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Variations in the Spectrum of Desulfoviridin from *Desulfovibrio gigas*

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Abstract

Desulfoviridin preparations from *D. gigas* showed variations in the position of the absorption maximum (the β-peak) in the 580-nm region of the spectrum. On treatment with $Na_2S_2O_4$ a preparation with a β-peak at 585 nm was affected rapidly, the 585-nm peak shifting to the 596-nm region; this was partially reversed by $K_3Fe(CN)_6$. Treatment of the original preparation with $K_3Fe(CN)_6$ resulted in a shift of the β-peak to 582–583 nm. Desulfoviridins with β-peaks from 580 to 583 nm were not rapidly affected by $Na_2S_2O_4$. The spectrum of the chromophore of desulfoviridin was also affected by $Na_2S_2O_4$ with the peak at 587 nm shifting to 597 nm; this effect was completely reversed by oxygen. There was no evidence to show that spectral variations in desulfoviridin preparations were due to the loss or acquisition of metal ions during growth or to the selection of mutants containing spectrally different desulfoviridins. It is suggested that during biosynthesis or purification, desulfoviridin may be structurally modified, allowing partial detachment of the chromophore, thus causing a change towards the spectral properites of the detached chromophore.

Introduction

Desulfoviridin has been characterized as the dissimilatory bisulphite (and/or sulphite) reductase of *Desulfovibrio* species (Suh and Akagi 1969; Lee and Peck 1971; Kobayashi *et al.* 1972; Jones and Skyring 1974). The extractable prosthetic group of the enzyme is an isobacteriochlorin (Postgate 1956; Murphy and Siegel 1973; Skyring 1975). Spectra of purified preparations of desulfoviridin from *Desulfovibrio gigas* NCIB* 9332 have major absorption peaks at 627–628 nm (α-peak) and 408–409 nm (Soret peak); however, the absorption peak in the 580–590-nm (β-peak) region of the spectrum has been given as 580 nm (Lee and Peck 1971), 581 nm (Jones and Skyring 1975), 583 nm (Murphy and Siegel 1973), and 588 nm (Jones and Skyring 1974). Another instance of unexplained variability in desulfoviridin spectra is that of the differences in the preparations from *D. vulgaris* NCIB 8303 by Postgate (1956) and by Lee *et al.* (1973); absorption maxima of the former preparation were at 630, 585, 411, and 390 nm whilst for the latter they were at 628, 580, 408, and 390 nm. The spectrum of desulfoviridin from another strain of *D. vulgaris* had absorption maxima at 630, 585, 410, and 390 nm (Kobayashi *et al.* 1972); this was almost identical to that recorded by Postgate (1956).

Generally, the spectrum of desulfoviridin does not significantly alter on treatment with sodium dithionite, $Na_2S_2O_4$ (Postgate 1956; Ishimoto and Koyama 1957; Lee and Peck 1971; Murphy and Siegel 1973; Lee *et al.* 1973; Jones and Skyring 1975). However, Jones and Skyring (1974) showed that with a desulfoviridin preparation having a β-peak at 588 nm a reaction occurred with $Na_2S_2O_4$ in which the extinction

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at 588 nm decreased and the effect was reversed with $K_3Fe(CN)_6$. The effect of $Na_2S_2O_4$ on this preparation of desulfoviridin was similar to that reported for P582, the sulphite reductase of *Desulfotomaculum nigrificans* (Trudinger 1970).

The present paper examines some possible reasons for variations in the position of the β-peaks in preparations of desulfoviridin from *D. gigas* and for variations in reaction with $Na_2S_2O_4$. Some metal ions react with tetrapyrrole molecules having marked effects on their spectral properties (Phillips 1963). Thus, in the present work the effects of the presence or absence of metal ions on the spectrum of desulfoviridin during biosynthesis and preparation as well as the effect of other growth conditions on its spectrum were investigated. In addition the influence of $Na_2S_2O_4$, $K_3Fe(CN)_6$ and oxygen on the spectra of both desulfoviridin and the extracted chromophore in aqueous solutions was examined.

