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CRYSTALLINE PSEUDOMONAS CYTOCHROME OXIDASE

II. SPECTRAL PROPERTIES OF THE ENZYME

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SUMMARY

In the crystalline preparation of Pseudomonas cytochrome oxidase, the α-band due to haem a_2 varied in both absorbancy and position with pH when the enzyme was reduced with $Na_2S_2O_4$. Thus it was at 655 mμ, 629 mμ and 652 mμ, and 625 mμ at pH 7.6, 7.0 and 5.6, respectively. The corresponding γ-band was invariably at 460 mμ. But, when the enzyme was reduced with ascorbate, the α-band did not show dependence on pH. The γ-band due to haem a_2 was very low in absorbancy compared with corresponding α-band.

In the presence of cyanide, the α-band due to haem a_2 appeared at 627 mμ and the corresponding γ-band was split into two parts, with maxima at 443 mμ and 472 mμ. In the presence of CO, the α-band due to haem a_2 was very depressed in absorbancy and the corresponding γ-band became invisible. Nitrite and NO had the same effect on $Na_2S_2O_4$-reduced enzyme. In the presence of these reagents, a peak appeared at 665 mμ, the γ-band at 400 mμ disappeared, and the enzyme solution became reddish brown. With ascorbate-reduced enzyme, a bump at 570 mμ appeared on addition of nitrite or NO. Hydroxylamine had a complicated effect on the enzyme. The reagents mentioned above scarcely affected the absorption spectrum due to haem *c* of the enzyme.

INTRODUCTION

In 1928, YAOI AND TAMIYA[1] found that cells of *Escherichia coli* and *Shigella dysenteriae*, which had been grown aerobically, showed an absorption band which differed from those of previously described cytochromes in that it was in the red region of the spectrum. KEILIN[2] attributed this band to a cytochrome component, which he designated a_2. WARBURG *et al.*[3,4], NEGELEIN AND GERISCHER[5], TAMIYA AND YAMAGUTCHI[6], and FUJITA AND KODAMA[7] independently found spectroscopic evidence that cytochrome a_2 was autoxidizable and could combine with carbon monoxide and cyanide. FUJITA AND KODAMA also showed that this cytochrome was widely distributed in other bacteria *e.g. Azotobacter chroococcum*, *Proteus vulgaris*, *Acetobacter pasteurianum*, *Eberthella typhosa*, and *Salmonella parathyphi*. The oxidized form in whole cells showed the atypical band at 645 mμ in the visible region of the spectrum.

Mainly from spectroscopic studies of cytochrome a_2 in whole cells, it is generally assumed that in organisms which do not possess cytochrome a, cytochrome a_2 functions as a cytochrome oxidase[8].

BARRETT[9] extracted and partially purified a green haemin from *Aerobacter aerogenes* and several other bacteria, all of which contain cytochrome a_2. He designated it haemin a_2. However, cytochrome a_2 has not been isolated from the bacteria described above.

In our laboratory, a cytochrome, which has haem a_2 and a haem c in its molecule, was extracted and purified from cells of *Pseudomonas aeruginosa* which were grown anaerobically in the presence of nitrate[10]. The cytochrome was designated as Pseudomonas cytochrome oxidase because it possesses the general properties of a cytochrome oxidase. Furthermore, it was found that this cytochrome acts as a nitrite reductase[11,12]. It is very likely that the cytochrome is the chemical entity of the nitrite reductase of *P. aeruginosa*, because, Pseudomonas cytochrome oxidase was obtained from cells of the organism which had been grown in the presence of nitrate but not from cells grown in the absence of nitrate[13]. Therefore, in cells grown aerobically in the absence of nitrate, there may be a cytochrome oxidase other than Pseudomonas cytochrome oxidase, and the former may be the cytochrome a_2 which possesses only haem a_2 in its molecule.

Recently, Pseudomonas cytochrome oxidase has been obtained in a crystalline state[14,15]. The crystalline preparation of the enzyme has the two haems and the two activities mentioned above. Although as mentioned above, a cytochrome which has only haem a_2 on its molecule has not been isolated, the Pseudomonas cytochrome oxidase has haem a_2 as well as a haem c. Thus, it may be possible to determine various properties of cytochrome a_2, with the Pseudomonas cytochrome oxidase preparation.

