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Primary sequence, oxidation-reduction potentials and tertiary-structure prediction of *Desulfovibrio desulfuricans* ATCC 27774 flavodoxin

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Flavodoxin was isolated and purified from *Desulfovibrio desulfuricans* ATCC 27774, a sulfate-reducing organism that can also utilize nitrate as an alternative electron acceptor. Mid-point oxidation-reduction potentials of this flavodoxin were determined by ultraviolet/visible and EPR methods coupled to potentiometric measurements and their pH dependence studied in detail. The redox potential E_2, for the couple oxidized/semiquinone forms at pH 6.7 and 25°C is −40 mV, while the value for the semiquinone/hydroquinone forms (E_1), at the same pH, −387 mV. E_2 varies linearly with pH, while E_1 is independent of pH at high values. However, at low pH (< 7.0), this value is less negative, compatible with a redox-linked protonation of the flavodoxin hydroquinone. A comparative study is presented for *Desulfovibrio salexigens* NCIB 8403 flavodoxin [Moura, I., Moura, J. J. G., Bruschi, M. & LeGall, J. (1980) *Biochim. Biophys. Acta 591*, 1–8].

The complete primary amino acid sequence was obtained by automated Edman degradation from peptides obtained by chemical and enzymic procedures. The amino acid sequence was confirmed by FAB/MS.

Using the previously determined tridimensional structure of *Desulfovibrio vulgaris* flavodoxin as a model [similarity, 48,6%; Watenpaugh, K. D., Sieker, L. C., Jensen, L. H., LeGall, J. & Dubourdieu M. (1972) *Proc. Natl Acad. Sci. USA 69*, 3185–3188], the tridimensional structure of *D. desulfuricans* ATCC 27774 flavodoxin was predicted using AMBER force-field calculations.

Flavodoxins are a group of small electron-transfer proteins [1–3] (≈ 15–23 kDa) isolated from different organisms, that contain a single FMN group bound non-covalently to the polypeptide chain. They can transfer two electrons at low and differentiated redox potentials.

The way in which a flavodoxin's peptide chain modulates the redox properties of the FMN cofactor has been the major focus of the tridimensional structural studies. Thermodynamic data have been discussed in terms of the binding of the cofactor to the polypeptide chain in different redox states of the protein. The pH dependence of the mid-point oxidation-reduction potentials has been extensively explored.

To infer on factors controlling the electron-transfer process, X-ray diffraction and NMR spectroscopy have been valuable tools to reveal the environment of the FMN group bound to the protein. Four tertiary structures are available, for *Clostridium beijerinckii* (former *Clostridium* CP) [4, 5], *D. vulgaris* Hildenborough [6–8], *Anacystis nidulans* [9] and *Chondrus crispus* [10, 11] flavodoxins from X-ray studies; and structures were proposed from two-dimensional and three-dimensional NMR studies for *Megasphaera elsdenii* [12–15] and *D. vulgaris* [16, 17] flavodoxins. Amino acid sequences are also available for *D. desulfuricans* ATCC 29577 [18] *Desulfovibrio salexigens* [19] and *Desulfovibrio gigas* ATCC 29464 and 19364 flavodoxins [20]. Cloning and gene expression on flavodoxins from *C. beijerinckii* [21], *D. vulgaris* [3, 22, 23], *D. salexigens* [19] and *D. gigas* (two strains) [20] have been reported.

In this study we present the isolation, purification and characterization of *D. desulfuricans* ATCC 27774 flavodoxin. Redox potentials were determined by potentiometric titrations coupled with visible and EPR techniques, at different pH values. The value of the dissociation constant of the FMN group was estimated by differential spectrophotometric titrations of FMN with apo-flavodoxin. The complete amino acid sequence of *D. desulfuricans* ATCC 27774 flavodoxin was obtained and its degree of similarity with other *Desulfovibrio* species flavodoxins is presented. A theoretical model of a tertiary structure obtained using the most similar sequence available (*D. vulgaris* flavodoxin) is predicted using molecular-modeling tools.

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Note. The novel amino acid sequence data published here have been submitted to the EMBL sequence data bank and are available under the accession number P80312.

MATERIALS AND METHODS

Growth of microorganisms

D. desulfuricans ATCC 27774 cells were grown using the medium described by Liu and Peck [24]. Low concentration of iron was a requirement for maximal flavodoxin expression, confirming previous findings indicating the replacement of flavodoxin by ferredoxin in a rich iron media [25]. Nitrate rather than sulfate was used as a terminal electron acceptor in order to obtain a higher yield of biomass. The cells were harvested by centrifugation and stored at −80 °C until use. The cells were thawed and resuspended in 10 mM Tris/HCl, pH 7.6, and broken by means of a French press (6205 kPa). Cells from *D. salexigens* strain British Guiana (NCIB 8403) were grown on 37 °C on lactate/sulfate medium [26, 27] and treated as previously indicated.

