI 0005-2728(95)00026-7

the polypeptide chain. This type of information is useful in view of the fact that the three-dimensional structure is known only for the wild-type enzyme and not for the deletion mutants.

2. Materials and methods

Wild-type and mutant lipoamide dehydrogenase was expressed in *E. coli* TG2 and purified as described before [8,9]. Enzyme concentrations were determined using the molar absorption coefficient of bound FAD, $\epsilon_{457} = 11.0$ $mM^{-1}\,cm^{-1}$. For the Δ14 mutant, $\epsilon_{450} = 11.1$ $mM^{-1}\,cm^{-1}$ was used. The buffer used was 50 mM potassium phosphate, 0.5 mM EDTA (pH 7.0). FMN and FAD were obtained from Boehringer (Mannheim, Germany).

Absorption spectra of 30 μM enzyme solutions in 1-cm-pathlength cuvettes (Hellma) between 300 and 600 nm were recorded on an Aminco DW-2000 spectrophotometer.

CD spectra were recorded on a Jobin-Yvon Mark V dichrograph. The control of the instrument was performed by an Asyst™-oriented interface between the spectrometer and a PC. For the region between 190 and 240 nm cuvettes with a pathlength of 0.1 cm (Hellma) were used in case of 2 μM enzyme solutions and of 0.01 cm (Hellma) in case of 30 μM enzyme solutions. The scan speed was 0.055 $nm\,s^{-1}$. CD spectra of 30 μM enzyme solution in 1-cm-pathlength cuvettes (Hellma) were obtained between 300 and 600 nm at a scan speed of 1 $nm\,s^{-1}$. In all CD experiments the buffer blank was separately recorded and subtracted from the sample spectrum. All spectra consisted of either 500 (190–240 nm) or 1000 (300–600 nm) data points and they were transferred to an Apple Macintosh computer and further processed. At least four CD spectra were averaged. The experiments were conducted at 22° C. Far UV-CD spectra between 190 and 240 nm were analysed for secondary structure using the CONTIN computer program [10].

Steady-state fluorescence experiments on protein-bound FAD were performed essentially as described elsewhere [11].

3. Results and discussion

3.1. Far-UV CD spectra

In Fig. 1, a typical example of an experimental spectrum of 2 μM wild-type lipoamide dehydrogenase is shown. The smooth spectrum is the fit to secondary structure content using the 16 basis CD spectra of 'known' proteins [10]. According to the (weighted) residuals fluctuating randomly around zero, the fit is excellent. In Table 1, multiple results have been collected. The α-helix content found by CD is in very good agreement with the one

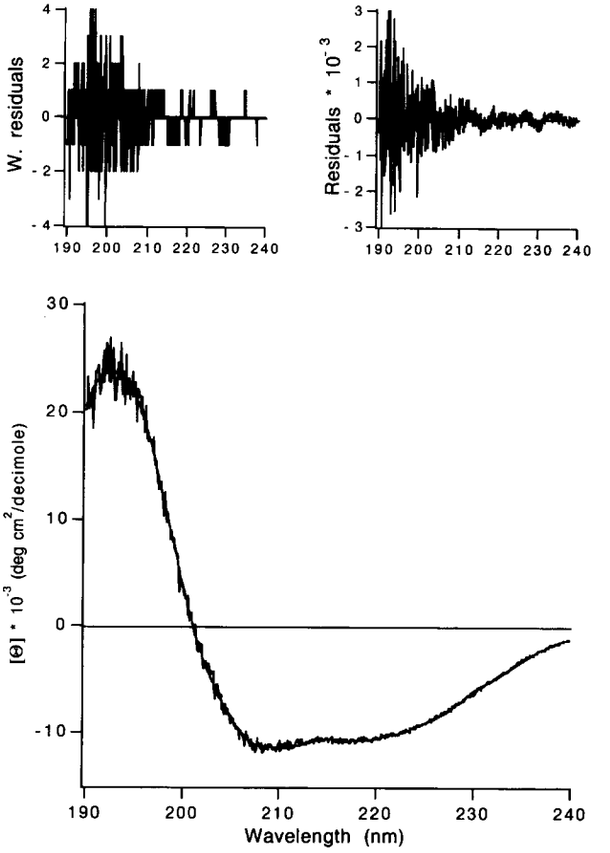

Fig. 1. Experimental (noisy) and fitted (smooth) far-UV CD spectra (500 data points) of 2 μM wild-type lipoamide dehydrogenase. In addition to a direct (non-weighted) analysis, the analysis was also performed over two separate regions of the spectrum (which is an option provided by the computer program): the more noisy spectrum between 190 and 195 nm and the less noisy region between 195 and 240 nm. The weighted and non-weighted residuals, shown in the top panels, indicate optimal fitting because of their random distribution around the zero-line. Both analyses yielded the same secondary structure content. The secondary structure content (averaged over at least four determinations) is given in Table 1.