Materials and Methods

Growth Conditions

The basic growth medium for *D. gigas* (NCIB 9332) consisted of $(NH_4)_2SO_4$, 5·0 g; NaCl, 10 g; $MgSO_4$, 0·5 g; $CaCl_2$, 0·1 g; K_2HPO_4, 2 g; KH_2PO_4, 1·0 g; Difco yeast extract, 1 g; Difco vitamin-free casamino acids, 4 mg; cysteine hydrochloride, 1 mg; *i*-inositol, 2 mg; thiamine hydrochloride, 400 μg; biotin, 10 μg; *p*-aminobenzoic acid, 200 μg; vitamin B_{12}, 2 μg; calcium pantothenate, 800 μg; pyridoxine hydrochloride, 400 μg; riboflavin, 200 μg; 2-mercaptoethanol, 0·01% (v/v); distilled water, 1 litre. Except where otherwise specified the oxidizable substrate was sodium lactate of concentration 3·5 g/l. In preparing the medium, aqueous K_2HPO_4 was added last and the medium was sterilized by filtration through a Millipore GS filter into 1-, 5-, or 20-litre growth vessels. The medium was incubated for 15–17 h at 35°C before addition of a 10% inoculum of *D. gigas* grown for 3 days in the basal medium. In other instances the cells were grown in modified Starkey's medium (Jones 1971).

To test the possible effects of growth media composition on the spectrum of desulfoviridin from *D. gigas*, the organism was grown in the following media which were made by adding the listed compounds to the basic medium. Unless otherwise stated the cultures were harvested 3 days after inoculation and the temperature of incubation was 34°C.

Medium	Compound added
1	$CuSO_4$, 40 μg
2	$FeCl_3$, 200 μg
3	$MnSO_4$, 400 μg
4	Na_2MoO_4, 200 μg
5	$ZnSO_4$, 400 μg
6	$CoCl_2$, 200 μg
7	All of the salts in media 1–6.
8	All of the salts in media 1–6; temperature 28°C.
9–14	All of the metal salts (media 1–6) except one; e.g. medium 9 contained $CuSO_4$, $FeCl_3$, $MnSO_4$, Na_2MoO_4 and $ZnSO_4$.
15–20	The metal salts at 10 times the concentrations shown for media 1–6. Incubation times varied from 3 to 7 days since lag periods in some media were long.
21	20 μg vitamin B_{12} instead of 2·0 μg as in the basal medium.
22	5·0 g Difco yeast extract instead of 1·0 g as in the basal medium.
23	3·5 g sodium pyruvate instead of sodium lactate as in the basal medium.
24	A sulphate-free medium in which 5·0 g NH_4Cl replaced $(NH_4)_2SO_4$ and 0·5 g $MgCl_2$ replaced $MgSO_4$ in the basal medium; 1·0 g Na_2SO_3 was also added.
25	The basal medium without trace metal additions.

Preparation of Extracts

For preparation of the extracts, the cells were harvested by centrifugation, washed and stored at −20°C in 0·1 M tris–HCl, pH 7·0. The culture was thawed, lysozyme added (1 mg/ml), and then

incubated at 35°C for 0·5–1 h; it was then chilled and treated with a sonic disintegrator for 30 s (Fisher ultransonic generator, 20±1 kHz tuned to 150 W); this material was then centrifuged at 190000 *g* for 30 min and the supernatant retained as the extract.