Protein purification

All the purification procedure of *D. desulfuricans* ATCC 27774 flavodoxin was carried out at 4 °C. The crude extract (total volume 1500 ml) obtained from 800 g cells was dialyzed and centrifuged at 19000×*g* during 30 min then at 180000×*g* for 75 min. The supernatant was applied to a DEAE-52 (Whatman) column (5 cm×40 cm) and the bound proteins eluted with a linear gradient from 10 mM to 500 mM Tris/HCl, pH 7.6 (total volume of 2 l). A yellow fraction eluting at 400 mM was collected, concentrated in a Diaflo apparatus (Amicon) with a YM5 membrane and centrifuged at 4500 *g* for 30 min. The sample was then filtered with a membrane of 0.22 μm, applied to a HPLC with a DEAE-52 TosoHaas column (20 cm×5.5 cm) and eluted with a linear gradient from 10 mM to 500 mM Tris/HCl, pH 7.0. Flavodoxin was collected around 380 mM. The same concentration/centrifugation procedure was then repeated. A final purification step was performed using TSK-GEL G-3000SW LKB HPLC column (60 cm×2.1 cm; gel filtration), eluted with 0.3 M Tris/HCl, pH 7.0. The flavodoxin was dialyzed against 10 mM Tris/HCl, pH 7, concentrated and stored at −20 °C. An A_{280}/A_{450} purity ratio of 3.7 was obtained. Liophilization procedure is avoided due to dissociation of the FMN group. Approximately 350 mg pure flavodoxin was obtained from 1 kg wet cells under the growth conditions used. *D. salexigens* flavodoxin was purified as indicated in reference [28].

Oxidation/reduction studies (mid-point oxidation-reduction potentials)

Mid-point oxidation-reduction potentials of *D. salexigens* and *D. desulfuricans* ATCC 27774 flavodoxins were determined by ultraviolet/visible potentiometric titrations performed under anaerobic conditions in an optical redox cell slightly modified from the design of Dutton et. al. [29, 30]. The solution potential was measured with a Crison 2002 potentiometer equipped with platinum (P1312 radiometer) and Ag/AgCl (K 8040 radiometer) electrodes and quoted relative to the normal hydrogen standard electrode. The following redox mediators were present at the final concentration of 5 μM: 1,4-naphthoquinone, methylene blue, triquat, phenosafranine, benzylviologen, methylviologen, dichloroindophenol, benzoquinone, anthraquinone-2-sulfonic acid, phenazinamethosulfate, dimethyltriquat, indigo tetrasulfonate, 2-hydroxy-1,4-naphthoquinone, 5-hydroxy-1,4-naphthoquinone, duroquinone, phenazine and safranine.

Solution redox potentials (in equilibrium) were varied by adding appropriate volumes of deareated dithionite as reductant. Ultraviolet/visible spectra were recorded during titration on a Shimadzu spectrophotometer 265FS. To study the pH dependence of the mid-point redox potentials of flavodoxin, different buffers were used at the following pH values: 0.05/0.05 M sodium citrate/sodium phosphate, pH 5.5; 0.1 M, Tris/HCl, pH 6.7−8.0; 0.1 M glycine/NaOH, pH 9.1. All experiments were performed under a purified argon atmosphere (passed through an oxygen trap from Chemical Research Supplies). Electrodes were calibrated with quinhydrone at pH 7.0 [31].

EPR redox titrations (at pH 8.0) were also performed for *D. desulfuricans* ATCC 27774 flavodoxin. The redox potentials were varied and measured as described above. The samples (protein at a concentration of 200 μM) were poised at different redox potentials and transferred under purified argon to EPR tubes and frozen in liquid nitrogen for subsequent quantification. Spectra were recorded at 200 K on a Bruker ESP 300 Spectrometer.

Estimation of dissociation constants for the complex apo-flavodoxin/FMN

Apoprotein was prepared by extracting the FMN from the holoprotein with trichloroacetic acid as described [32]. Apo-flavodoxin was collected by centrifugation and resuspended after neutralization with Tris/HCl, pH 9. This solution was freshly prepared before conducting the association assay. Commercial FMN (Sigma) or flavodoxin trichloroacetic acid FMN extracted and repurified by gel filtration (Sephadex G-10) were used. Differential spectrophotometric titration was used to determine the dissociation constant [2, 3, 33]. All solutions were buffered at pH 7.0 with 0.1 M Tris/HCl. Aliquots of apoprotein were added to one cuvette while the same volume of buffer was added to the reference cuvette. Temperature was maintained at 25 °C. The titration curve was followed by the difference of optical absorbance ($A_{491}-A_{437}$).

Sequence of *D. desulfuricans* ATCC 27774 flavodoxin

N-terminal sequence analyses of native flavodoxin and its derived peptides were performed on an Applied Biosystem Sequencer model 477A, coupled to an Applied Biosystem 120 Analyzer following the manufacturer's instructions.

Cleavage of flavodoxin (10 mg/ml in 70%, by vol., formic acid) at methionine residues was performed with cyanogen bromide (100 mg/ml final concentration) for 4 h at room temperature, in the dark, under a nitrogen atmosphere. The reaction mixture was then diluted with water and lyophilized.

Proteolytic digestion of flavodoxin (5 mg/ml in 100 mM sodium phosphate, pH 7.8) was performed with α-chymotrypsin (Sigma), endoproteinase Glu-C (Boehringer Mannheim), endoproteinase Arg-C (Boehringer Mannheim) at an enzyme/substrate ratio of 1:50 (by mass) at 37 °C overnight. Alternatively, digestion with endoproteinase Glu-C was carried out in 50 mM ammonium bicarbonate, pH 8.0.