As for the prosthetic group of cytochrome a_2, LEMBERG AND WYNDHAM[16] considered that it resembled biliviolin iron complexes. From a study of the difference spectrum of the CO-compound of cytochrome a_2, CHANCE[17] concluded that the spectrum of its prosthetic group lacked a distinct γ-band. But these ideas were refuted by BARRETT[9] and by our previous report[10]. This paper deals with the spectral properties of the crystalline preparation of Pseudomonas cytochrome oxidase. The properties of the prosthetic groups will be described in the next paper of this series[18].

MATERIALS AND METHODS

Pseudomonas cytochrome oxidase was obtained in a crystalline state by the method previously described[15]. Twice recrystallized enzyme was dissolved in 0.2 M phosphate buffer (pH 7.0) at arbitrary concentrations and the green solution obtained was used as the enzyme solution. Yeast lactate dehydrogenase was purified by the method of YAMANAKA *et al.*[19] with the slight modification by YAMASHITA[20].

Spectrophotometric determinations were performed in a Cary recording spectrophotometer, model 14. To determine the absorbancy anaerobically, a Thunberg type cuvette was used.

RESULTS

Normal absorption spectra

As previously reported[15], Pseudomonas cytochrome oxidase showed a com-

plicated absorption spectrum especially in the visible region of the spectrum. There were absorption peaks at 280 mμ, 412 mμ, 525 mμ, and 635 mμ in the oxidized form, and at 418 mμ, 523 mμ, 549 mμ, and 554 mμ in the reduced form. In the latter form, there was a bump at 460 mμ ,and the band at 635 mμ in the oxidized form changed into a plateau from 620 mμ to 650 mμ with a bump around 650 mμ. As previously reported[16], the bump at 460 mμ represents the γ-band of haem a_2. Thus, the bump may be designated as the γ-band of "cytochrome a_2". The γ-band at 460 mμ is clearly shown in the difference spectrum (Fig. 1). It is very noticeable

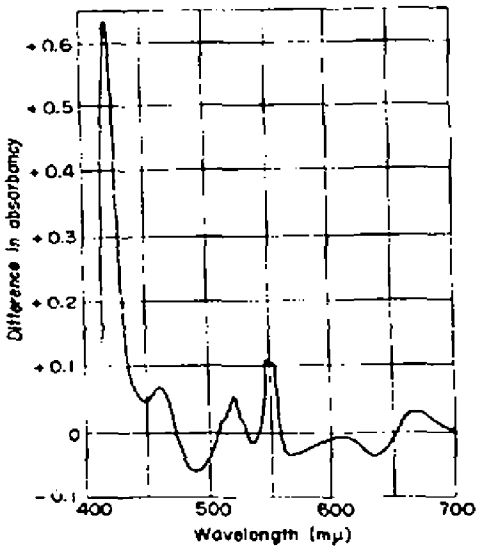

Fig. 1. Difference spectrum of Pseudomonas cytochrome oxidase. The concentration of Pseudomonas cytochrome oxidase was $8.0 \cdot 10^{-6}$ M. Spectrophotometric measurements were made in 0.2 M phosphate buffer (pH 7.0) in air. The absorption curve shows the difference, $Na_2S_2O_4$-reduced minus oxidized.

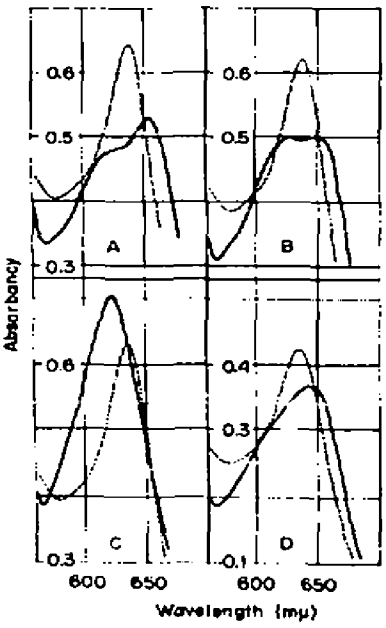

Fig. 2. Effect of pH on the absorption spectrum of Pseudomonas cytochrome oxidase in the red region. The concentration of Pseudomonas cytochrome oxidase was $2.0 \cdot 10^{-6}$ M (A), $2.7 \cdot 10^{-5}$ M (B,C) and $1.8 \cdot 10^{-5}$ M (D). The pH was 7.6 (A), 7.0 (B), 5.6 (C), and 7.0 (D). The enzyme was dissolved in 0.2 M phosphate buffer at each pH, except for D when 0.17 M phosphate buffer was used. - - - - - -, Oxidized; ———, reduced with $Na_2S_2O_4$ in A, B and C, and with yeast lactate dehydrogenase and lactate in D. Spectrophotometric measurements were performed in air in A, B, and C, but *in vacuo* in D.

