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REACTIONS OF P582 FROM *DESULFOTOMACULUM NIGRIFICANS* WITH SUBSTRATES, REDUCING AGENTS AND CARBON MONOXIDE

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SUMMARY

Desulfotomaculum nigrificans contains a CO-binding pigment (P582) which may be responsible for sulphite, hydroxylamine and nitrite reduction in this organism. In crude extracts P582 reacted with $NaBH_4$ or $Na_2S_2O_4$ as evidenced by a decrease in absorbance at 582 nm. The extent to which P582 reacted with $NaBH_4$, but not $Na_2S_2O_4$, diminished progressively during purification of the pigment. In the presence of Na_2SO_3, however, $NaBH_4$ caused a decrease in the absorbance of the purified pigment at 582 nm and the appearance of an absorbance maximum in the region of 610 nm: a CO complex was also formed in the presence of CO, $NaBH_4$ and Na_2SO_3. Similar spectral changes in the presence and absence of CO, were produced by dithiothreitol and reduced methyl viologen although not at equal rates.

It is concluded that the spectral changes were a consequence of reduction of P582 and did not reflect specific interactions between the chromophore and sulphur compounds.

Methyl viologen-reduced P582 appeared to be reoxidized by Na_2SO_3 and NH_2OH. KNO_2 or NO reacted with reduced P582 and a spectrum with an absorbance maximum at 593 nm was obtained.

INTRODUCTION

The separation and partial purification of a CO-reacting pigment (P582) from the dissimilatory sulphate-reducing bacterium, *Desulfotomaculum nigrificans*, has been described previously[1]. Presumptive evidence was obtained that P582 was responsible for reduced methyl viologen-linked sulphite, nitrite and hydroxylamine reductase activities in the bacterium. Subsequently Skyring and Trudinger[2] found that there was complete coincidence of the brown P582 bands with reduced methyl viologen-linked sulphite-reductase bands (as determined by H_2S production) when crude extracts of *D. nigrificans* and partially-purified P582 were electrophoresed on polyacrylamide gels under a number of different conditions.

The purest preparations of P582 underwent spectral changes, and formed a

CO complex, in the presence of $Na_2S_2O_4$ (ref. 1). Several other reducing agents failed to substitute for $Na_2S_2O_4$. It has since been found that the effects of $NaBH_4$ *plus* Na_2SO_3 on the spectrum of purified P582 in the presence or absence of CO, are similar to those caused by $Na_2S_2O_4$. These results raised the question as to whether the spectral changes were simply a consequence of reduction of P582 or whether sulphur-compounds *per se* were essential.

There have been a number of reports indicating specific effects of sulphite on the spectra of sulphite reductases. Siegel and Kamin[3] obtained spectral evidence for the formation of an enzyme–sulphite complex in the presence of NADPH with purified sulphite reductase from *Escherichia coli*. Asada *et al.*[4], working with purified spinach sulphite reductase, reported that sulphite caused a shift in the absorption maximum in the red region of the CO complex of the $NaBH_4$-reduced enzyme from 598 nm to 607 nm. The two latter enzymes have spectral and other properties which resemble those of P582[1].

Lee and Peck[5] studied bisulphite reductase from *Desulfovibrio gigas*, an enzyme which appears to be identical with the green porphyroprotein, desulfoviridin[6,7]. They reported that the sequential addition of sulphite and $NaBH_4$ to the enzyme caused the appearance of an intense absorption maximum at 310 nm which they suggested might reflect the formation of an enzyme–substrate complex.

In this paper we examine further the reactions of P582 with substrates, reducing agents and CO in an attempt to determine the nature of the spectral changes observed.

MATERIALS AND METHODS

The organism, growth conditions, preparation of extracts, reagents and spectrophotometric procedures were as described earlier[1]. Additional reagents used were DEAE-cellulose (Whatman DE-52), hydroxylapatite (Biorad-HT; Biorad Laboratories, Richmond, California) and N_2O (Commonwealth Industrial Gases, Australia). NO was prepared by acidification of $NaNO_2$ and washed with 4 M KOH[8].

Anaerobic reactions were carried out in 1 cm pathlength cuvettes equipped with stopcocks through which reagents were injected. $Na_2S_2O_4$ solutions were prepared immediately before use in 0.1 M Tris–HCl buffer (pH 8) containing 1 mM EDTA. They were sparged with O_2-free Ar.