Peptides were isolated by reverse-phase HPLC on either a Pep-S (Pharmacia) or Lichrospher RP-100 (Merck) column (25 cm×0.4 cm, C_{18}, 5-mm particle size) eluting at 1 ml/min with a gradient of 0.1% trifluoroacetic acid in (A) water and (B) acetonitrile, following the absorbance at 220 nm.

Amino acid analyses were performed using a Beckman 6300E analyzer after sample hydrolysis with 6 M HCl at

110°C for 24, 48 and 72 h in sealed evacuated tubes. Values for threonine, serine and tyrosine were corrected after extrapolation to zero-time hydrolysis. Cysteine and methionine were recovered as cysteic acid and methionine sulfone, respectively, after performic oxidation [34, 35].

FAB mass spectra of peptides were recorded with a mass spectrometer Mat 900 (Finnigan MaT) equipped with a liquid secondary-ion ionization system. Nominal accuracy was ±0.5 Da in the range 500–2500. The molecular mass of native apo-flavodoxin was determined by laser-desorption MS using either a Lasermat (Finnigan Mat) or a Kratos Kompact MALDI-3 instrument. Equine heart cytochrome *c* (12360.1 Da) and soybean trypsin inhibitor (20090.6 Da) were used as calibration standards. Accuracy claimed by the manufacturers was ±0.1%.

Prediction of tridimensional structure by similarity modeling

A computational prediction of the tertiary structure of the flavodoxin was performed using a personal Iris-Silicon Graphics workstation. SYBYL 5.5 (Tripos Associates) and AMBER force field [36]. *D. vulgaris* Hildenborough flavodoxin was chosen as the template structure due to its high degree of similarity, and its atom coordinates were obtained from the Brookhaven Protein Data Bank [37].

RESULTS AND DISCUSSION

Flavodoxins have been identified and isolated from several members of the *Desulfovibrio* genus [18, 26, 33]. Most have been characterized in terms of molecular mass, amino acid composition and sequences, as well as visible spectroscopy (oxidized, one-electron-reduced and two-electron-reduced forms). *D. vulgaris* flavodoxin structure was elucidated by X-ray and two-dimensional ^{1}H NMR [6–8, 16, 17].

Many studies have shown structural and physical similarities within this family, but differences exist, mainly concerning the span of redox potentials and details on the structure around the FMN-binding site [38]. Comparison of amino acid sequences indicate that all *Desulfovibrio* species flavodoxins shared a common FMN-binding site with Trp and Tyr flanking residues. Lifetime fluorescence of bound FMN in *D. gigas* flavodoxin is consistent with a more polar FMN-binding site [39].

D. desulfuricans ATCC 27774 flavodoxin spectral features, redox potentials and K_d values for the apo-flavodoxin/FMN complex

Fig. 1 indicates the spectral characteristics of the three redox forms of *D. desulfuricans* ATCC 27774 flavodoxin. These visible spectra closely resemble those of other flavodoxins isolated from different organisms including *Desulfovibrio* species [26, 33]. The absorption coefficients were calculated to be 37.0 (oxidized, 275 nm), 10.3 (oxidized, 452 nm) and 4.2 $mM^{-1}\,cm^{-1}$ (semiquinone, 580 nm). The visible spectrum B (Fig. 1) shows a highly stable semiquinone form derived from the full conversion of the FMN moiety. Fig. 2 shows the EPR semiquinone isotropic signal with noticeable hyperfine structure. The linewidth of the radical species observed in the semiquinone state narrows when the aqueous solvent is exchanged with a deuterated one (1.9 mT to 1.5 mT), indicating the presence of a neutral radical form

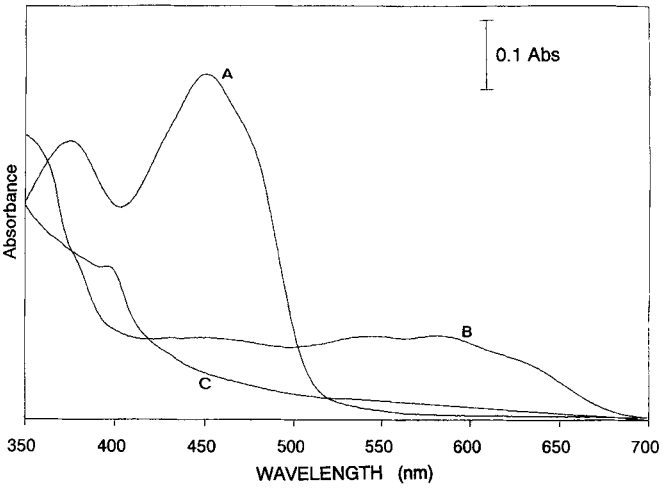

Fig. 1. Ultraviolet and visible spectra of *D. desulfuricans* ATCC 27774 flavodoxin, (A) native-oxidized, (B) semiquinone and (C) hydroquinone forms. Spectra were obtained at pH 7.6 in 10 mM Tris/HCl. The spectra were obtained poising the protein solution at adequate redox potentials (see Materials and Methods) in the presence of dye mediators. The small contribution observed in the fully reduced state (spectrum C) at 400 nm is due to the reduced forms of the mediators present. The semiquinone-form spectrum represents a redox state of the protein where this form attains maximal intensity.