that the absorbancy at 635 mμ in the oxidized form was depressed by reduction of the enzyme with $Na_2S_2O_4$. The depressed absorbancy reverted to the original height on reoxidation with $K_3Fe(CN)_6$. Therefore, the depression of the band at 635 mμ by reduction with $Na_2S_2O_4$ was not caused by decomposition of the haem a_2. The band at 635 mμ was not influenced by variation in pH, whereas the spectrum of $Na_2S_2O_4$-reduced enzyme in the red region varied considerably with pH. As shown in Fig. 2, at pH 7.6, an absorption band appeared at 655 mμ, and at pH 5.6, an

absorption band appeared at 625 mμ on reduction with $Na_2S_2O_4$. At pH 7.0, there were two peaks at 629 mμ and 652 mμ which appeared to be a plateau at lower concentrations of the enzyme. When ascorbate was used in place of $Na_2S_2O_4$ to reduce the enzyme, an asymmetrical peak at 645 mμ appeared, whereas the corresponding γ-band appeared at 460 mμ. With ascorbate, there was no such variation in the position of the α-band of haem a_2 with pH as was seen with $Na_2S_2O_4$ although the absorbancies of the α-band and the γ-band at 460 mμ became higher as the pH became acidic. The ratio of $A^{red}_{460m\mu}/A^{red}_{645m\mu}$ increased as the pH decreased from 8.2 to 5.0 (Table I). When the enzyme was reduced with yeast lactate dehydrogenase and lactate, a peak appeared at 649 mμ similar to that observed with ascorbate (see Fig. 2D). Even under quite anaerobic conditions the peaks at 645 mμ and 649 mμ described above were not altered. However, addition of $Na_2S_2O_4$ under these conditions resulted in the same absorption spectrum as that produced by addition of $Na_2S_2O_4$ only. In the case of $Na_2S_2O_4$-reduction, evacuation of the enzyme solution did not change the plateau appearing at pH 7.0, in the red region of the spectrum. At pH 5.6, as described above, the peak appearing at 625 mμ on $Na_2S_2O_4$ reduction, was higher than the peak at 635 mμ of the oxidized form. However, especially at pH 7.0, the peak at 635 mμ was depressed by reduction with $Na_2S_2O_4$ and this resulted in the appearance of two peaks in the difference spectrum in the red region, as shown in Fig. 1. In all cases mentioned above, the γ-band due to haem a_2 appeared constantly at 460 mμ, and the $A^{red}_{460m\mu}/A^{oxi}_{635m\mu}$ ratio was invariably approx. 2.2 (Table II). The ratio of $A^{red}_{460m\mu}/A^{red}_{625m\mu}$ (at

TABLE I

EFFECT OF PH ON THE $A^{red}_{460m\mu}/A^{red}_{645m\mu}$ RATIO OF ASCORBATE-REDUCED PSEUDOMONAS CYTOCHROME OXIDASE

pH	$A^{red}_{460m\mu}/A^{red}_{645m\mu}$
8.2	2.1
7.0	2.3
6.0	2.5
5.0	2.9

TABLE II

VARIATION IN THE α- AND γ-BANDS OF HAEM a_2 OF PSEUDOMONAS CYTOCHROME OXIDASE

pH	λ_{max} (mμ) γ-band	α band		$A^{red}_{\gamma}/A^{oxi}_{\alpha}$	$A^{red}_{\alpha}/A^{oxi}_{\alpha}$
	Reduced	Oxidized	Reduced		
7.6	460	635	655	2.15	0.83
7.0	460	635	629, 652	2.28	0.81
6.4	460	635	625	2.24	1.0
5.6	460	635	625	2.15	1.1

* When there were two peaks, the higher one was selected.

pH 5.6) was 1.9. These ratios were very small and not comparable with those of cytochrome *a* (see ref. 21). The peaks at 418 mμ, 549 mμ and 554 mμ of the reduced form of the enzyme did not vary with pH.