Preparations of P582

The initial purification steps were as described earlier[1] except for the following modifications: (a) $(NH_4)_2SO_4$ precipitation was omitted; (b) Whatman DE-52 was substituted for Whatman DE 11 and P582 was eluted by a 0.2 M–0.5 M gradient of KCl in 0.01 M Tris–HCl buffer (pH 8) containing 1 mM EDTA instead of by stepwise elution; (c) Sephadex G-200 was used in place of Biogel P200. The materials at the three stages of purification are designated Fractions DE-52, Sephadex 1 and Sephadex 2, respectively.

A small amount of P582 was further purified as follows. After the second filtration through Sephadex G-200 the material was dialysed against 0.01 M sodium phosphate buffer (pH 7.2) and about 75 mg of the dialysed protein was absorbed onto a 12.5 cm × 2.5 cm column of Biogel HT. The column was washed with a

gradient of 0.05 M to 0.1 M (200 ml/200 ml) sodium phosphate buffer (pH 7.2) and finally with 150 ml of 0.2 M sodium phosphate buffer (pH 7.2). Approximately one third of the recovered P582 was eluted as a peak between about 0.08 and 0.085 M sodium phosphate during the gradient elution (Fraction HT1) and the remainder (Fraction HT2) was eluted as a broad trailing fraction by 0.2 M phosphate. Fraction HT1 had 23% of the P582 originally applied to Biogel HT. It was concentrated to 0.5 ml by ultrafiltration over a Diaflo UM 20E membrane, applied to a colum of Sephadex G-200 (37 cm × 2.5 cm) and eluted with 0.1 M Tris–HCl buffer (pH 8) containing 0.5 M KCl and 1 mM EDTA. The protein eluted between 92 and 123 ml of eluant was collected. This material is designated Fraction Sephadex 3: it contained 11% of the protein and 16% of the P582 originally applied to Biogel HT. Fraction Sephadex 3 had a ratio $A_{280\ nm}/A_{392\ nm}$ of 2.32 and specific activities (nmoles methyl viologen oxidized/min/mg protein at 50 °C) for reduced methyl viologen-linked sulphite and hydroxylamine reduction of 88 and 3750, respectively. The material also had nitrite reductase activity but reliable specific activities could not be obtained (*cf.* ref. 1).

RESULTS AND DISCUSSION

Effects of $NaBH_4$ and substrates on spectrum of P582

The addition of $NaBH_4$ appeared to cause no spectral changes to P582 in Fraction Sephadex 3. Occasionally small losses (<5%) of absorbance at 392 nm were obtained on the addition of large amounts (2–5 mg) of $NaBH_4$ but these appeared to be due to irreversible degradation of the pigment probably as the result of excessive frothing. In the presence of Na_2SO_3, however, $NaBH_4$ caused a marked decrease in the absorption at 582 nm and the appearance of a shoulder at about 610 nm (Fig. 1, Curve 2). The subsequent addition of CO caused the appearance of absorbance maxima at 596, 551 and about 404 nm (Fig. 1, Curve 3). None of the spectral changes was affected by the order of addition of reagents.

The spectrum of the mixture of P582 with Na_2SO_3 and $NaBH_4$ showed intense absorbance in the ultraviolet region with a maximum at 305 nm. A solution of Na_2SO_3 *plus* $NaBH_4$, however, gave a similar ultraviolet spectrum which Panson and Weill[9] have demonstrated to be due to the formation of dithionite by chemical reduction of sulphite by $NaBH_4$. The latter reaction may also explain the cooperative effect of Na_2SO_3 and $NaBH_4$ on P582 since similar spectra to those shown in Curves 2 and 3 of Fig. 1 were obtained by adding $Na_2S_2O_4$ to Fraction Sephadex 3 under H_2 and CO, respectively.

No significant changes under H_2 or CO were caused by the following: Na_2SO_3; $NaBH_4 + NH_2OH$ or $NaBH_4 + KNO_2$. A spectrum similar to that of Curve 3 in Fig. 1 but displaced slightly towards the blue end of the spectrum was, however, obtained on adding $NaBH_4 + KNO_2 + Na_2SO_3$ (see below).