Preparation of Partially Purified Desulfoviridin

Polyacrylamide gel electrophoresis (Skyring and Trudinger 1972) of crude extracts often resulted in a desulfoviridin band contaminated with a *c*-type cytochrome. To remove cytochromes, the crude extract was made 0·1 M with respect to KCl and treated with DE52 ion-exchange cellulose (Whatman) equilibrated in 0·1 M KCl, 0·01 M tris–HCl, 0·001 M EDTA buffer, pH 7·8. The desulfoviridin and cytochromes were absorbed onto the cellulose, which was then washed successively using a batchwise procedure with 0·1, 0·2 and 0·3 M KCl–0·01 M tris–HCl, 0·001 M EDTA, pH 7·8 buffers. The contaminating cytochrome was removed by the 0·2 M KCl buffer and the desulfoviridin, eluted by the 0·3 M KCl buffer, contained little or no cytochrome. When run in polyacrylamide gels the desulfoviridin bands were free of cytochromes as recognized by the absence of peaks near 553 and 421 nm when treated with $Na_2S_2O_4$. Some preparations were further purified by the method of Jones and Skyring (1974); these are referred to as 'G200' preparations.

Spectrophotometry

All spectra reported here were obtained with G200 preparations at 20–22°C; the buffers used are given in the figure legends. These preparations were filter-sterilized and stored at 4°C; as judged by fluorescence when exposed to 365-nm u.v. light, indicating a loss of chromophore from the protein (Postgate 1956), they were stable for several weeks. A Varian 635 spectrophotometer was used in the normal mode, employing cuvettes of 1-cm path length and 1-ml capacity, or in the gel scanning mode. The spectra of G200 preparations of desulfoviridin at pH 7·0 were the same as those spectra obtained for the same preparation when in the polyacrylamide gels at pH 9·1. All spectra were corrected for base line variations.

Results

The Effect of Growth Conditions on the Spectrum of Desulfoviridin

Of the 25 growth conditions used, none could be relied upon to consistently produce a desulfoviridin for which the β-peak was at a wavelength longer than 580 nm. It was noted, however, that for several preparations of desulfoviridin the position of the β-peak was at longer wavelengths than 580 nm and the ratio of the extinction coefficients of the β- and α-peaks, $E_{\beta\text{-peak}} : E_{\alpha\text{-peak}}$, was highest for those preparations with a β-peak at 585–586 nm. This was consistent with published results; the ratio $\Delta E(588\text{–}700) : \Delta E(627\text{–}700)$* for the preparation of Jones and Skyring (1974) was 0·53 whereas it was 0·42 for other preparations (Lee and Peck 1971; Jones and Skyring 1975). Irrespective of the position of the β-peak, none of the preparations fluoresced in 365-nm u.v. light.

During these experiments, two batches of cells derived from the same mother culture and grown under apparently identical conditions yielded desulfoviridin preparations with β-peaks at 580 and 583 nm respectively. Selection of mutants with spectrally different desulfoviridins during one growth cycle would therefore seem unlikely.

In Vitro *Effect of Metal Ions on the Spectrum of Desulfoviridin*

In the presence of 10 μg/ml of $FeCl_3$, $MnCl_2$, $CoCl_2$ and $ZnSO_4$ (with or without $Na_2S_2O_4$), the spectrum of partially purified desulfoviridin (β-peak at 581 nm) was not significantly affected. However, irrespective of the position of the β-peak, small

* $\Delta E(588\text{–}700)$ is the extinction at 588 nm minus that at 700 nm. Similarly $\Delta E(627\text{–}700)$ is the difference between the extinctions at 627 and 700 nm.

quantities of copper sulphate (4·0 μg Cu/ml) in the presence of $Na_2S_2O_4$ caused an immediate and progressive decrease in E_{627}, and increases in E_{640}, E_{600} and E_{554}; this effect was independent of the order in which $CuSO_4$ and $Na_2S_2O_4$ were added (Fig. 1*A–E*). Subsequent treatment with $K_3Fe(CN)_6$ only partially eliminated these spectral changes and did not reverse the absorption loss at 627 nm (Fig. 1*F*). The $Na_2S_2O_4$ and $K_3Fe(CN)_6$ were added as crystals in small amounts (300–500 μg). The peaks at 554 and 600 nm present in the original difference spectrum did not reappear to the same extent on subsequent reduction with $Na_2S_2O_4$. These difference spectra are interpreted as the result of a concomitant elimination of part of the desulfoviridin spectrum and the appearance of the copper–chromophore complex which has an α-peak in the 616-nm region and a β-peak in the 554-nm region of the spectrum.