Effect of cyanide

The cytochrome oxidase and nitrite reductase activities of Pseudomonas cytochrome oxidase with reduced Pseudomonas cytochrome *c*-551 as the electron donor were strongly inhibited in the presence of 10^{-3} M KCN (see refs. 12, 15, 22). But the latter activity was inhibited less than the former. In crude preparation of the enzyme, no spectral change was seen on addition of CN^- (ref. 22). The absorption spectrum of the crystalline preparation was affected strikingly by CN^-, as shown in Fig. 3. In the presence of 10^{-3} M KCN at pH 7.0, a peak appeared at 627 mμ

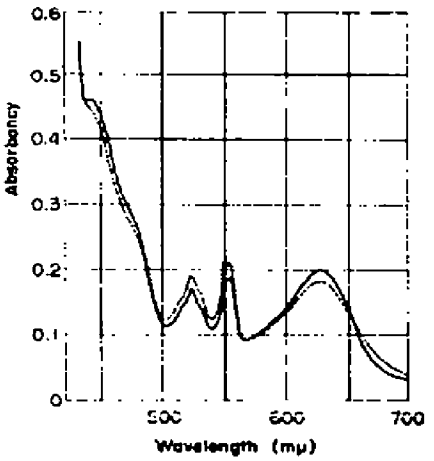

Fig. 3. Effect of CN^- on the absorption spectrum of Pseudomonas cytochrome oxidase. The concentrations of Pseudomonas cytochrome oxidase, ascorbate and KCN were $7.0 \cdot 10^{-6}$ M, $3.1 \cdot 10^{-2}$ M and $1.9 \cdot 10^{-3}$ M, respectively. Spectrophotometric measurements were performed in 0.19 M phosphate buffer (pH 7.0) in air. ——, reduced with ascorbate; - - - - -, reduced with $Na_2S_2O_4$.

on $Na_2S_2O_4$-reduction, instead of a plateau as in the absence of CN^-, and the enzyme solution became greener. This peak at 627 mμ was similar to that appearing at pH 5.0 in the absence of CN^-. However, there was a big difference between the two spectra. Thus, the γ-band due to haem a_2 appeared at 460 mμ on reduction with $Na_2S_2O_4$ in the absence of CN^-, whereas no γ-band appeared in the presence of CN^-, but there was a bump at 443 mμ and a shoulder at 472 mμ. As Fig. 3 shows, the γ-band due to haem a_2 was split into two peaks. This was clearly shown in the difference spectrum (Fig. 4). Thus, the absorption spectrum of the crystalline preparation of the enzyme in the presence of CN^- was very similar to that of the crude enzyme in the absence of CN^- (see refs. 10, 23). The bump at 443 mμ appearing in the presence of CN^- was more distinctive when the enzyme was reduced with ascorbate than when it was reduced with $Na_2S_2O_4$ (Fig. 3). No spectral change in the

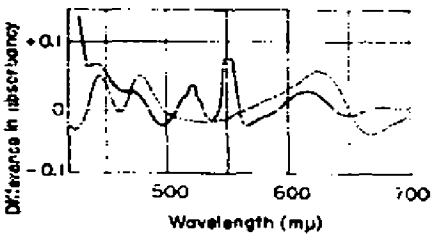

Fig. 4. The difference spectrum of Pseudomonas cytochrome oxidase in the presence of CN^-. The concentrations of Pseudomonas cytochrome oxidase and KCN were $7.3 \cdot 10^{-6}$ M and $1.2 \cdot 10^{-3}$ M, respectively. Spectrophotometric determinations were carried out in 0.18 M phosphate buffer at pH 7.0, in air. ———, Difference, (with CN^- and $Na_2S_2O_4$) (minus oxidized); - - - - -, difference, (with CN^- and $Na_2S_2O_4$) minus (with $Na_2S_2O_4$).

oxidized form of the enzyme was observed in the presence of CN^-, and the peak at 418 mμ in the reduced form was not affected by the reagent.