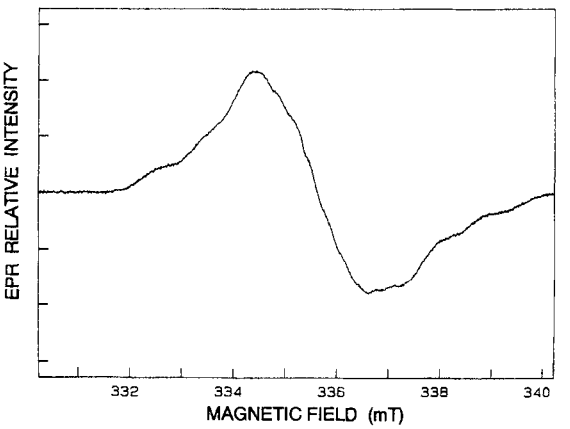

Fig. 2. EPR spectrum of the semiquinone form of *D. desulfuricans* ATCC 27774 flavodoxin. Experimental conditions: temperature, 200 K; microwave power, 2.37 mW; modulation amplitude, 0.1033 mT/point.

[40]. Redox potentials were determined, at pH 8.0, following the increase and decrease of this signal under non-saturating conditions at 120 K. The fitting of the experimental data (not shown) with two independent one-electron Nernst equations gives $E_1 = -100$ mV and $E_2 = -410$ mV.

Detailed analysis of the pH dependence of the mid-point redox potentials was made by visible redox titrations performed as described in Materials and Methods. Fig. 3 compiles the measured redox potentials and its pH dependence (including data for *D. salexigens* flavodoxin). The redox potentials of both flavodoxins in the studied pH range show a characteristic behavior, as already reported for other flavodoxins [1–3, 33]. E_2 has a linear slope of −59 mV, while E_1 is pH independent (at high pH) until a pK_a value around 6.7 is reached. It then becomes pH dependent, indicating the existence of a pH-linked process.

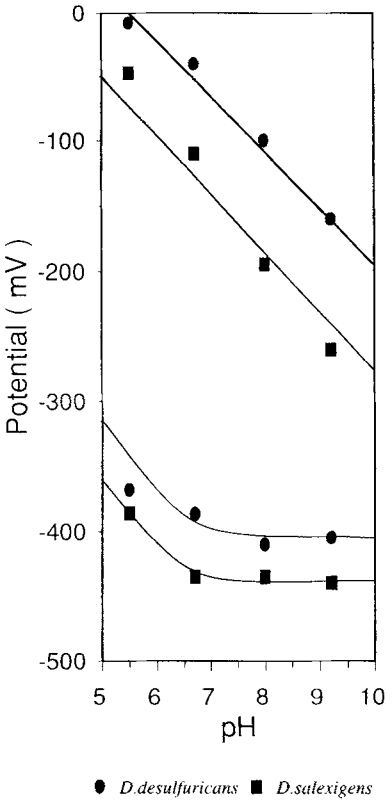

Fig. 3. pH dependence of the oxidation-reduction potentials of *D. desulfuricans* ATCC 27774 (●) and *D. salexigens* (■) flavodoxins. Experimental details as indicated in Materials and Methods.

The origin of this pH-redox-linked process has been discussed by different authors and its origin has not yet been completely assigned [2, 3]. The ratios of K_d values for quinone, semiquinone and hydroquinone of riboflavin versus those of FMN were used to show that the phosphate group of FMN has an important role on the binding of the flavin to the apoprotein [3]. The large shift of redox potential of the semiquinone/hydroquinone couple was proposed to be due to unfavorable electrostatic interactions between the negative charges of the phosphate group of FMN and the negative charge of N1 of the flavin hydroquinone [2, 41]. Ludwig and coworkers [2] also supported the existence of charge-charge repulsions but attributed it to the presence of a negative charge on a protein residue only. This conclusion was also reached based on NMR arguments [42–44]. However, the problem is not yet completely understood since when Vervoort and co-workers [41–43] introduced an extra negative charge into the FMN group they observed that the E_1 value became more negative whilst the removal of the phosphate group produced the opposite effect. Altogether, the presence of a nearby negative charge as well as the existence of the phosphate group, seems to be important in controlling the E_1 values.

The peculiarity of *D. desulfuricans* ATCC 27774 lies in the fact that, at pH 6.7, the E_2 value is about 70 mV more positive than *D. salexigens* E_2 and about 130 mV more positive than *D. gigas* E_2 (Moura, I., unpublished results) while, at the pH-independent zone, its E_1 value is 30/35 mV more positive than the typical −440 mV [3]. As a consequence, *D. desulfuricans* ATCC 27774 flavodoxin shows the widest redox-potential span (E_2-E_1) yet known in this family of proteins.

To calculate the dissociation constant of the complex of apo-flavodoxin with FMN, the addition of apo-flavodoxin to FMN was followed spectrophotometrically, as indicated in Materials and Methods. The experimental data were fitted with a theoretical curve based on a simple 1 : 1 binding model (Fig. 4). A K_d value of 0.1 nM was determined, as well as for *D. vulgaris* flavodoxin, K_d values of 0.24 nM (recombinant protein) and 0.2 nM (wild-type protein) [3].

