Conformational changes in *Chondrus crispus* flavodoxin on dissociation of FMN and reconstitution with flavin analogues

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The apoflavodoxin produced by precipitation of *Chondrus crispus* flavodoxin with trichloroacetic acid migrates as a single molecular species on non-denaturing PAGE, but at a much lower R_m than the flavoprotein. Values of s and D were significantly lower than for the flavodoxin, but their substitution in the Svedberg equation indicated the molecular mass was closely similar to that of the flavodoxin. This was confirmed by meniscus-depletion sedimentation-equilibrium studies. The Stokes radius of the apoflavodoxin was 3.65 nm, compared with 2.33 nm for the flavodoxin, and estimates of frictional coefficient ratio suggested the apoprotein was in extended conformation compared with the roughly globular shape of the flavodoxin. The K_d for FMN binding was 2.8×10^8 M, and the electrophoretic and physicochemical properties of the reconstituted flavoprotein were closely similar to those of the native flavodoxin. FAD, iso-FMN and thio-FMN were also bound effectively, but methyl-FMN and riboflavin were bound only weakly, if at all. The reconstituted flavoproteins were active to various extents in mediating electron transfer from NADPH to cytochrome *c* catalysed by flavodoxin–NADP⁺ oxidoreductase, the highest activity being with the thio-FMN flavodoxin.

INTRODUCTION

Flavodoxins are electron-transfer proteins of small molecular mass that contain as the redox-active component a single molecule of non-covalently bound FMN [1]. In the micro-organisms in which they occur they are generally synthesized only in response to iron-limitation, whereupon they replace the usual constitutive low-potential electron carrier, the iron–sulphur protein ferredoxin [2].

Among the few eukaryotes in which flavodoxin is found the macroalga *Chondrus crispus* is unusual in that this protein is constitutive and ferredoxin is absent [3]. This flavodoxin has been characterized in terms of molecular properties [3], efficacy in electron transfer [3,4], redox properties [5], amino acid sequence [6] and X-ray-diffraction studies of the crystallized protein to give the tertiary structure to 0.235 nm resolution [7].

The apoflavodoxin produced by dissociation of FMN from the holoprotein by several methods migrated at much lower R_m in analytical PAGE, with multiple bands being evident after some treatments [8]. This was consistent with aggregation of the apoflavodoxin after dissociation of the FMN, and was in marked contrast with the behaviour of two cyanobacterial flavodoxins, where the R_m value of the apoflavodoxin was not significantly different to that of the respective flavodoxin. However, we have now demonstrated that on dissociation of FMN *Chondrus crispus* flavodoxin undergoes a remarkable change in conformation, and have gone on to demonstrate the reversibility of this on binding of FMN and other flavins.

METHODS

Flavodoxin was isolated from *Chondrus crispus* as described previously [3] and purified to homogeneity, as assessed by analytical PAGE [9] at pH 8.2 on 15% (w/v) acrylamide gels in which 2.6% of the total acrylamide was *NN'*-methylene-bisacrylamide. The detection of protein in the gels was by staining with Coomassie Brilliant Blue G-250, with destaining in acetic acid/ethanol/water (8:27:65, by vol.).

Apoflavodoxin was prepared by precipitation with trichloroacetic acid at 4 °C and with light excluded [10,11]. The

flavodoxin, usually 6 mg in 2 ml of 0.15 M-Tris/HCl buffer, pH 7.8, containing 50 mM-dithiothreitol, was adjusted to 3% (w/v) trichloroacetic acid. The precipitated apoprotein was pelleted by centrifugation at 4000 *g* for 10 min, the supernatant was discarded, and the pellet was dissolved in 1.5 ml of 0.15 M-Tris/HCl buffer, pH 7.8, containing 0.1 mM-EDTA. For reconstitution studies 0.5 ml of apoflavodoxin solution was added to 1.5 ml of the same buffer containing 0.5 mM-FMN or a flavin analogue. After 2 h at 4 °C the solution was centrifuged at 1400 *g* for 10 min to pellet any undissolved material, and the supernatant was dialysed for 5 h against 250 ml of the Tris buffer containing EDTA, and then for 1 h each against three changes of Tris buffer without EDTA.