Effect of CO

The cytochrome oxidase activity of Pseudomonas cytochrome oxidase was strongly inhibited by CO (see ref. 22). In the crude preparation of the enzyme, it was found that the absorbancy in the red region of the spectrum was depressed in the presence of CO. This finding was confirmed in the crystalline preparation. Although the depression of the absorbancy in the red region of the spectrum caused by CO was

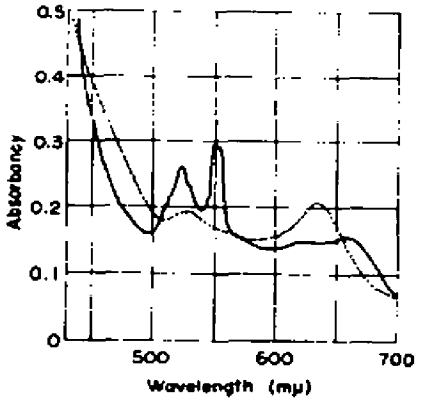

Fig. 5. Effect of CO on the absorption spectrum of Pseudomonas cytochrome oxidase. The concentration of Pseudomonas cytochrome oxidase was $1.1 \cdot 10^{-5}$ M. Spectra were measured in 0.2 M phosphate buffer (pH 7.0) in 100% CO, using a Thunberg-type cuvette. - - - - -, Oxidized; ———, reduced with $Na_2S_2O_4$.

similar to that observed when the enzyme was reduced with $Na_2S_2O_4$ at pH 7.0 in the absence of CO, as already mentioned, the absorbancy was much more strikingly depressed in the presence of CO than in its absence (Fig. 5). The most striking difference between the two cases was that, in the absence of CO, a γ-band was seen at 460 mμ whereas in the presence of CO, no γ-band appeared. The difference spectrum between the normal reduced form and the reduced form in the presence of CO

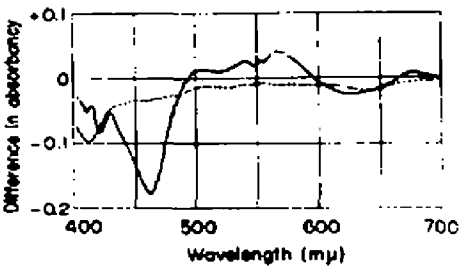

Fig. 6. The difference spectrum of Pseudomonas cytochrome oxidase in the presence of CO. The concentration of Pseudomonas cytochrome oxidase was $1.0 \cdot 10^{-5}$ M. Spectra were measured in 0.2 M phosphate buffer (pH 7.0) using a Thunberg-type cuvette. - - - - -, Difference, (oxidized in CO) minus (oxidized); ——, difference, (reduced with $Na_2S_2O_4$ in CO) minus (reduced with $Na_2S_2O_4$)

shows these facts very clearly (Fig. 6). Fig. 6 also shows that CO affected the spectrum of the oxidized form. But the effect of CO on the spectrum of the oxidized form was much less striking than on that of the reduced form.

As previously reported, the nitrite reduction catalysed by Pseudomonas cytochrome oxidase was not inhibited by CO even in the dark[12,15]. This finding suggests that the cytochrome oxidase and nitrite reductase activities are due to the different active sites on the enzyme. As shown in Fig. 7, enzyme reduced with ascor-

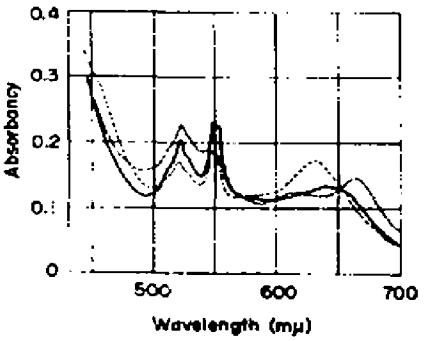

Fig. 7. Effect of NO_2^- on the absorption spectrum of Pseudomonas cytochrome oxidase in CO. The concentrations of Pseudomonas cytochrome oxidase, ascorbate and KNO_2 were $7.7 \cdot 10^{-6}$ M, $1.9 \cdot 10^{-2}$ M and $7.7 \cdot 10^{-3}$ M, respectively. Spectra were measured in 0.18 M phosphate buffer (pH 7.0) in 100% CO, using a Thunberg-type cuvette. ——, Reduced with ascorbate; - - - - -, NO_2^- was added to the ascorbate-reduced enzyme; and · · · · ·, $Na_2S_2O_4$ was added after addition of NO_2^-.

bate was partially oxidized on addition of NO_2^- in the presence of CO, and the peak at 635 mμ was almost completely restored to that of the oxidized state. This result may be explained by assuming that NO_2^- reacted with another site than that combining with CO, and that the enzyme was oxidized. However, when $Na_2S_2O_4$ was added to the above reaction mixture, the absorption spectrum was the same as that of $Na_2S_2O_4$-reduced enzyme in the presence of NO_2^- and absence of CO (Fig. 7; see Fig. 12). The peak at 665 mμ was caused by the reaction of reduced enzyme with NO as described below. Thus, it was shown that CO has much less affinity for the reduced enzyme than NO, and perhaps than NO_2^-.