Dissociation constants for the complexes of apo-flavodoxin with the semiquinone and hydroquinone forms were calculated, K_{dsq} (semiquinone) = 24 nM and K_{dhq} (reduced form) = 4.8 mM, using the E_1 and E_2 values determined, the K_d values for the complex FMN (oxid) and apo-flavodoxin and the values of E_1 and E_2 reported for protein-free FMN [33]. These values indicate that the semiquinone form is more tightly bound to the protein moiety, as observed for other flavodoxins, with a relationship between K_{dsq}/K_{dox} = 240, which is one order of magnitude higher than the usually reported value and the consequence of a more positive E_2 value. The ratio between $K_{dsq}/K_{dhq} = 2\times10^4$ is similar to those previously found [33, 45–47].

D. desulfuricans ATCC 27774 flavodoxin amino acid sequence

The determined primary structure of *D. desulfuricans* ATCC 27774 flavodoxin is presented in Fig. 5, along with details on peptide analysis. The sequence is compared in Fig. 6 with five other known sequences of flavodoxins isolated from *Desulfovibrio* species (*D. vulgaris* Hildenborough [33], *D. desulfuricans* ATCC 29577 [18], *D. salexigens* [19], *D. gigas* ATCC 19364 and *D. gigas* ATCC 29494 [20]). For this purpose two programs were used: MACAW [48] and PC-Gene™ version 6.7 package [49] with the SWISS-PROT 26 (August 1993) data bank [50]. The multisequential alignment and the PROSITE program [51] has determined a flavodoxin signature at positions 6–22:

ILFGSSTGNTESIAQKL22

which is known from X-ray structural analysis to be the binding region of the FMN phosphate side chain [4–7]. The six compared flavodoxins had a consensus length of 149 amino acids, sharing 25.5% identity and 45.6% similarity. Table 1 indicates the degree of identity between each pair of these proteins. The alignment was also used to determine the most significant sequence position of each amino acid on the six flavodoxins, allowing the calculation of the number of identical amino acids in one sequence position. This number (from 6 to 1) is represented in Fig. 6 with different linewidths (thicker line to the most conserved position). It was also used to help visualize the spatial protein regions that had been more or less conserved in the different known *Desulfovibrio* flavodoxins on the tertiary backbone structure of *D. vulgaris* (Fig. 7).

Both aromatic residues, Trp60 and Tyr98, that flank the flavin isoalloxazine ring in the crystal structure of *D. vulgaris* are present in *D. desulfuricans* 27774 flavodoxin. The FMN-binding site is also preserved in *Desulfovibrio* sp. flavodoxins. However, variability is found in these flanking regions when other flavodoxins are considered and the presence of two aromatic residues is not always verified: *C. beijerinckii* Met56 and Tyr98; *M. elsdenii* Met56 and Tyr98 and *A. nidulans* Trp57 and Trp94 (see Table 2).

The molecular mass of apo-flavodoxin determined by mass spectrometry (combined 15420Da ± 19) is higher (by