In contrast with Fraction Sephadex 3, $NaBH_4$ alone reacted with P582 in cruder preparations to cause a loss of absorption at 582 nm. The extent of the spectral change, relative to that caused by $Na_2S_2O_4$, decreased with increasing purity of P582 (Table I): in all cases the spectral changes were reversed by subsequent aeration of the pigment. The extent of reaction of $Na_2S_2O_4$ relative to the absolute P582 content was similar at all stages of purification.

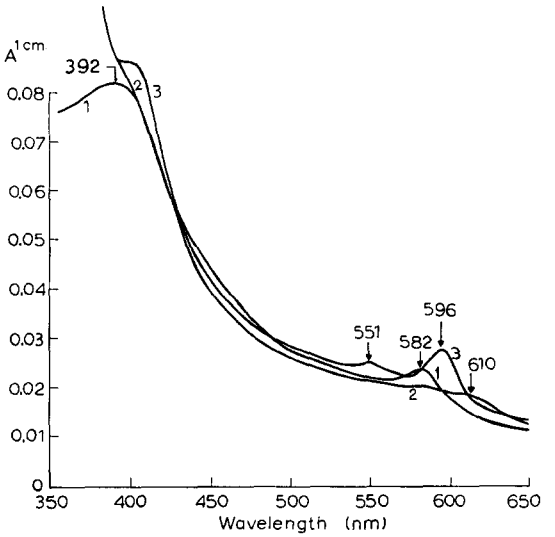


Fig. 1. Absolute spectra of Fraction AB (0.1 mg protein per ml in 0.1 M Tris–HCl buffer (pH 8.0), 0.5 M KCl, 1 mM EDTA). Spectrum 1, oxidized P582; 2, P582, 45 min after treatment with approx. 1 mg each of $NaBH_4$ and Na_2SO_3 under H_2; 3, as 2 after flushing with CO. Absorbance maxima (in nm) are indicated.

TABLE I

RELATIVE EXTENTS OF REDUCTION OF P582 BY $NaBH_4$ AND $Na_2S_2O_4$ AT VARIOUS STAGES OF PURIFICATION

$NaBH_4$ (1–2 mg) was added to 1 ml of P582 preparation after gassing with H_2. $A_{610-582\ nm}$ was determined from the reduced *minus* oxidized difference spectrum after the spectrum became stable About 2 mg of $Na_2S_2O_4$ was then added and $A_{610-582\ nm}$ again measured after the spectrum stabilized. Control experiments showed that a second addition of $NaBH_4$ in place of $Na_2S_2O_4$ caused no further reduction of P582 and that the extent of P582 reduction by $Na_2S_2O_4$ alone was similar to that caused by $Na_2S_2O_4$ *plus* $NaBH_4$.

Preparation	$A_{610-582\ nm}$		
	$NaBH_4$	$Na_2S_2O_4$	$\frac{Na_2S_2O_4}{NaBH_4}$
Crude extract	0.057	0.092	1.6
Fraction DE52	0.025	0.062	2.5
Fraction Sephadex 1	0.018	0.068	3.9

The reason for the variable reaction of P582 with $NaBH_4$ is not clear. Although the determination of rates of reduction of P582 by $NaBH_4$ was difficult due to the production of gaseous H_2 there were no obvious differences in these rates with the different P582 preparations. Thus the possibility that an additional component was required for the $NaBH_4$–P582 reaction which was separated from the pigment during purification, appears unlikely. A second possibility was that P582 existed in two (or more) forms which separated during purification and which were differentially reduced by $NaBH_4$. Recently Chambers (unpublished) has detected by gel electrophoresis two forms of P582, each with sulphite reductase activity, in semi-purified preparations. They have not yet been detected in crude extracts and whether they represent

distinct biochemical entities or an artefact caused by the purification procedure remains to be determined. The extent of reduction of P582 by $NaBH_4$ was not influenced by the presence of 1 mM cysteine but was decreased by about 50% by 1 mg/ml of bovine serum albumin. These compounds have been reported to promote enzymic activity by spinach sulphite reductase[3].

Course of reaction of P582 with $Na_2S_2O_4$ and CO

The reaction of P582 with 1.1 mM $Na_2S_2O_4$, and the formation of the CO complex, at pH 8 and 30 °C as a function of time are shown in Fig. 2. When CO was added prior to $Na_2S_2O_4$ the course of the reaction appeared to be similar to that of the P582–$Na_2S_2O_4$ reaction. There was no reaction between P582 and CO in the absence of $Na_2S_2O_4$ over a period of several hours. The rate of formation of the CO complex was considerably increased when CO was added to $Na_2S_2O_4$-treated P582 (Fig. 2, Curve 3).