The association constant for flavin binding was determined at 25 °C by monitoring the fluorescence at 535 nm after excitation at 450 nm as 5 μ l additions (approx. 1.6 nmol) of apoflavodoxin were made to a solution of 3 μ M oxidized FMN in 0.1 M-sodium phosphate buffer, pH 7.7, containing 0.1 mM-EDTA [10,11]. The FMN used was that released from the flavodoxin by trichloroacetic acid treatment or from FAD purified by DEAE-cellulose chromatography and then treated with snake-venom phosphodiesterase. The purity of the FMN was checked by t.l.c. [3] and h.p.l.c., and it was concentrated as necessary by using a Florisil column (10 cm $\times$ 1 cm; 60–100 mesh) equilibrated with 5% (v/v) acetic acid and developed with aq. 5% (v/v) pyridine. The eluate was adjusted to pH 6.5 and the solvent was removed by rotary evaporation. Apoflavodoxin concentration was measured from the end-point of the titration [11]. Correction to the data was made for residual fluorescence after addition of a 2-fold excess of apoflavodoxin.

The purity of flavins used in reconstitution experiments was assessed by h.p.l.c. After passing through a Whatman GFF filter, samples of 20 μ l (0.1–0.5 nmol of flavin) were injected on to an ODS Hypersil (5 μ m-particle-size) column that had been equilibrated with 5 mM-NaH₂PO₄/methanol (4:1, v/v) at pH 5.5. The column was developed with this buffer at a flow rate of 2 ml/min, the A_{450} of the eluate being monitored. This procedure gave a good separation of all the flavins used (Fig. 1) except for FMN and thio-FMN, where retention time differed by only 5 s;

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Fig. 1. Separation of flavins by h.p.l.c.

A mixture of about 250 nmol of each of the flavins was applied to an ODS Hypersil column. On development the order of elution of the flavins after the solvent peak (s) is FAD (peak a), FMN and thio-FMN (peak b), iso-FMN (peak c), methyl-FMN (peak d), riboflavin (peak e) and an impurity in the iso-FMN (peak f).

however, the absorption spectra are sufficiently different to avoid any confusion.

As judged by this method, the FAD contained some 2% FMN, the thio-FMN contained a trace of FMN, and the iso-FMN and methyl-FMN were pure. The iso-, methyl- and thio-FMN analogues used were generously donated by the Fachbereich Biologie, University of Konstanz, Konstanz, Germany.

The efficacy of the reconstituted flavoproteins in electron transfer was assessed by ability to mediate cytochrome *c* reduction with NADPH as the electron donor [12] in the presence of flavodoxin-NADP⁺ oxidoreductase from *Chondrus crispus*. The reaction mixture contained 0.05 μ mol of cytochrome *c*, 0.12 μ mol of NADPH, 15 units of the oxidoreductase and reconstituted flavoprotein (0.5–12 μ M) in 1 ml of 0.15 M-Tris/HCl buffer, pH 7.8. The K_m of the oxidoreductase for NADPH was 56 μ M and that for flavodoxin was 4.4 μ M. The reduction of cytochrome *c* was monitored by measuring ΔA_{550} (ϵ 19 100 M⁻¹·cm⁻¹) after addition of flavodoxin.

Protein concentration determination was by the Folin phenol method [13], flavodoxin determination was based on an ϵ_{464} value of 10 700 M⁻¹·cm⁻¹ [3], and that of FMN on an ϵ_{445} value of 12 500 M⁻¹·cm⁻¹.

Ultracentrifuge methods were as described elsewhere [3,14].

RESULTS AND DISCUSSION

As demonstrated in earlier work [3–8], *Chondrus crispus* flavodoxin is readily purified to homogeneity, as assessed by PAGE (Fig. 2a). The apoflavodoxin produced by trichloroacetic acid treatment also gave a single band on electrophoresis in 15% (w/v) polyacrylamide gels, though this had an R_m of about 0.3 compared with 0.73 for the native flavodoxin. The apoflavodoxin was stable for several weeks at -20 °C in the presence of dithiothreitol; in the absence of reductant this molecular species was accompanied by several minor bands of R_m 0.31–0.43, which are probably products of oxidation, and a band of lower R_m (0.14), which may represent an aggregation product. The distinction in electrophoretic behaviour of the flavodoxin and apoflavodoxin was not observed if SDS/PAGE was used (results not shown).