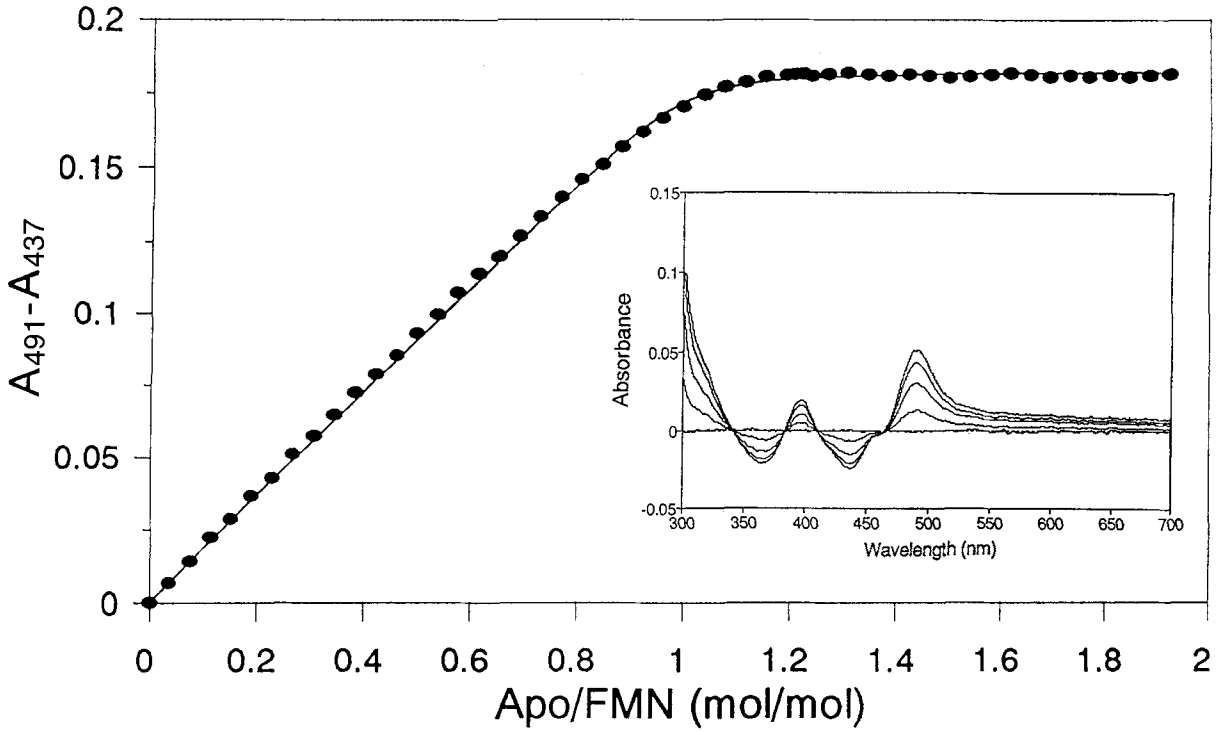

Fig. 4. Determination of the dissociation constant of the FMN group to *D. desulfuricans* ATCC 27774 apo-flavodoxin. Squares are experimental points and the line indicates the theoretical fitting. Differential spectrophotometric titration of 43.8 μM FMN (in 20 mM Tris/HCl, pH 7.6, with 417 μM apo-flavodoxin at 25°C. The insert shows equal volumes of FMN introduced into both cuvettes. Difference spectra were recorded after each addition of apo-flavodoxin to the sample cuvette and an equal volume of buffer to the reference cuvette. The fraction of bound FMN was calculated by the change in absorbance at 496 nm caused by the binding of apoprotein. The dissociation constant was calculated from the experimental curve after correction for dilution.

93Da ±19) than that calculated from its amino acid sequence (15327.2Da; the molecular mass of the holoprotein estimated by gel filtration is 16 kDa). This indicates that either the sequence of apo-flavodoxin is longer or that it contains a modified residue. Every position within the apo-flavodoxin sequence was confirmed by FAB/MS analysis of at least two different peptides, except the polypeptide stretch 56–62. However, this portion was sequenced from a CNBr-obtained peptide providing an unambiguous assignment of all the residues except Cys57, which gave a blank. A cysteine residue at this position is assumed based on its conservation in other known flavodoxin sequences. Recently, we have succeeded in crystallizing *D. desulfuricans* ATCC 27774 flavodoxin and the crystals diffract better than 2.2 nm (in collaboration with A. Romero and M. J. Romão). Resolution of the three-dimensional structure indicates the presence of one or two extra amino acid residues in the C-terminus.

Molecular modeling studies on *D. desulfuricans* ATCC 27774 flavodoxin

Using *D. vulgaris* flavodoxin tridimensional structure we have tried to predict a plausible tertiary structure for the *D. desulfuricans* ATCC 27774 flavodoxin. A similar tertiary-structure prediction for *D. salexigens* flavodoxin was reported recently following an analogous methodology [52]. These calculations are based on the assumption that if a certain class of proteins shows a high degree of similarity in function, cofactor content and amino acid sequences then a somewhat strong tertiary similarity is also expected [53, 54]. Cases are known that even with a very weak similarity in the amino acids sequences and/or function, the overall tridimensional folding pattern can be quite similar. This is the case with the remarkably similar folding pattern of the riboflavin synthetase [55] and N-carbamoyl synthetase [56, 57] and that found in flavodoxins.

Before using this method, the amino acid sequence of both *D. vulgaris* and *D. desulfuricans* flavodoxins were aligned. Since no insertions or deletions were required, and due to the high degree of similarity between the two proteins, the alignment was straightforward, and in agreement with the alignment of all other *Desulfovibrio* flavodoxins.

In the tertiary structure of *D. vulgaris*, only the side chains of the different amino acids were changed according to the alignment, conserving all the atoms in the main chain and the α-β carbon bond, to maintain the relative orientation of the side chain to the α carbon.

The resultant computational mutated structure was then released of its energetic tensions using the AMBER force field including all hydrogen atoms.

The strategy of energy minimization was developed as proposed in [58] and consisted of four sequential steps. In the first step a rigidity constraint of the main-chain atoms was defined, which also includes all the atoms of the FMN group. Energy minimization was performed ignoring electrostatic interactions, only taking into account the steric repulsions. A second step was performed with the same constraints, but considering electrostatic interactions. After these two steps the constraints are removed both from the main-chain and the FMN-side-chain atoms. The atoms of the isoalloxazine ring were kept rigid and always coplanar, in agreement with X-ray and NMR observations on flavodoxins