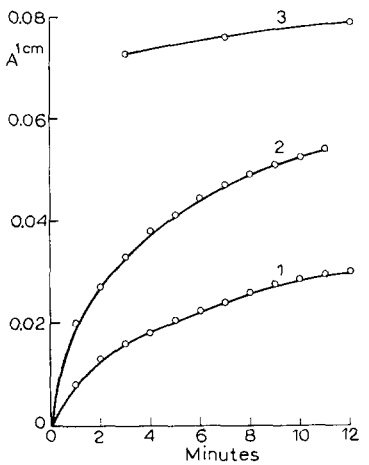


Fig. 2. Course of reaction of P582 with $Na_2S_2O_4$ under H_2 and CO. P582 (Fraction DE-52; $A_{280\ nm}/A_{392\ nm}$, 3.2; 2.6 mg protein) in 0.9 ml of 0.1 M Tris–HCl buffer (pH 8) was gassed with either H_2 or CO. 10 μl of 0.1 M $Na_2S_2O_4$ was injected and changes in the absorbance (ΔA) recorded at 30 °C. Curve 1: shows the decrease in $\Delta A_{582\ nm}$ under H_2 (maximum ΔA was −0.041 after about 30 min); Curve 2: shows the increase in $\Delta A_{596\ nm}$ under CO (maximum ΔA was 0.088 after 30 min); Curve 3: as 2 except that cuvette was flushed with CO after 50 min incubation of P582 with $Na_2S_2O_4$ under H_2: changes in absorbance were recorded from 2 min after addition of CO.

In the presence of 3.3 mM and 20 mM $Na_2S_2O_4$ the rates of reaction under H_2 or CO were, respectively, about 20% and 100% higher than in the presence of 1.1 mM $Na_2S_2O_4$. The general shape of the reaction curves and the maximum changes in absorbance were not significantly affected by $Na_2S_2O_4$ concentration.

At pH 8.0 the rates of the $Na_2S_2O_4$–P582 reaction and CO-complex formation at 30 °C and 10 °C relative to those at 50 °C were about 20% and 2%, respectively.

Effect of dithiothreitol

It was previously reported that dithiothreitol had no effect on the spectrum of P582[1]. Slow reactions between P582 and dithiothreitol under H_2 and CO have now

been observed. The spectral changes were similar to those caused by $Na_2S_2O_4$ under the same conditions but with 2 mM dithiothreitol the rates of change were about one thirtieth of those obtained with an equivalent concentration of $Na_2S_2O_4$.

Effect of reduced methyl viologen

Reduced methyl viologen acts as an electron donor for the enzymic activities of P285 and, therefore, might be expected to reduce the pigment. Fig. 3 (Curve 3) shows the effect of $Na_2S_2O_4$-reduced methyl viologen on P582. An equivalent amount of $Na_2S_2O_4$ and reduced methyl viologen was added to the reference to offset the pronounced blue colour of reduced methyl viologen. The spectrum showed a distinct maximum at 612 nm, which appeared within 1 min as compared with the shoulder in the spectrum of $Na_2S_2O_4$-treated P582 (Fig. 3, Curve 2) which developed over a period about 2–2.5 h.

In attempts to study the effect of reduced methyl viologen alone on P582, it proved impossible to add stoichiometric amounts of the dye to both sample and reference cuvettes owing to the instability of reduced methyl viologen. An arbitrary amount of Zn-reduced methyl viologen, therefore, was injected into a stoppered