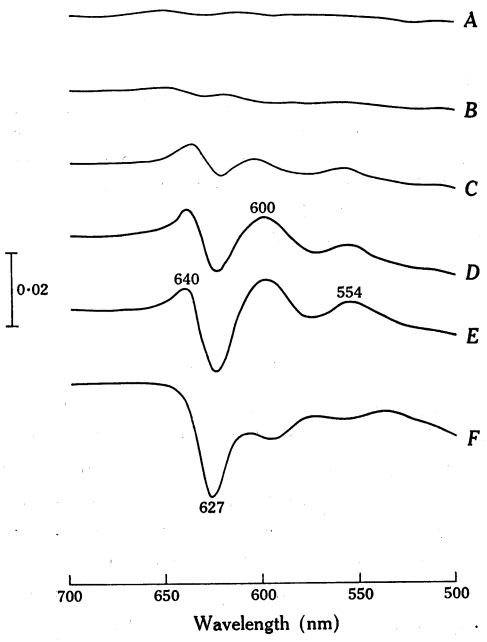

Fig. 1. Difference spectra showing the effect of copper plus $Na_2S_2O_4$ plus $K_3Fe(CN)_6$ on desulfoviridin. The E_{627} for desulfoviridin in this experiment was 0·108; the buffer was 0·05 M tris–HCl, 0·05 M phosphate, pH 7·0. Additions were to the sample cuvette only. *A*, Base line; *B*, after addition of 4 μg Cu/ml (as $CuSO_4$) for 10 min; *C*, *D*, *E*, after addition of $Na_2S_2O_4$ for 3, 7, and 17 min respectively; *F*, after addition of $K_3Fe(CN)_6$. The vertical line indicates absorbance units.

Effect of $Na_2S_2O_4$ on the Spectrum of Desulfoviridin

Spectra of desulfoviridin preparations with a β-peak at 580–583 nm were barely affected by $Na_2S_2O_4$. However, preparations with β-peaks at 585 nm or longer wavelengths did react with $Na_2S_2O_4$ and this reaction continued with time; extinctions at 627 nm decreased and the extinction at 590–596 nm increased (Fig. 2*A* and *B*). The effect of $Na_2S_2O_4$ on the 585-nm peak was reversed with $K_3Fe(CN)_6$ but the extinctions at 627 and 585 nm were not restored to their previous value (Fig. 2*C*). Prolonged (2-h) $Na_2S_2O_4$ treatment of a desulfoviridin preparation with a β-peak at 580 nm also caused similar but less intense changes in the spectrum. Irrespective of the position of the β-peak, preparations of desulfoviridin fluoresce under 365-nm u.v. light within 5–10 min of $Na_2S_2O_4$ treatment; it was considered that this was not due to pH changes of the buffer (1 mg/ml $Na_2S_2O_4$ added to 0·5 M tris–HCl reduced the pH from 7·00 to 6·93 and the pH of 0·1 M tris–HCl was reduced from 7·00 to 6·59).