derived from the three-dimensional (X-ray) structure which amounted to 30% [2]. The amount of β-sheet in case of the solution CD spectra (50%) is distinctly higher than found in the protein crystal (27% [2]). It has been shown that the prediction of β-sheet content (antiparallel and parallel) is not as good as when data are obtained up to 178 nm [12]. Prediction of α-helix content remains reliable when the experimental data are truncated at 190 nm [12]. In the remaining section we will therefore discuss the relative changes observed in the amount of β-strands in wild-type and mutant proteins. Apparently, with respect to β-sheet content, there is better agreement between the two deletion mutants and the crystalline wild-type enzyme. This is illustrated in Fig. 2 in which the fitted spectra of the three proteins at 30 μM concentration have been given. Note the resemblance of the spectra of the two deletion mutants which is reflected by a similar secondary structure content

Table 1
Secondary structure content of wild-type and deletion mutant lipoamide dehydrogenase

Enzyme	α-Helix (%)	β-Sheet (%)	β-Turn (%)	Remainder (%)
Wild-type (2 μM)	30 (2)	55 (3)	15 (2)	1 (3)
Wild-type (30 μM)	33 (1)	48 (3)	9 (3)	10 (3)
Δ9 (2 μM)	30 (1)	37 (5)	12 (3)	21 (4)
Δ9 (30 μM)	32 (2)	34 (3)	12 (1)	22 (2)
Δ14 (2 μM)	17 (1)	47 (3)	22 (2)	13 (2)
Δ14 (30 μM)	32 (1)	33 (2)	13 (1)	22 (1)

Values between parentheses are the standard errors resulting from multiple determinations.

given in Table 1. For the two deletion mutants the amount of β-sheet is distinctly lower than that of the wild-type enzyme. It has been established in lipoamide dehydrogenase from *Pseudomonas* species (showing up to 84% sequence identity as compared to the *A. vinelandii* lipoamide dehydrogenase) that the last 10 C-terminal residues (which are poorly defined in the electron density map of the *A. vinelandii* enzyme) can be observed in the crystal structure [13]. The C-terminus is folded back towards the active site and residues 467–472 interact with the other subunit. Deletion of 9 residues results in loss of two intersubunit hydrogen bonds (Tyr16-His470′; Glu24-Ala468′). These interactions lead apparently to a higher β-sheet structure in the wild-type enzyme. Only the Δ14 mutant enzyme exhibited strongly concentration-dependent spectra. This is illustrated in Fig. 3, showing experimental and fitted CD spectra of Δ14 lipoamide dehydrogenase at two different concentrations. At lower concentration, the α-helix content considerably decreases (from 32 to 17%, see Table 1). This large change is equivalent to a gross conformational change and is probably connected to the decreased stability of the dimer when 14 residues are deleted [9,14]. Deletion of 14 residues 'disrupts' helix 4.11 formed by residues 454–462, while residues 467–472 are directly involved in subunit-subunit interaction [13]. The lower percentage of α-helix content of the Δ14 enzyme at 2 μM concentration is not due to monomerization with subsequent FAD release. This conclusion is supported by the following observations: (i) upon dilution of the Δ14 enzyme to 2 μM, there is no change in the optical flavin spectrum which is typical for the dimeric state [11]; (ii) from relative fluorescence quantum yield measurements of protein-bound FAD as a function of enzyme concentration, an apparent K_d value of 0.25 ± 0.1 μM is estimated for the monomer-dimer equilibrium of the Δ14 protein. Although this value is about two orders of magnitude higher than the corresponding value for the wild-type enzyme [11], this suggests that, at 2 μM concentration, the majority of the Δ14 protein molecules is in the dimeric state.