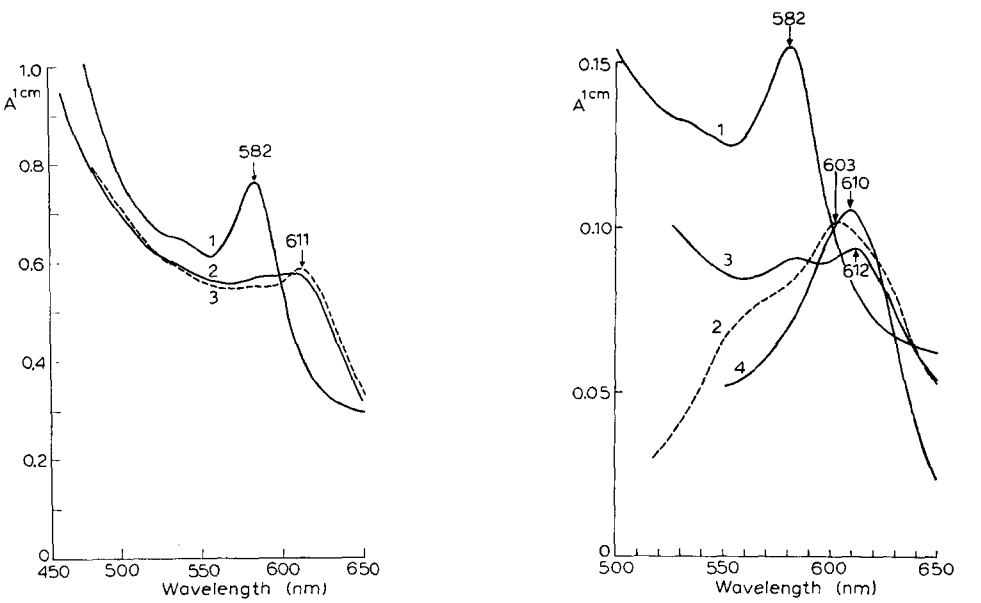


Fig. 3. Effects of $Na_2S_2O_4$ and $Na_2S_2O_4$-reduced methyl viologen on the spectrum of P582 (Fraction DE-52, 5.7 mg protein per ml). Spectrum 1, oxidized P582; 2, P582 + 2 mg per ml of $Na_2S_2O_4$, after stabilization (2.5 h at 20 °C); 3, P582 + 2 mg per ml $Na_2S_2O_4$ in presence of 0.002% methyl viologen after 1 min: reference cuvette contained $Na_2S_2O_4$ and 0.002% methyl viologen in buffer. Absorbance maxima (in nm) are indicated.

Fig. 4. Spectra of P582 reduced by reduced methyl viologen. P582 (Fraction DE-52; $A_{280\text{ nm}}/A_{392\text{ nm}}$, 3.3; 1.6 mg protein per ml) was gassed with H_2 and an arbitrary amount of Zn-reduced methyl viologen added. Methyl viologen was added to a solution of $Na_2S_2O_4$ in Tris–HCl buffer (pH 8) in the reference cuvette until the concentrations of reduced methyl viologen in sample and reference cuvettes were of the same order. Spectrum 1, oxidized P582; 2, 8.7 μM methyl viologen (reduced by $Na_2S_2O_4$) in absence P582; 3, P582 + reduced methyl viologen against reference containing a slight excess of reduced methyl viologen; 4, P582 + reduced methyl viologen against reference containing a slight deficiency of reduced methyl viologen. Absorbance maxima (in nm) are indicated. Note that for spectrum 4 the base line is offset by 0.09 absorbance unit. A maximum at 610 nm is 0.195.

cuvette containing P582, and the reduced methyl viologen concentration in the reference cuvette balanced as closely as possible by titration of methyl viologen into a buffered solution of $Na_2S_2O_4$.

The spectra obtained when reduced methyl viologen was in slight excess in either the reference or sample cuvettes are shown in Fig. 4, Curves 3 and 4, respectively. Reduction of P582 by reduced methyl viologen is indicated by absorption maxima at 610–612 nm. These maxima were in a similar region to those in the spectra of P582 treated with $NaBH_4$ *plus* Na_2SO_3, $Na_2S_2O_4$ *plus* methyl viologen or with dithiothreitol and were further to the red end of the spectrum than those of either P582 or reduced methyl viologen (Fig. 4, Curves 1 and 2, respectively). The absorbance maxima at 610–612 nm appeared within 2–3 min at 25 °C after addition of reduced methyl viologen to P582 and did not increase with time. This, together with the results with $Na_2S_2O_4$-reduced methyl viologen reported above (Fig. 3), indicates that reduced methyl viologen reacted with P582 at a considerably faster rate than other reducing agents such as $Na_2S_2O_4$, $NaBH_4$ *plus* Na_2SO_3 and dithiothreitol. It is possible that the apparently greater reducing efficiency of reduced methyl viologen was due to its property of donating one electron per molecule.