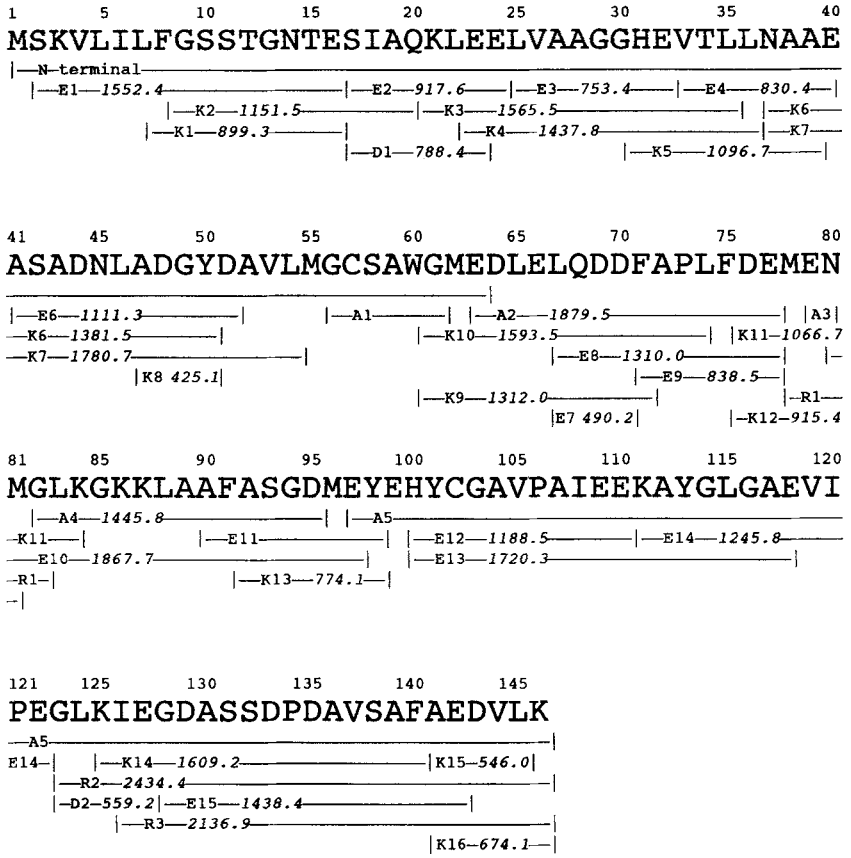

Fig. 5. Amino acid sequence of *D. desulfuricans* ATCC 27774 flavodoxin. The obtained peptides and their molecular masses are shown. A, cyanobromogen; K, α-chymotrypsin; E, endoproteinase Glu-C in sodium phosphate buffer; D, endoproteinase Glu-C in ammonium bicarbonate buffer; R, endoproteinase Arg-C. Molecular masses were determined by FAB/MS.

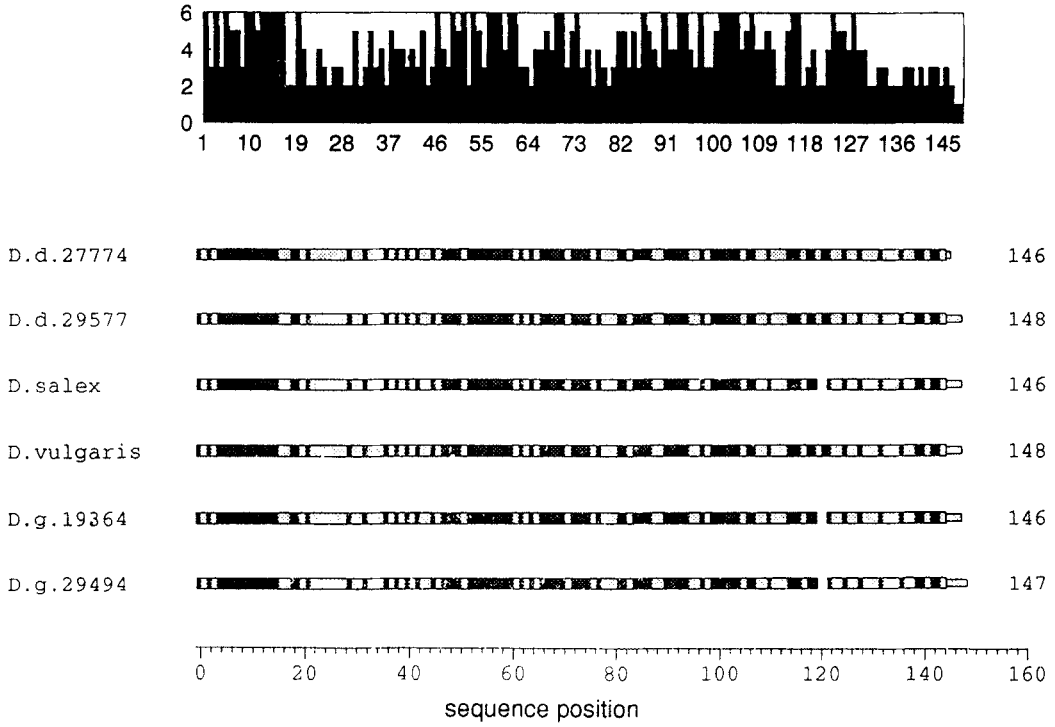

Fig. 6. Comparison of *Desulfovibrio* species flavodoxin amino acid sequences. The degrees of similarity of amino acid sequences flavodoxins are given in Table 1. d, *desulfuricans*; salex, *salexigens*; g, *gigas*.

Table 1. Matrix comparison of amino acid sequence similarity in flavodoxins isolated from sulfate reducers.

	Similarity of flavodoxins from				
	D. gigas 19364	*D. gigas* 29494	*D. vulgaris* Hld	*D. salexigens*	*D. desulfuricans* 29577
	%				
D. desulfuricans 27774	44.5	45.2	48.6	53.4	75.3
D. desulfuricans 29577	43.8	43.5	43.8	44.5	
D. salexigens	54.1	56.9	53.4		
D. vulgaris Hld	56.2	56.5			
D. gigas 29494	65.8				