The reduced enzyme was brown in the presence of CO. When the CO was evacuated and the enzyme solution was allowed to stand overnight, it became green, the colour of the normal enzyme solution. Therefore, CO seemed to be removed from the enzyme solution by evacuation. This idea was supported by the absorption spectrum, since the γ-band at 460 mμ appeared (Fig. 8). However, on introduction of air into the enzyme solution, the enzyme solution became brown and the γ-band at 460 mμ

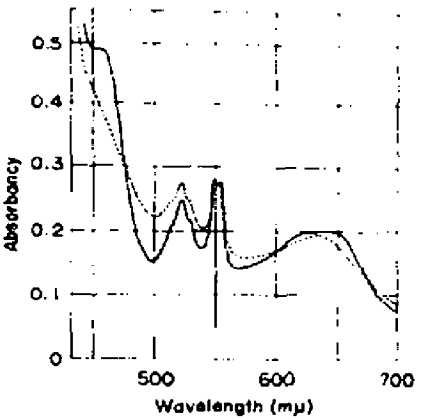

Fig. 8. Change in the absorption spectrum on evacuation of a solution of Pseudomonas cytochrome oxidase containing CO. The concentration of Pseudomonas cytochrome oxidase was $1.0 \cdot 10^{-4}$ M. Spectra were measured in 0.2 M phosphate buffer (pH 7.0). —·—·—, Allowed to stand overnight after a considerable evacuation of the enzyme solution containing CO (see Fig. 5); - - - - -, after introduction of air into the above sample.

was lost, although the absorbancy in the red region of the spectrum was less depressed than in the presence of a 100% CO-atmosphere, and the peaks at 549 mμ and 554 mμ remained strongly reduced. The peak at 478 mμ was not affected by CO.

Effect of NO_2^- and NO

Reduced Pseudomonas cytochrome oxidase was rapidly oxidized in the presence of air. It is anaerobically oxidized in the presence of nitrite, as the enzyme acts as a nitrite reductase. As shown in Fig. 9, enzyme which had been reduced with yeast lactate dehydrogenase and lactate was rapidly partially oxidized on addition of NO_2^- under anaerobic conditions. Fig. 9 shows that the haem a_2 moiety was completely oxidized but that the haem *c* moiety remained partially reduced. On further addition of nitrite the enzyme was completely oxidized. On the other hand, when nitrite was added to enzyme which had been reduced with ascorbate, a peak appeared at 630 mμ, which was evidently different from the peak of the oxidized form at 635 mμ (Figs. 9 and 10). It was very noticeable that ascorbate-reduced enzyme showed a peak at 570 mμ on addition of nitrite. When $Na_2S_2O_4$ was added to enzyme solution which had reacted with ascorbate and then with nitrite, there was no reduction of the haem *c* moiety (Fig. 11). The same absorption spectrum was observed when NO in N_2 was bubbled through enzyme which had been reduced with ascorbate. This is to be ex-