Under CO, P582 reacted with reduced methyl viologen to give a well defined absorption maximum at 596 nm indicating the formation of the CO complex (Fig. 5). The reaction was freely reversible: the original oxidized spectrum was regenerated by exposure to O_2 for 1–2 min.

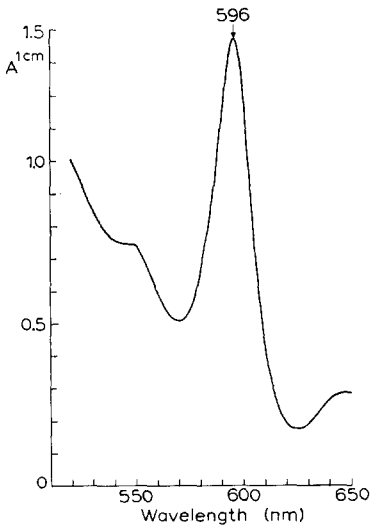


Fig. 5. Spectrum of reduced methyl viologen-reduced P 582 under CO. The experiment was carried out as described in Fig. 4 except that H_2 was replaced by CO. The spectrum was obtained with a slight excess of methyl viologen in the reference cuvette. The absorbance maximum (in nm) is indicated.

Reaction of substrates with reduced P582

Spectra of P582 were examined after its reduction by Zn-reduced methyl viologen followed by enzymic reoxidation of the latter by substrates. The experiments were carried out at pH 8 and room temperature with a DE-52 fraction of P582.

Sufficient Zn-reduced methyl viologen was added to cause extensive reduction of P582 (*cf.* Fig. 4). Subsequently 1 mg of the appropriate substrate was added in a final volume of 3 ml. With NH_2OH the final spectrum was that of oxidized P582. In the case of Na_2SO_3, at the time that reduced methyl viologen was reoxidized the pigment was partly reduced but it was fully reoxidized after a further incubation period of about 1 h. The formation of the CO complex of P582 at pH 8 in the presence of 2–3 mM $Na_2S_2O_4$ was inhibited by 10 mM NH_2OH.

With KNO_2 as the substrate, the spectrum, after reoxidation of reduced methyl

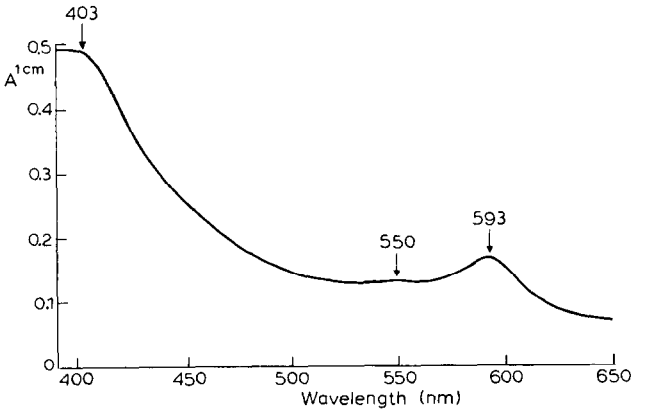


Fig. 6. Spectrum of reduced methyl viologen-reduced P582 in the presence of KNO_2. Fraction DE-52 ($A_{280\ nm}/A_{392\ nm}$, 3.3; 1.5 mg protein per ml) in 0.1 M Tris–HCl buffer (pH 8.0) containing 4.4 mM KNO_2 was flushed with Ar and an arbitrary amount of Zn-reduced reduced methyl viologen added. The spectrum was recorded after complete oxidation of methyl viologen. Absorbance maxima (in nm) are indicated.