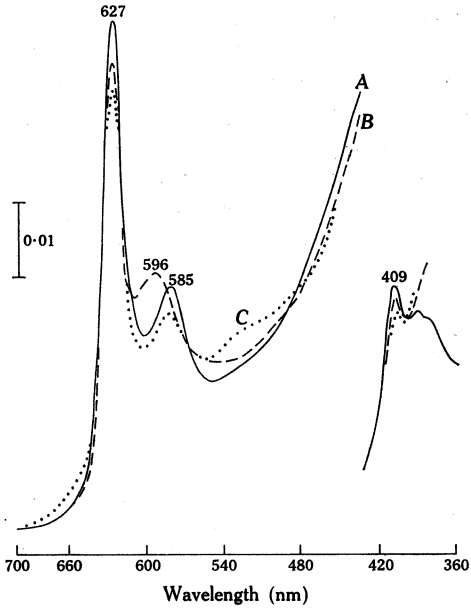

Fig. 2. Absorption spectra of desulfoviridin with a β-peak at 585 nm treated successively with $Na_2S_2O_4$ and $K_3Fe(CN)_6$. The buffer was 0·5 M tris–HCl, pH 7·2. *A*, Desulfoviridin (——); *B*, after 20-min treatment with $Na_2S_2O_4$ (– – –); *C*, the $Na_2S_2O_4$ treatment was continued for another 10 min and then a small amount of $K_3Fe(CN)_6$ was added (· · ·). The vertical line indicates absorbance units.

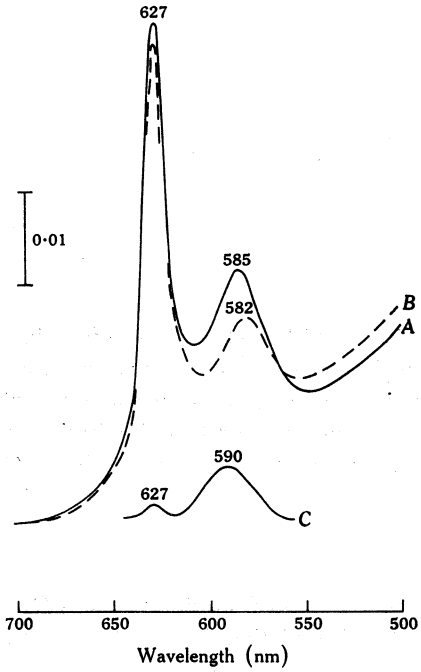

Fig. 3. Absorption spectra of desulfoviridin with a β-peak at 585 nm treated with $K_3Fe(CN)_6$. The buffer was 0·5 M tris–HCl, pH 7·2. *A*, Desulfoviridin (——); *B*, desulfoviridin immediately after treatment with a very small amount of $K_3Fe(CN)_6$ (– – –); *C*, the difference spectrum of *A* minus *B*. The vertical line indicates absorbance units.

Effect of $K_3Fe(CN)_6$ on the Spectrum of Desulfoviridin

Treatment of desulfoviridin having a β-peak at 585 nm with $K_3Fe(CN)_6$ caused an immediate decrease in extinctions at 626–627 nm (about 8%) and at 588–589 nm, the latter exhibiting the greater change. As a result of these spectral changes, the β-peak had moved to 582–583 nm and the ratio $E_{583} : E_{627}$ changed to 0·405 compared with 0·481 for the ratio $E_{585} : E_{627}$ of the preparation prior to treatment with $K_3Fe(CN)_6$ (Fig. 3*A* and *B*). A difference spectrum, obtained by subtracting spectrum *B* from *A* in Fig. 3, has peaks at 627 and 590 nm (Fig. 3*C*). After 3 h treatment with $K_3Fe(CN)_6$ the E_{627} had decreased by about 40%. Oxygen did not appear to affect the spectrum of desulfoviridin.