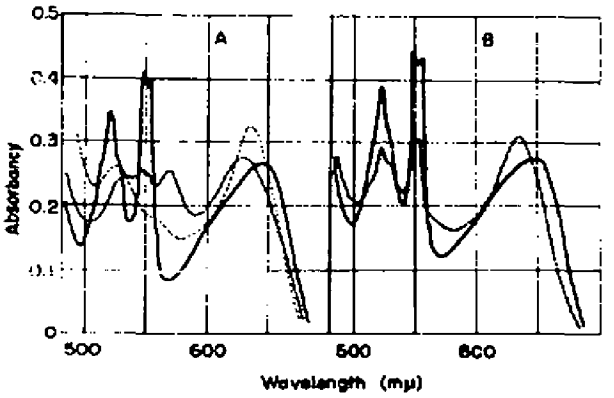

Fig. 9. Effect of NO_2^- on the absorption spectrum of Pseudomonas cytochrome oxidase reduced with ascorbate (A) and with yeast lactate dehydrogenase (B). (A) The concentrations of Pseudomonas cytochrome oxidase, ascorbate and KNO_2 were $1.8 \cdot 10^{-5}$ M, $2.2 \cdot 10^{-3}$ M and $1.7 \cdot 10^{-2}$ M, respectively. Spectra were measured in 0.13 M phosphate buffer (pH 7.0), in N_2. - - - - -, Oxidized; ——, reduced with ascorbate; - - - - - -, NO_2^- added to the ascorbate-reduced enzyme. (B) The concentrations of Pseudomonas cytochrome oxidase, lactate and KNO_2 were $2.0 \cdot 10^{-5}$ M, $4.2 \cdot 10^{-3}$ M and $1.7 \cdot 10^{-2}$ M, respectively. Yeast lactate dehydrogenase was diluted to a concentration which did not interfere with spectral measurements of Pseudomonas cytochrome oxidase. Spectra were measured in the same way as for A. ———, reduced with a yeast lactate dehydrogenase system for 25 min; - - - - -, NO_2^- added to the above sample.

pected because NO is the reduction product of NO_2^- (see ref. 12). When NO_2^- was added to $Na_2S_2O_4$-reduced enzyme, a peak appeared at 665 mμ, and the enzyme solution changed from green to reddish brown (Fig. 12). In this case the peak at 570 mμ did not appear. The same spectrum and the reddish brown colour were observed also when NO in N_2 was bubbled through a solution of $Na_2S_2O_4$-reduced enzyme. It was noteworthy that in the presence of CO this peak at 570 mμ did not appear when NO_2^- was added to ascorbate-reduced enzyme, whereas the peak at 635 mμ appeared on addition of NO_2^- (see Fig. 7). The spectrum of the oxidized

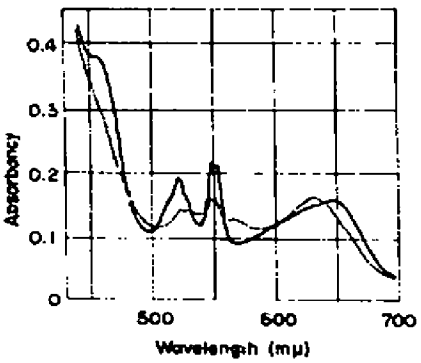

Fig. 10. Effect of NO_2^- on the absorption spectrum of Pseudomonas cytochrome oxidase reduced with ascorbate. The concentrations of Pseudomonas cytochrome oxidase, ascorbate and KNO_2 were $7.2 \cdot 10^{-5}$ M, $2.0 \cdot 10^{-3}$ M and $8.0 \cdot 10^{-4}$ M, respectively. Spectrophotometric measurements were performed in 0.18 M phosphate buffer (pH 7.0), in N_2 using a Thunberg-type cuvette. ———, Reduced with ascorbate; - - - - -, NO_2^- added after reduction with ascorbate for 5 min.

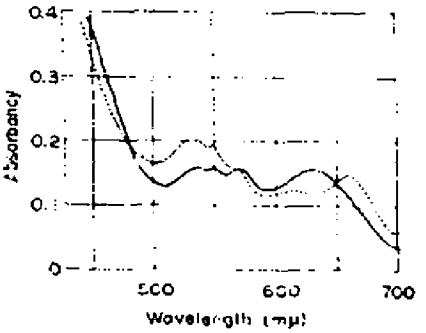

Fig. 11. Reduction by $Na_2S_2O_4$ of Pseudomonas cytochrome oxidase which had reacted with ascorbate in NO. The concentrations of Pseudomonas cytochrome oxidase and ascorbate were $8.7 \cdot 10^{-6}$ M and $1.8 \cdot 10^{-3}$ M, respectively. Spectra were measured in 0.18 M phosphate buffer (pH 7.0), in NO. · · · —, Reduced with ascorbate. - - - -, further reduced with $Na_2S_2O_4$.

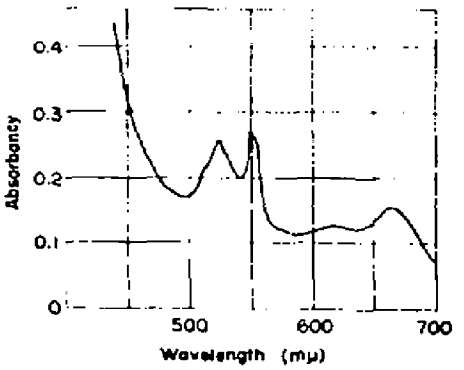

Fig. 12. Absorption spectrum of Pseudomonas cytochrome oxidase which had been reduced with $Na_2S_2O_4$ in NO. The concentration of Pseudomonas cytochrome oxidase was $9.0 \cdot 10^{-6}$ M. Spectra were measured in 0.2 M phosphate buffer (pH 7.0). The absorption curve shows the absorption spectrum of $Na_2S_2O_4$-reduced enzyme in NO.