Fig. 2. Electrophoresis of flavodoxins formed by replacement of the native flavin by alternative flavin prosthetic groups

The separations on 15% (w/v) polyacrylamide cylindrical gels are of native flavodoxin (lane a), apoflavodoxin (lane b) and products of association of apoflavodoxin with FMN (lane c), FAD (lane d), iso-FMN (lane e), methyl-FMN (lane f) and thio-FMN (lane g). The position of the anion front is shown by a wire inserted in each gel.

Molecular properties of the apoflavodoxin

During sedimentation-velocity studies using the analytical ultracentrifuge the apoflavodoxin, at 3.1 mg/ml in 0.15 M-Tris/HCl buffer, pH 7.4, migrated as a single symmetrical peak with a sedimentation coefficient ($s_{20,w}$) significantly lower than that exhibited by the flavodoxin (Table 1). This suggested that the apoflavodoxin was not present as an aggregate, which had been the interpretation [8] of the markedly slower migration on PAGE compared with the holoprotein. The diffusion coefficient ($D_{20,w}$) was also smaller than that previously found for the flavodoxin (Table 1). The molecular mass derived from these data lacks some precision, since ideally the coefficient at a range of protein concentrations should be extrapolated to infinite dilution. Nevertheless, substitution of the corrected coefficients in the Svedberg equation gave a molecular mass consistent with the apoflavodoxin being a monomeric species. Meniscus-depletion sedimentation-equilibrium experiments left no doubt that this was the correct conclusion.

An alternative approach to investigating the difference in conformation between the apoflavodoxin and flavodoxin is through estimates of the Stokes radius. This can be done by using

Table 1. Molecular properties of *Chondrus crispus* flavodoxin and the derived apoflavodoxin

Property	Flavodoxin	Apoflavodoxin
Sedimentation coefficient* (s)	2.15×10^{-13}	1.41×10^{-13}
Diffusion coefficient* (cm ² ·s ⁻¹)	9.1×10^{-7}	5.4×10^{-7}
Molecular mass (Svedberg equation) (Da)	21 300	23 500
Molecular mass (sedimentation equilibrium) (Da)	20 800	21 000
Stokes radius (nm)	2.33	3.55

* Data at 20 °C corrected to water as solvent.

Fig. 3. Stokes radius of the apoflavodoxin

The relationship of $\sqrt{(-\log K_{av})}$ versus Stokes radius is shown for the standard proteins used: ●, BSA (a), ovalbumin (b) and cytochrome *c* (d); ○, *Chondrus crispus* flavodoxin (c). The experimentally derived function of K_{av} for the apoflavodoxin is shown by the arrow.

the data on molecular mass and sedimentation coefficient [15,16], but also independently from gel-filtration behaviour. For the latter approach chromatography on a Sephadex G-100 column was used. Void and total volumes were determined with Blue Dextran and $(\text{NH}_4)_2\text{SO}_4$ respectively, and the column was calibrated with four proteins with well-established molecular characteristics, namely BSA, ovalbumin, cytochrome *c* and *Chondrus crispus* flavodoxin itself. The resultant plot of $\sqrt{(-\log K_{av})}$ versus Stokes radius [17] was linear (Fig. 3). Stokes radius (*a*) for these proteins was determined from the relationship $M = 6\pi \cdot \eta \cdot N \cdot a \cdot s \cdot (1 - \bar{v}\rho)$.

The apoflavodoxin had a K_{av} value of 0.21, which indicates a Stokes radius of 3.65 nm; direct calculation from the experimentally derived values of *M* and *s* gave a value of 3.53 nm. Thus, although the molecular mass of the apoprotein is about 20000 Da, its K_{av} value is very close to that for serum albumin (molecular mass 66000 Da). The extent of unfolding of the protein on dissociation of FMN can be found by calculating the frictional ratio f/f_0 by using the relationship $f/f_0 = M^{2/3} \cdot (1 - \bar{v}\rho) / 6\pi \cdot \eta_0 \cdot N \cdot s \cdot (4\pi \cdot N / 3\bar{v})^{1/3}$ given in ref. [15]. Comparing apoflavodoxin and flavodoxin the values thus estimated were 1.9 and 1.3 respectively. Using the Stokes radius for apoflavodoxin determined from gel filtration and the relationship $f/f_0 = a / (3\bar{v} \cdot M / 4\pi \cdot N)^{1/3}$ the value of the frictional coefficient for apoflavodoxin was 2.0. Values close to unity indicate spherical molecules, and a higher value an increased axial ratio. Comparison with representative data [16] suggests that the frictional ratio of the apoflavodoxin is consistent with the axial ratio of approx. 20 or 30 depending on whether the molecule is a prolate or oblate ellipsoid respectively. For comparison, the size of the flavodoxin itself is known from X-ray crystallography [7] to be 3.3 nm × 2.8 nm × 4.0 nm.