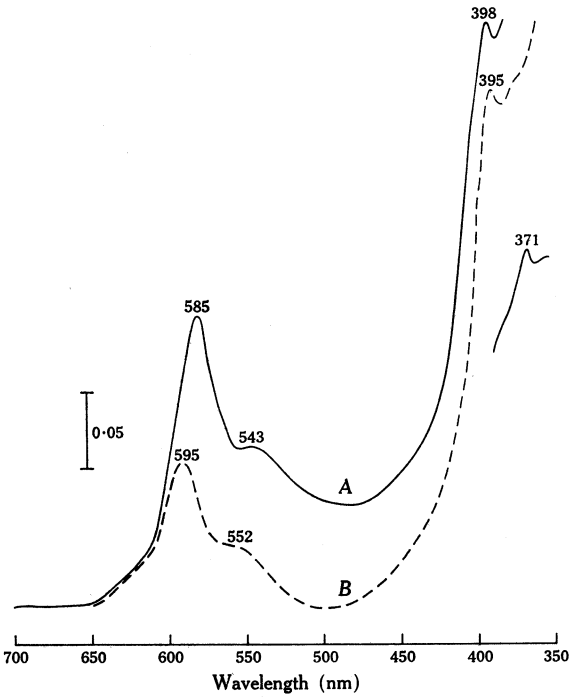

Fig. 4. Absorption spectra of the desulfoviridin chromophore located as a band in a polyacrylamide gel. *A*, Spectrum of the chromophore (——); *B*, spectrum of the chromophore after the gel had been soaked for 12 min in a dilute solution of $Na_2S_2O_4$ (– – –); by this time some diffusion had occurred. The vertical line indicates absorbance units.

Effect of pH on the Spectrum of Desulfoviridin

No difference in the spectra of desulfoviridin could be detected in buffers between pH 6·0 and 8·5. Prolonged treatment at high pH values did, however, cause the release of some of the chromophores as evidenced by fluorescence in u.v. light at 365 nm. All experiments in the present investigation were conducted in buffers which did not exhibit any significant change in pH due to the addition of $Na_2S_2O_4$ or $K_3Fe(CN)_6$.

Spectrum of the Desulfoviridin Chromophore

Irrespective of the position of the β-peak, incubation of desulfoviridin at 50°C for 2 h, with or without proteolysis by pronase*, released the chromophore; freezing

* Calbiochem, Pronase, B grade, 45 000 proteolytic units per gram.

desulfoviridin in tris–HCl or phosphate buffer (pH 7) at −20°C sometimes resulted in detachment of the chromophore. The chromophore was separated from residual protein by gel electrophoresis and the spectrum of the chromophore in the gel is shown in Fig. 4*A*. These spectra are similar to those given for the neutral form of the chromophore in pyridine (Murphy and Siegel 1973). Similar spectra were obtained when the chromophore was released by freezing and thawing.

Effect of $Na_2S_2O_4$ on the Chromophore of Desulfoviridin

By soaking polyacrylamide gels containing a band of the desulfoviridin chromophore in a dilute solution of $Na_2S_2O_4$, the spectrum changed (Fig. 4*B*) so that in the visible region of the spectrum there were peaks at 595 and 552 nm.

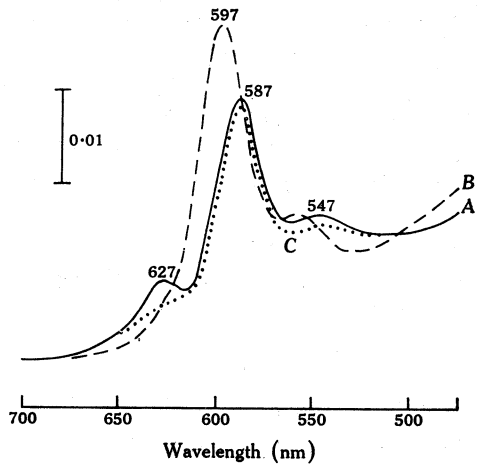

Fig. 5. Absorption spectra of desulfoviridin hydrolysed by pronase at 50°C in 0·5 M tris–HCl, pH 7·2. *A*, Desulfoviridin after 2 h at which time a decrease in E_{627} no longer occurred (——); *B*, after reaction with $Na_2S_2O_4$ (– – –); *C*, after shaking with air (· · ·). Although there was some overall absorption loss, treatments *B* and *C* were repeated on the same sample to give the same spectral shifts. The vertical line indicates absorbance units.

