CHEMICAL STUDY OF TWO FLAVODOXINS EXTRACTED FROM SULFATE REDUCING BACTERIA

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SUMMARY : In this paper is presented a preliminary study of the structure of two sulfate reducing bacteria flavodoxins : Desulfovibrio gigas and Desulfovibrio vulgaris.

Their respective spectroscopic characteristics have been investigated together with their amino acid composition and the nature of the flavin linked to the protein. This co-factor, that has been shown to be FMN, can be splitted off easely. In the case of the flavodoxin from D. gigas, reassociation between apoprotein and FMN is described. The two flavodoxins are very similar to the flavodoxins described from other organisms.

Desulfovibrio gigas flavodoxin has been purified and some of its physiological properties have been studied by Le Gall and Hatchikian (1). It is involved (2) like Clostridium pasteurianum flavodoxin (3) in the phosphoroclastic reaction. Furthermore this electron carrier is an intermediate in the sulfite reduction between hydrogenase and sulfite reductase (1). In the two processes flavodoxin replaces ferredoxin (Garraia et al., Bacteriol. Proc., p. 133, 1968; 2). Gel filtration gives a molecular weight of about 16,000. Emission spectra studies show that it does not contain any metal atom (unpublished data). The recrystalized protein is electrophoreticaly homogenous. Like many other flavoproteins it is photoreductible (1), producing under nitrogen, with or without EDTA, a blue radical form according to the classification of Massey and Palmer (4). This semiquinoid species may be obtained also with molecular hydrogen and hydrogenase, in the dark (5).

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We have purified also the flavodoxin from another sulfate reducing bacterium, Desulfovibrio vulgaris, strain 8303. The procedure is essentially the same that has been used for D. gigas flavodoxin. The two proteins give identical elution volumes after migration on Sephadex columns.

MATERIAL AND METHODS

Spectral characteristics. A Cary 14 spectrophotometer was used. Extinction coefficients of the oxidized proteins were determined by two methods. The first method consists in comparing the absorbances (at the 450 mμ maximum) of a flavodoxin solution before and after a treatment with Trichloro acetic acid (ε_{FMN} in TCA 3 % = 11,100). In the other method the molarity of the flavodoxin solution was determined by amino acid analysis. Absorbance of a 1 mg per ml flavoprotein solution was measured and then a 0,3 ml aliquote was taken, hydrolysed during 40 hours and analysed. Extinction coefficients for the semiquinone were estimated by plotting absorbance at 456 mμ versus absorbance at 580 mμ. The semiquinone forms were obtained by light irradiation in the presence of EDTA under nitrogen at pH 6 or with an hydrogenase preparation under hydrogen (5).

Amino Acid Analysis. Analyses were performed on an automatic amino acid analyser Beckman model "Unichrom", according to Moore and Stein (6). Samples were hydrolysed in 6 N constant boiling hydrochloric acid in evacuated sealed tubes at 110° C in an oven for 18 to 50 or 60 hours. Cystein was determined as cysteic acid after performic oxidation followed by 20 h hydrolysis (7). After preliminary experiments on D. gigas cytochrome c_3, RNase, calibration mixture, a theoretical yield of 93 % was used. Calculations were made by reference with stable amino acids, aspartic and glutamic acids, alanine, sometimes valine or leucine. Ellman's method (8) was used for sulfhydril groups determination. Tryptophan was determined by the method of Spies and Chambers (9) or by amino acid analysis after alcaline hydrolysis (10, 11) or after acid hydrolysis in the presence of thioglycolic acid to prevent tryptophan degradation (12). In this latter case a tryptophan yield of 83 % was used and in the case of alcaline hydrolysis, tryptophan was calculated by reference with alanine, leucine and isoleucine.

Flavin determination by paper chromatography. Dissociation was performed using denaturing agents like 6 M guanidine (13) or lowering pH with HCl

or TCA. Flavin was separated on a Sephadex G 15 column (10 x 150 mm). After elution the flavin was chromatographied on Whatman n° 1 paper according to Kilgour, Felton and Huennekens (14). FMN and FAD were used as markers. Spots were located by their fluorescence.

Dissociation and reversible recombination of D. gigas flavodoxin. The method of Warburg and Christian (15) modified by Baudras (16) was used. 10 mg of protein were dissolved in a centrifuge tube in 3 ml 0.05 M Tris-HCl buffer pH 7.6 containing 0.001 M EDTA. Ammonium sulfate was added up to one third of saturation ; the solution was cooled between - 10° C and - 15° C. After the addition of 2 ml 1 N HCl and complement of ammonium sulfate up to saturation, the solution was centrifuged at - 15° C during 5 minutes at 30,000 g. This operation was repeated twice. The pellet was then dissolved in 2 ml 0.05 M. Tris-HCl pH 8.1 ; 0.001 M EDTA. Salts were removed by chromatography at 4° C on a Sephadex G 15 column (25 x 200 mm) previously equilibrated with 0.05 M Tris-HCl pH 7.6 ; 0.001 M EDTA.

Recombination was tested by three methods :

- displacement of the yellow peak (abosrption maximum at 445 mμ for free FMN and at 456 mμ for flavodoxin).