A similar extreme change in conformation seemingly occurred on the dissociation of FAD from glucose oxidase [18]. For flavodoxins, c.d. [10,19,20] and kinetic and thermodynamic studies [19] have suggested that the *Azotobacter vinelandii* protein undergoes some change in conformation on removal of flavin. However, this change may be considerably less than observed here for *Chondrus crispus* flavodoxin, and it is also apparent that

for some other flavodoxins [8] there is no significant change in conformation on dissociation of FMN.

Reconstitution of flavodoxin

Reconstitution of flavodoxin with FMN, conveniently detected by PAGE (Fig. 2, lane c), was readily achieved. Thus, despite the large conformational change involved, the apoflavodoxin rebinds FMN to form a flavodoxin that behaves like the native flavodoxin on electrophoresis. The absorption spectrum was also identical, with peaks at 275, 283 (shoulder), 292 (shoulder), 383, 464 and 487 nm (shoulder); however, the A_{464}/A_{383} absorbance ratio was somewhat lower for the reconstituted flavoprotein.

The K_a for apoflavodoxin and FMN was determined for the *Chondrus crispus* protein and for comparison also for the *Nostoc* strain MAC flavodoxin, where no conformational change on dissociation of FMN was detected. The quenching of FMN fluorescence as a function of added apoflavodoxin was measured (Fig. 4); points at the junction of the linear portions of the plot are attributable to equilibria of free FMN and flavodoxin, which permits estimation of K_a from $\alpha/\text{FMN} = K_a \cdot (1 - \alpha)$, where α is the mol of FMN bound/mol of apoflavodoxin. For *Chondrus crispus* apoflavodoxin the equilibrium titration (Fig. 4 inset) gave a value for K_a of 2.9×10^8 M, confirmed by a second titration, which gave a K_a value of 2.7×10^8 M. Similar experiments for *Nostoc* strain MAC apoflavodoxin gave a K_a value of 2.0×10^8 M (s.d. ± 0.9 , $n = 3$). These values are very similar to the K_a values reported for flavodoxins of the larger molecular-mass group from other sources, e.g. 2.2×10^8 M for *Azotobacter vinelandii* flavodoxin [19].

From the FMN-binding data and the E_m (midpoint potential) values for the oxidized-semiquinone and semiquinone-hydroquinone interconversions the K_a values for the reduced forms can be calculated by using the E_m data for FMN [21].

The K_a values at pH 7 for FMN-semiquinone and FMN-hydroquinone binding to apoflavodoxin are calculated to be

Fig. 4. Determination of the association constant for FMN and the apoflavodoxin from *Chondrus crispus*

FMN was titrated with apoflavodoxin at 25 °C and flavin fluorescence in arbitrary units was monitored. The inset shows those experimental points where free FMN and flavodoxin were in equilibrium plotted as functions of α , the mol of FMN bound per mol of apoflavodoxin.

5.5×10^9 M and 4×10^5 M respectively. This is in accord with the general observation for flavodoxins that binding of the semi-quinone is stronger than that for the oxidized form, whereas on two-electron binding there is a substantial decrease in binding affinity for the hydroquinone [22]. Here $K_{sq.}/K_{ox.}$ is about 20, whereas $K_{sq.}/K_{hq.}$ is approx. 10^4 .

Reconstitution with other flavins

For the flavin analogues, success in binding to the apo-flavodoxin was monitored by PAGE; these data are summarized in Fig. 2, except for riboflavin, where no interaction could be demonstrated. Flavodoxins have been divided into two classes dependent on their ability to bind riboflavin [1,23], and on the basis of this evidence *Chondrus crispus* flavodoxin appears to be placed in that category where a phosphate group on the flavin is required to trigger a conformational change before binding a flavin.