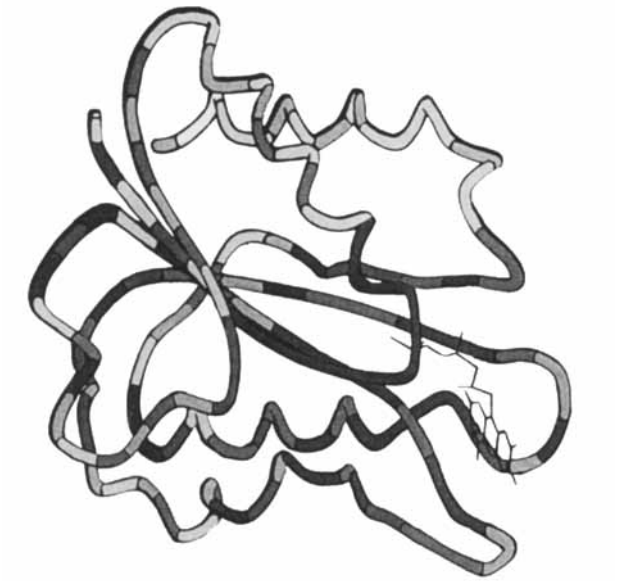

Fig. 7. Tridimensional similarity between *Desulfovibrio* species flavodoxins based on the *D. vulgaris* structure. Shadowing refers to higher or lower degrees of amino acid similarity when comparing the six amino acid sequences (darkest, six conserved amino acids until white, no conserved amino acid in a certain sequence position; see also Fig. 6).

[2, 4–8, 42–44]. The third and fourth steps were performed considering the same type of interactions used in the first and second steps, respectively.

The predicted structure of *D. desulfuricans* ATCC 27774 flavodoxin is essentially conserved in relation to the original model of *D. vulgaris* flavodoxin. The maintenance of the aromatic side chains in the same spatial position, coming from non-aligned amino acid in the primary structure was previously noted [59]. It was also shown that in spite of finding a similar general electrostatic potential on the surface of the flavodoxin (with a very acidic character), the α-helices do show a higher mutational frequency in this outer layer, exposed to the solvent, than in its inside.

A similarity of 48.6% between our primary sequence and the template, together with the mutisequential alignment of all six known sequences of *Desulfovibrio* flavodoxins, gave us confidence in our general assumption, especially near the FMN-binding region.

Analysis of the *D. desulfuricans* ATCC 27774 flavodoxin tertiary structure

The tertiary-structure prediction indicates two major mutational changes in amino acid position, Ser11 and Met62.

In all flavodoxins isolated from *Desulfovibrio*, as well as in many other species, Thr11 is conserved, but in *D. desulfuricans* ATCC 27774 a serine residue is found in this position. This is the region that binds the FMN ribityl 5′-phosphate chain. However, the mutation should not affect the binding of the cofactor, since an OH group is present in the side chain. Therefore, the H bond can be conserved as well as all others H bonds around the FMN group, as found in *D. vulgaris* template structure.

The region of Gly61–Asp62 is found to be important as the binding region for the semiquinone through an additional H bond. The effect of the flavodoxin polypeptide chain on the redox potentials of the bound FMN was predicted thermodynamically to be caused by an increase of the association constant value, in the semiquinone state (relative to the oxidized and hydroquinone states), making the oxidized/semiquinone oxidation/reduction potential more positive and the semiquinone/hydroquinone more negative, hence producing a stabilization of the semiquinone form. Several X-ray studies [2, 6, 58, 60, 61] have focused on the existence of

Table 2. Comparison of flanking amino acid sequences in flavodoxins. The sequence from *A. nidulans* was not completed, W98 was deduced from the X-ray structure [9].

Species	FMN upper amino acid	FMN lower amino acid	Closest negatively charged group	Peptide that H bonded to FMN in the semiquinone
C. beijerinckii	Met56	Trp90	Glu59	Gly57–Asp58
A. nidulans	Trp57	Trp94	–	–
M. elsdenii	Trp56	Tyr98	Glu60	Gly58–Ser59
C. crispus	Trp56	Tyr98	–	Asn57–Thr58
D. vulgaris	Trp60	Tyr98	Asp63[a]	Gly61–Asp62
D. desulfuricaus ATCC 27774	Trp60	Tyr98	Glu63[a]	Gly61–Met62

[a] Not in the same position as the amino acid in *C. beijerinckii* or *M. elsdenii* [7].

three redox states in flavodoxins. They indicate significant conformational changes in the oxidized/semiquinone redox step, while only minor alterations in the semiquinone/hydroquinone reduction, in which the control mechanism is considered to be mainly electrostatic [2].

X-ray structures of the semiquinone [5, 6, 58] had revealed that the most important conformational changes are in Gly57−Asp58 in *C. beijerinckii* or in Gly61−Asp62 in *D. vulgaris*, both in such way that an additional H bond will be established between the glycine oxygen (O57 or O61) and the FMN group.

The overall control of the redox potentials [12, 13, 58] is achieved by the sum of subtle changes in the protein structure. Our prediction, being only based on the model of oxidized *D. vulgaris* flavodoxin, as well as the employed calculation method, is not accurate enough to explain in what way a different molecular environment affects the FMN binding to the apoprotein in terms of precise distances and angles which dramatically influence H bonds and electrostatic interactions.

Mutation of Asp62 in *D. vulgaris* to Met62 in *D. desulfuricans* ATCC 27774 should be noticed, taking into account the higher stabilization of the semiquinone state and the more positive value of both E_1 and E_2 redox potentials relative to other flavodoxins with known redox potentials.

The determination of a model structure of *D. desulfuricans* ATCC 27774 flavodoxin from X-ray crystallography is underway, and a comparison with the present predicted model will be of interest (in collaboration with M. J. Romão and A. Romero, unpublished results).

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