In another experiment desulfoviridin was degraded with pronase for 2 h at 50°C when, on the basis of change in extinction at 627 nm, the release of the chromophore was completed. Fig. 5*A* shows the spectrum of the chromophore with a more pronounced peak in the 627-nm region of the spectrum than was noted in the spectrum of the chromophore in a high pH gel (see Fig. 4*A*); presumably some protonated form was present in this solution. The effect of $Na_2S_2O_4$ was similar to that observed for the gel preparation (compare Figs 4*B* and 5*B*). Except for the fact that the 627-nm peak reappeared only as a shoulder, the effect of $Na_2S_2O_4$ was completely reversed by resaturation with oxygen (Fig. 5*C*). By using small quantities of $K_3Fe(CN)_6$ some reversible reaction was achieved.

Since any significant change in pH due to the addition of $Na_2S_2O_4$ and $K_3Fe(CN)_6$ could not be demonstrated, these changes were considered to be due to redox reactions.

Discussion

Lee *et al.* (1973) treated desulfoviridin of *D. gigas* with 8 M urea (over a 2-h period) and sodium dodecyl sulphate and suggested that the observed spectral changes represented an intermediate stage in the oxidation of the chromophore of desulfoviridin. However, Kobayashi *et al.* (1972) reported that sodium dodecyl sulphate releases the u.v. fluorescent chromophore of desulfoviridin and it is probable that the preparation

of Lee *et al.* (1973) was similarly affected. The present results and those of Kobayashi (1972) suggest that Lee *et al.* (1973) were probably recording spectral changes associated with the degradation of desulfoviridin and not with the spectrum of an intermediate form of the enzyme.

It was initially thought that the metal ion content (or lack thereof) of the medium in which the particular batch of *D. gigas* cells was grown, in some way affected the spectrum of desulfoviridin isolated from those cells. The results, however, were erratic and it was concluded that the spectral variations were not due to a loss or acquisition of metal ions during growth. These growths experiment also suggested that mutant selection was unlikely, and from electrophoretic data the production of more than one kind of desulfoviridin also seemed unlikely.

Except for copper, reaction of desulfoviridin with metal ions did not cause any variation in β-peak positions of desulfoviridin. The shifts in spectrum and the faint u.v. fluorescence during copper plus $Na_2S_2O_4$ treatment suggested that a copper–isobacteriochlorin complex was formed. However, copper contamination does not appear to be an explanation for the 588-nm β-peak of desulfoviridin reported by Jones and Skyring (1974) since the spectral change caused by $Na_2S_2O_4$ was reversible with $K_3Fe(CN)_6$, and the 627-nm peak showed no immediate change in extinction on reduction. In the present experiments the effects of copper plus $Na_2S_2O_4$ were not reversed by $K_3Fe(CN)_6$.

If the spectral changes observed in the present study (see Figs 4 and 5) are in fact due to oxidation–reduction reactions and not protonation reactions, then the chromophore, when it is released by relatively gentle procedures, must be in a partially reduced state since it was reduced by $Na_2S_2O_4$ and oxidized by $K_3Fe(CN)_6$.

From the effects of $Na_2S_2O_4$, $K_3Fe(CN)_6$ and oxygen on the spectrum of desulfoviridin and its chromophore it is suggested that during the biosynthesis or purification of desulfoviridin structural alterations sometimes occur in or around the chromophore causing the spectral properties typical of the detached chromophore to become more apparent; this results in a higher wavelength for the β-peak and a greater $E_{\beta\text{-peak}}$: $E_{\alpha\text{-peak}}$ ratio in the desulfoviridin. In such desulfoviridins (e.g. the preparation of Jones and Skyring 1974) the chromophore is more susceptible to observable reaction with $Na_2S_2O_4$ and $K_3Fe(CN)_6$ of the kind observed with the detached chromophore. Thus, on reduction a shift of the β-peak to wavelengths between 590 and 594 nm occurs and on oxidation a shift of the β-peak to lower wavelengths occurs. In these situations, however, the chromophore is not totally detached from the protein since these preparations of desulfoviridin maintain their spectra throughout purification, and they do not fluoresce in u.v. light.

Acknowledgments

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