Reconstitution of flavoprotein with FAD to yield an FAD-flavodoxin (λ_{max} 455 nm) was, as judged by densitometry of the gels, better than 90%. FAD was also bound by *Azotobacter vinelandii* apoflavodoxin [19], though not by the apoprotein from *Megasphaera elsdenii* [22]. Formation of an iso-FMN-flavodoxin (λ_{max} 463 nm) was similarly effective, as it was for the flavodoxins from these other organisms. X-ray-crystallographic studies of *Megasphaera elsdenii* flavodoxin containing iso-FMN suggested that this flavin was bound somewhat differently to FMN. In contrast, only weak binding of methyl-FMN to the apoflavodoxin occurred (λ_{max} 446 nm), and here a minor band of lower R_m (Fig. 2, lane f) was evident and may represent an aggregate of the apoprotein. This flavin was bound by the apoflavodoxins from *Azotobacter* and *Megasphaera*. A thio-FMN-flavodoxin (λ_{max} 464 nm) was formed at a yield of better than 50% under the experimental conditions used; this flavin was also bound by the apoflavodoxin from *Azotobacter vinelandii* [24].

Activity of reconstituted flavoproteins

The ability of the reconstituted flavoproteins to mediate in electron transfer from NADPH to cytochrome *c* catalysed by the native flavodoxin-NADP⁺ oxidoreductase was assessed (Fig. 5). The background to the use of modified flavins as replacement for the native flavin in studies of structure-function relationships in flavoproteins has been discussed elsewhere [25]. In interpreting the data it should be noted that, because of uncertainty regarding molar absorption coefficients of flavodoxins containing substituted flavins, the protein concentration is based on the Folin phenol method. Referral to Fig. 2 shows that this will underestimate activity when reconstitution of the flavoprotein has been incomplete; this is significant in the cases of methyl-FMN and thio-FMN, where reconstitution was not more than 10% and 60% respectively.

The basis of comparison in Fig. 5 is the activity of the native flavodoxin, with the confirmation that apoflavodoxin is inactive. The turnover number in this assay was 5000 min^{-1} . Flavodoxin reconstituted from apoflavodoxin and FMN gave some 90% of the activity of the native protein. FAD-flavodoxin was about 70% as active as the native flavodoxin, though it was less active on this comparative basis at low flavoprotein concentration. For *Desulfovibrio gigas* FAD-flavodoxin the activity in a sulphite reductase assay was only 28% of that of the native flavodoxin [26].

Although the *Chondrus crispus* apoflavodoxin bound iso-FMN effectively, the product showed low activity in the assay, as found for this reconstituted flavoprotein from *Megasphaera elsdenii*, where activity in a phosphoroclastic assay was about one-third of that of the native flavodoxin [22,27], ascribed to changed redox behaviour. Methyl-FMN-flavodoxin also showed de-

Fig. 5. Activity of flavodoxins formed by replacement of the native flavin by alternative flavin prosthetic groups

The ability of the flavoprotein to mediate electron transfer from NADPH to cytochrome *c* in the presence of *Chondrus crispus* flavodoxin-NADP⁺ oxidoreductase was measured. The activity of the native flavodoxin (○) is compared with that of flavoproteins formed from apoflavodoxin and FMN (●), FAD (▲), methyl-FMN (□), iso-FMN (■) and thio-FMN (△). The data for apoflavodoxin (○) are also shown.

creased activity in our assays, but here the low proportion of flavoprotein actually present must be considered, and taking this into account the activity at low protein concentrations is probably comparable with that of the native flavodoxin. In contrast, even though not fully reconstituted from *Chondrus crispus* apoflavodoxin, the thio-FMN-flavodoxin was more active by about 45% than the native flavodoxin. In the larger-molecular-mass group of flavodoxins there are comparable data for the protein from *Azotobacter vinelandii* [24], where reactivity in photochemical reduction to the semiquinone was investigated; the methyl-FMN- and 2-thio-FMN-flavodoxins were appreciably more reactive than the native FMN-flavodoxin, whereas the iso-FMN-containing form was reduced more slowly.

There have been few detailed studies of the molecular characteristics of the apoprotein formed when flavin is dissociated from a flavoprotein, and indeed it is a common assumption that the apoprotein is in essentially the same conformation as the holoprotein. For two cyanobacterial flavodoxins this was seemingly the case [8], though for *Azotobacter vinelandii* flavodoxin c.d. and other data [20] suggested the apoflavodoxin secondary structure may have differed somewhat. The information now presented for *Chondrus crispus* flavodoxin shows clearly that the secondary structure of this apoflavodoxin differs very markedly from that of the flavodoxin. It therefore presents an attractive simple model for studying the mechanism of folding of a peptide to accommodate a prosthetic group.

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