- comparison of the rate of formation of the semiquinone, in the native and in the reconstituted protein, by irradiation. The semiquinone formation was measured by the appearance of the 580 mμ peak.

- respirometric method of Warburg for testing the coupling activity between molecular hydrogen and sulfite reductase (1).

FMN used was of commercial grade whereas FAD was of grade II.

RESULTS

Nature of the flavin. After dissociation from the apoproteins, flavins were chromatographied in the following solvent systems :

(a) : 5 % Na_2HPO_4, 7 H_2O in water

(b) : n-butanol-acetic acid-water (40/10/50)

In solvent (a) the Rf were as follows : 58 for FMN, 46 for FAD, 58 for flavodoxins prosthetic group. In solvent (b) they were : 10 for FMN, 3.5 for FAD, 10 for the tested flavins. Another proof that FMN is actually the prosthetic group of the flavodoxins will be given by the recombination experiments with the D. gigas group flavodoxin.

-Table I-

EXTINCTIONS COEFFICIENTS AND ABSORPTION MAXIMA OF THE TWO FLAVODOXINS

D. gigas			D. vulgaris		
wave length nm	Extinction coefficient M^{-1} cm^{-1}		wave length nm	Extinction coefficient M^{-1} cm^{-1}	
	Oxidized	Semiquinone		Oxidized	Semiquinone
273	47,000		273	48,000	
349		8,700	349		9,000
374	8,200		375.5	8,700	
456.5	10,200		456-457	10,700	
580		4,100	580		4,700

Spectral characteristics. Wave-length maxima and corresponding extinction coefficients of the two proteins are given in Table I. From the table it can be seen that the two flavodoxins have similar spectra. The two proteins possess a shoulder at 486 mμ in the oxidized form. The semiquinone forms have both two shoulders at 620 and 381 mμ. During reduction to the semiquinone, two isobestic points appear between the oxidized and the semiquinone forms at 364 and 506 mμ for D. gigas flavodoxin and at 371 and 507 mμ for D. vulgaris flavodoxin. Comparison of the absorption maxima of pure FMN with those of the two flavodoxins indicates that there is a large shift of the yellow peak towards the red (11 to 12 mμ). This fact provides a test for the association of FMN with the apoprotein.

Amino acid analysis. The results are presented in Tables II and III. The proposed amino acid compositions were calculated from the average of the four different hydrolysates except that serine and threonine were extrapolated to zero time and valine was the average of the two longer hydrolyses. Methionine content was given by extrapolating to zero time and valine was the average of the two longer hydrolyses. Methionine content was given by extrapolating to zero time and after performic oxidation. Tyrosine residues were strongly degraded after performic oxydation. After hydrolysis in the presence of thioglycolic acid, more aspartic acid and proline and less of certain

TABLE II

AMINO ACID COMPOSITION OF D. VULGARIS FLAVODOXIN

Amino acid	Amino acid residues per mole of protein: After Acid hydrolysis 18 hrs	25 hrs	40 hrs	60 hrs	Average	Performic oxydation	Hydrolysis in presence of thioglycolic acid	Proposed amino acid composition
Asp	19.70	19.32	19.96	20.22	19.80	20.35	22.5	20
Thr	6.10	5.85	6.11	5.70	6	5.7	6.3	6
Ser	6.40	5.41	5.88	5.12	7	4.8	6.8	7
Glu	16.80	16.76	17.12	17.00	16.92	17.07	17.4	17
Pro	3.80	3.90	4.25	4.38	4.08	4.2	5.4	4
Gly	18.33	18.70	18.50	18.44	18.50	18.7	18.8	19
Ala	17.38	18.25	17.82	18.11	17.90	17.85	17.90	18
Cyst						4.70		5
Val	9.35	9.47	9.30	9.93	9.62	9.45	8.3	10
Met	0	0	0	0	0	0	0	0
Ileu	8.40	8.95	8.61	8.71	8.67	9.0	8.2	9
Leu	12.62	12.74	12.62	12.66	12.66	12.3	10	13
Tyr	5.08	4.80	4.90		4.93	1.5	4.80	5
Phe	5.85	5.50	5.85	5.50	5.68	5.32	5.7	6
Tryp							1.5	1-2
Lys	3.90	3.89	3.95		3.92		3.65	4
Hist	0.92	0.94	0.96		0.94		0.99	1
Arg	6.06	6.00	6.10		6.05		6.07	6
TOTAL								151-152

hydrophobic residues were found. The similarities of the amino acid composition of the two proteins is to be noted. They both have about 150 residues, a rather large proportion of acidic residues (35 to 37), 11 alcaline residues, 65 to 69 hydrophobic residues and 9 to 12 aromatic residues. They both have 5 cystein residues. The main difference is to be found in the number of histidine and methionine : D. gigas flavodoxin has 0 histidine and 2 methionine, D. vulgaris flavodoxin has 1 and 0 respectively. 0.2 free sulfhydril residue was found in the native D. gigas protein, in the oxidized state. Tryptophan