3.2. Visible and near UV spectra

It has been pointed out by Edmondson and Tollin [5] that CD spectra in the flavin-absorbing region can be

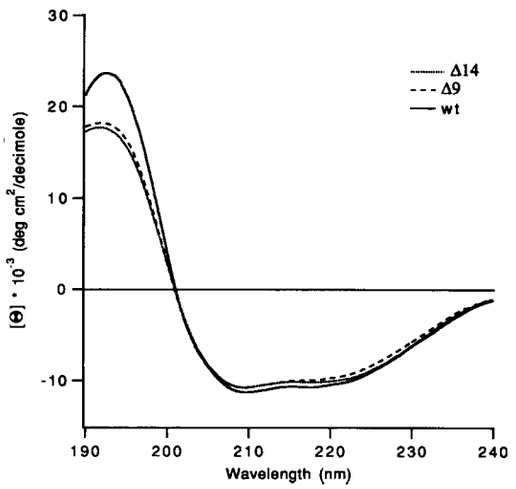

Fig. 2. Fitted far-UV CD spectra of 30 μM solutions of wild-type, Δ9 and Δ14 mutant lipoamide dehydrogenase. The secondary structure content (averaged over at least four determinations) of the three enzyme solutions is given in Table 1.

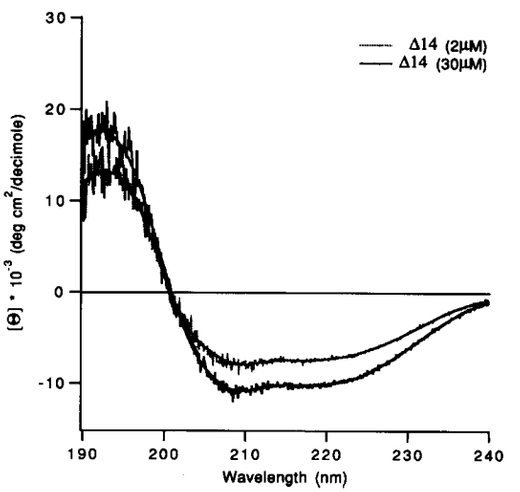

Fig. 3. Experimental and fitted far-UV CD spectra of Δ14 mutant lipoamide dehydrogenase at two different concentrations. Dotted line: 2 μM; solid line: 30 μM. The secondary structure content (averaged over at least four determinations) is given in Table 1.

considered as fingerprints for the type of flavoprotein. The CD spectra of flavodoxins possess characteristic features for this class of electron transfer proteins, while a flavoprotein with reductase function has its own characteristic CD spectrum. In other words, CD spectra (rather than absorption spectra) report about the flavin microenvironment which can be subtly altered depending on the catalytic function. The same arguments apply for lipoamide dehydrogenase, which hardly shows optical activity in the lowest energy singlet-singlet transition (visible absorption band) and a strong positive Cotton effect in the near-UV absorption band. Alongside other spectral techniques, CD spectra have been used to characterize site-selected mutations in lipoamide dehydrogenase [4]. In Fig. 4, both absorption and CD spectra are presented for wild-type lipoamide dehydrogenase and the two deletion mutants. Wild-type and Δ9 mutant have indistinguishable light absorption spectra, but slightly altered CD spectra in the second, near-UV absorption band with positive Cotton effects. The first, visible absorption band is virtually optically inactive in both enzymes. The dimeric Δ14 lipoamide dehydrogenase already has an absorption spectrum with a blue-shifted maximum characteristic for a flavin in more polar environment, which is in agreement with solvent exposure found with fluorescence relaxation spectroscopy [3]. The CD spectrum of dimeric Δ14 lipoamide dehydrogenase is completely altered. It shows a negative Cotton effect in the first band and a positive Cotton effect in the second absorption band. Such a CD spectrum is reminiscent of a reductase like ferredoxin-NADPH oxidoreductase [5] or of the bioluminescent lumazine protein from *Photobacterium leiognathi* recombined with riboflavin [15]. The latter binding site is rigid but fully exposed to the solvent. The optical activity observed is induced by the protein. Evidence for this is provided by the CD spectra of free FMN and FAD in aqueous buffer which give rise to effects about 10-times weaker (see Fig. 5). These spectra were obtained more than 25 years ago [16,17]. Free FAD in aqueous buffer is not a good reference for flavoproteins with FAD as prosthetic group, since bound FAD is always present in an fully extended conformation in which the adenine is located far away from the flavin. The excitonic band at higher energy (second, near-UV absorption band, see Fig. 5) is probably due to interaction between adenine and flavin, since in FAD about 80% of the molecules are in internally complexed form [18,19]. FMN is then a more appropriate model compound for comparison with the dichroic properties of (extended) FAD in flavoproteins. The optical activity of the lumiflavin chromophore, which is very weak in aqueous solution, becomes induced upon binding of the lumiflavin part of FAD to the protein.