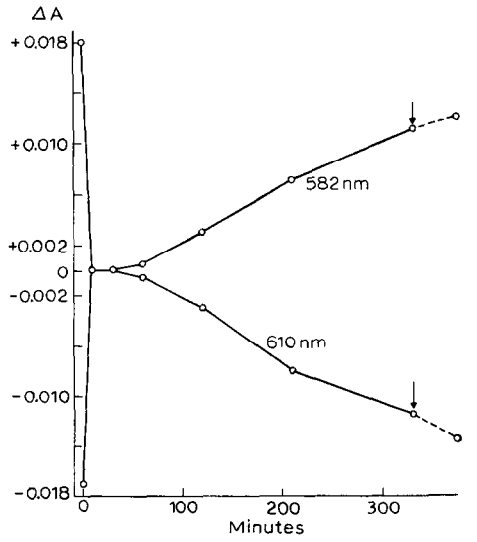


Fig. 7. Anaerobic reoxidation of $Na_2S_2O_4$-reduced P582. P582 (Fraction DE-52; $A_{280\ nm}/A_{390\ nm}$, 3.2; 1.2 mg protein in 0.9 ml) was incubated anaerobically at 50 °C with 1 μmole of $Na_2S_2O_4$. The curves show the ΔA at 582 nm or 610 nm relative to 596 nm, the isosbestic point for the oxidized and reduced spectra. The cuvettes were aerated at the times indicated by the arrows.

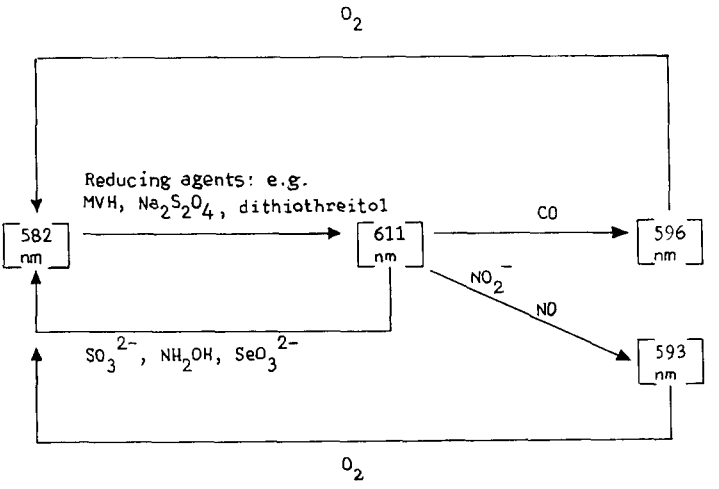
viologen, was characterized by absorbance maxima at 593, 550 and about 403 nm (Fig. 6) and resembled those obtained by adding KNO_2 to P582 in the presence of $Na_2S_2O_4$ or $NaBH_4$ *plus* Na_2SO_3. A similar spectrum was also obtained when P582 was flushed with NO, but not N_2O, in the presence of Zn-reduced methyl viologen. Aeration of the KNO_2–reduced methyl viologen-treated pigment slowly regenerated the oxidized form; at room temperature regeneration was about 50% at 6 h and complete within 24 h.

The selenium analogue of sulphite, Na_2SeO_3 (0.5 mM) caused an immediate and almost complete reoxidation of P582 which had been reduced by 3 mM $Na_2S_2O_4$. It also inhibited the formation of the CO complex in the presence of 3 mM $Na_2S_2O_4$.

$Na_2S_2O_4$-reduced P582 was slowly reoxidized after prolonged incubation under anaerobic conditions (Fig. 7). Recovery of the original oxidized spectrum was incomplete: this was probably due to denaturation since subsequent aeration caused only minor changes to the spectrum. $Na_2S_2O_4$ in solution thus acts as both a reductant and oxidant of P582. The nature of the actual oxidant is problematical: sulphite is a decomposition product of dithionite but other possibilities are the dithionite ion itself and the sulphoxylate radical ion ($SO_2^{\cdot-}$) which is in equilibrium with dithionite in solutions[10].

CONCLUSIONS

The various spectral changes undergone by P582 are summarized as follows:



The four reducing agents studied, Na_2SO_4, $NaBH_4$ *plus* Na_2SO_3, dithiothreitol and reduced methyl viologen (MVH) all caused shifts in the absorption maximum of P582 from 582 nm to 610–612 nm under H_2 and to 596 nm under CO, although not at equal rates or to equal extents. Reduced methyl viologen-reduced and $Na_2S_2O_4$-reduced P582 had similar spectra in the presence of KNO_2. We conclude, therefore, that all the spectral changes were consequences of reduction of P582 and that they do not reflect specific interactions between P582 and sulphur compounds.

Reoxidation of reduced methyl viologen-reduced P582 by sulphite or hydroxyl-

amine is consistent with the notion that the chromophore takes part in the transfer of electrons from reductant to oxidant during the reduction of these two substrates.

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