- TABLE III -

AMINO ACID COMPOSITION OF D. GIGAS FLAVODOXIN

Amino acid residues per mole of protein

AMINO ACID	After acid hydrolysis					Performic Oxydation	Hydrolysis in presence of thioglycolic acid	Proposed amino acid Composition
	18 hrs	25 hrs	40 hrs	60 hrs	Average			
Asp	17.16	17.0	17.15	17.03	17.08	17.3	20	17
Thr	8.88	9.05	8.65	8.22	9.2	7.3	8.3	9
Ser	7.15	6.83	6.70	5.80	8	6.9	6.3	8
Glu	17.87	18.0	17.35	17.90	17.78	17.9	18.8	18
Pro	6.17	6.0	6.24	6.32	6.18	5.11	6.5	6
Gly	15.07	14.92	14.6	14.37	14.49	12.6	13.55	14-15
Ala	14.93	15.06	15.21	14.60	14.95	15.0	15.0	15
Cyst						4.72		5
Val	14.15	16.0	15.45	16.32	15.89	15.2	16.0	16
Met	1.76	1.72	1.55	1.60	1.90	2		2
Ileu	4.55	4.96	5.05	5.11	4.92	6.2	4,3	5
Leu	13.53	13.58	13.88	13.38	13.60	11.3	13.0	14
Tyr	4.91	4.82	4.95	4.61	4.83	2	4.5	5
Phe	2.96	3.12	2.98	3.11	3.04	2.8	3.6	3
Tryp					0.93*		0,85	1
Lys	8.29	8.00	8.13		8.14		8.50	8
Hist	N.D.	N.D.	N.D.		0		0	0
Arg	2.70	2.93	2.87		2.83		2.60	3
TOTAL								149-150

* Spies and Chambers method

N.D. Not Detected

determination after alcaline hydrolyses gave non reproducible results, between 1 and 2 residues for the two proteins.

Dissociation and recombination of the prosthetic group. These experiments were carried out on D. gigas flavodoxin. After treatment, the apoprotein dilution was of 0.75 mg per ml (apoflavodoxin ε_{277} = 17,500) ; 90 % of

TABLE IV

BIOLOGICAL ACTIVITY OF RECONSTITUTED FLAVODOXIN

Hydrogen uptake after 30 minutes ($mm^3 H_2$)				
Blank without flavodoxin	Native flavodoxin 0,067 µM	FMN reconstituted protein 0,034 µM	FMN reconstituted protein 0,068 µM	FAD reconstituted protein 0.068 µM
- 8	- 92	- 32	- 69	- 26

Addition of free flavins did not increase hydrogen uptake.
Each cup contained : phosphate buffer pH 6, 5 % : 150 µM ; $MgCl_2$: 20 µM ; Na_2SO_3 : 20 µM. Particules : 12 mg of proteins ; soluble proteins, DEAE cellulose treated : 10 mg.
In the center well : 0.05 ml 20 % $CdSO_4$ and 0.05 ml 20 % KOH. Temperature : 37° C. Atmosphere : hydrogen.

the protein was dissociated. Equimolar addition of FMN or FAD in the apoprotein solution gave absorption maxima respectively at 456 mµ and 451 mµ. In photoreducting 0.13 µM of reconstituted FMN flavodoxin we got a quite normal 580 mµ signal. The rate of formation of the semiquinone was similar to that of the native flavodoxin and the extent was about 70 %. With the FAD reconstituted flavodoxin the extent of semi reduction is less than 20 %. Biological activity of reconstituted proteins are exposed in table IV. It can be concluded that recombination of the apoprotein with FMN gave a yield of about 70 % and that FAD is not the normal co-factor. For calculating these rates undissociated flavodoxin of the apoprotein solution was taken in account.

DISCUSSION

Besides the two flavoproteins from Desulfovibrio, two other flavodoxins have been studied up to now, in Clostridium pasteurianum (3, 13) and in Peptostreptococcus elsdenii (17, 18). The spectra, molecular weights are similar. All contain FMN as prosthetic group. They are able to replace ferredoxin in some ferredoxin-dependent reactions. They do not contain iron (not tested for D. vulgaris flavodoxin). Their amino acid composition show similarities with a high aspartic and glutamic acid content and equivalent proportions of alcaline, hydrophobic and aromatic residues. However D. vulgaris flavodoxin

differs from the three others as it is the only one to contain histidine and no methionine. The most important fact is that sulfate reducing bacteria flavodoxins contain 5 cystein residues against 1 or 2 for the others ; such a difference may involve differences in conformation if some of these cystein residues were involved in S-S bridges. If the spectra of the four flavodoxins are compared, it appears that the combination of the FMN with the apoproteins have not the same effect on the absorption spectra. For the yellow peak around 450 mμ, the shifts are very different : the Desulfovibrio flavodoxins exhibit an important shift (10 to 12 mμ) towards the red, whereas C. pasteurianum flavodoxin gives a small shift towards the blue (2 mμ). The flavodoxin from P. elsdenii shows no shift at all. These different spectral properties may reflect differences in the binding of FMN to the apoproteins and in the tightness of the binding.

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