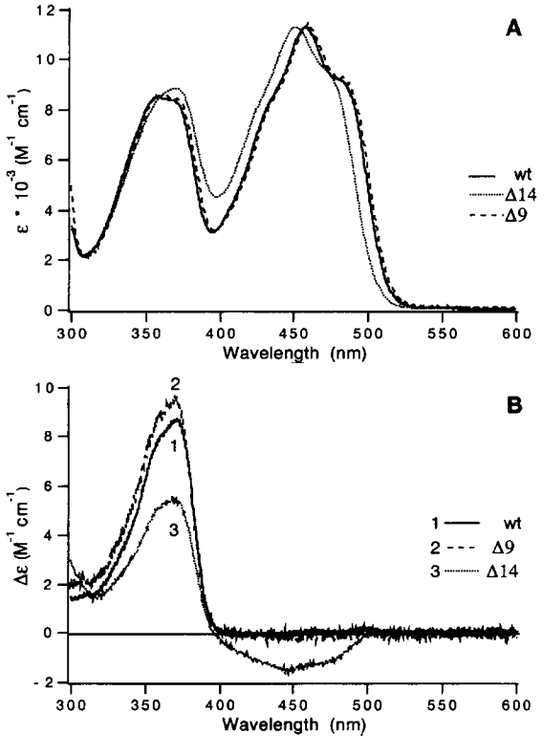

Fig. 4. Visible and near-UV absorption (A) and CD (B) spectra (1000 points) of 30 μM solutions of wild-type, Δ9 and Δ14 mutant lipoamide dehydrogenase. Solid line (curve 1): wild-type; dashed line (curve 2): Δ9; dotted line (curve 3): Δ14.

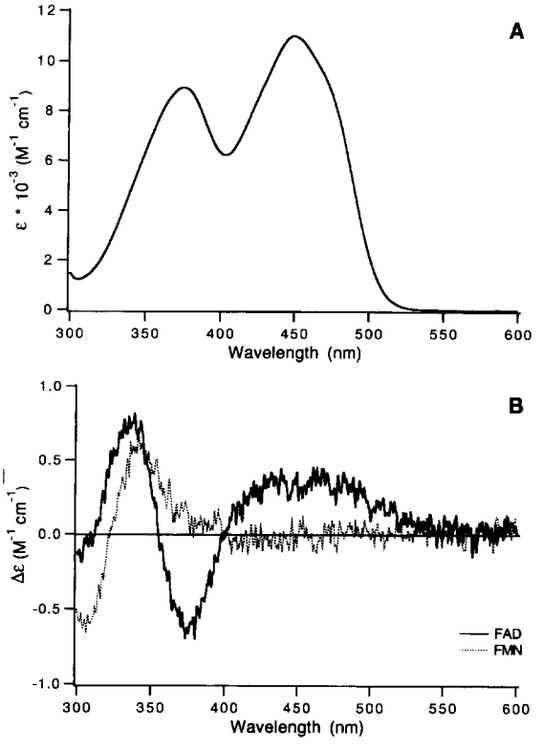

Fig. 5. Visible and near-UV absorption spectrum of FAD (A) and CD spectra (B) of 30 μM FAD and FMN solutions in aqueous buffer (pH 7). Solid line: FAD; dashed line: FMN. Note that the CD scale is expanded as compared to the scale in Fig. 4B.

4. Conclusions

Despite the removal of two residues of helix 4.11 in the Δ14 mutant, the α-helix content from CD data of the three lipoamide dehydrogenase preparations is the same as derived from the X-ray structure of the wild-type (30%).

The percentage of β-strand secondary structure from CD data of the deletion mutants is similar to that derived from the X-ray structure of the wild-type (27%); the CD data of the wild-type enzyme indicate more β-structure.

The flavin 'fingerprint' CD spectra show that removal of two intersubunit hydrogen bonds, as in the Δ14 mutant, is required to make the flavin more accessible to solvent. The CD spectrum of the Δ14 mutant resembles that of a flavoprotein with reductase function.

Acknowledgements

Adrie de Jager and Cornelis Schillemans are thanked for hardware and software adaptations required for Asyst interfacing of the Jobin-Yvon Mark V dichrograph. We thank Dr. S.W. Provencher for making available the CONTIN program.

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