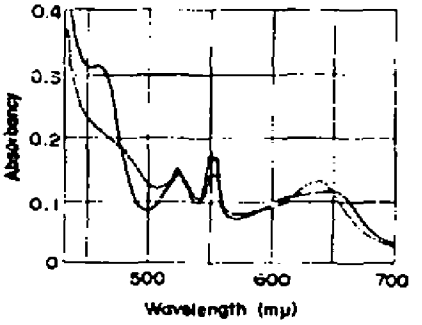

Fig. 13. Effect of NH_2OH on the absorption spectrum of Pseudomonas cytochrome oxidase in the presence of ascorbate. The concentrations of Pseudomonas cytochrome oxidase, ascorbate and NH_2OH were $3.7 \cdot 10^{-6}$ M, $5.6 \cdot 10^{-3}$ M and $1.7 \cdot 10^{-2}$ M, respectively. Spectra were measured in 0.18 M phosphate buffer (pH 7.0), in air. - - - - -, Reduced with ascorbate in the presence of NH_2OH; ———, further reduced by addition of $Na_2S_2O_4$ to the above reaction mixture.

enzyme was not affected with NO_2^- or NO, under the conditions tested. The peak at 418 mμ was not affected by these reagents.

Effect of NH_2OH

In the oxidized form, the absorption spectrum of Pseudomonas cytochrome oxidase was not affected by NH_2OH. When NH_2OH was added to enzyme which had been reduced with ascorbate, a broad shoulder around 450–500 mμ appeared in the absorption spectrum. On addition of $Na_2S_2O_4$ to the above mixture the same absorption spectrum was seen as when the enzyme was reduced with $Na_2S_2O_4$ in the absence of NH_2OH (Fig. 13). When the enzyme was reduced with $Na_2S_2O_4$ in the presence of NH_2OH without ascorbate, the bump at 460 mμ became obscure and

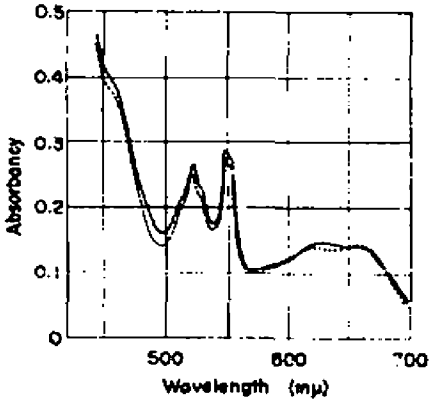

Fig. 14. Effect of NH_2OH on the absorption spectrum of Pseudomonas cytochrome oxidase in the absence of ascorbate. The concentrations of Pseudomonas cytochrome oxidase and NH_2OH were $9.6 \cdot 10^{-6}$ M and $1.8 \cdot 10^{-3}$ M, respectively. Spectrophotometric measurements were carried out in 0.18 M phosphate buffer (pH 7.0) in air. ———, Reduced with $Na_2S_2O_4$; - - - - -, ascorbate added to the above mixture.

the absorbancy around 630–660 mμ was much depressed (Fig. 14). Addition of ascorbate did not change the above absorption spectrum; the distinctive bump at 460 mμ did not appear.

DISCUSSION

As previously reported[14,15], the great difference in the absorption spectra of crude and crystalline preparations of Pseudomonas cytochrome oxidase was that the peak at 625 mμ observed in the reduced form of the crude preparation became a plateau in the crystalline preparation. Thus, in the difference spectrum of the crystalline preparation of the enzyme, two peaks were observed in the red region of the spectrum at 610 mμ and 670 mμ. If such difference spectra were found in whole cells, this would lead to the incorrect conclusion that these two peaks were due to the α-peaks of two cytochromes. However, as will be described later, it is doubtful whether Pseudomonas cytochrome oxidase shows the same spectrum as the crystalline preparation in the cell. In the difference spectrum, there is a peak at 460 mμ. This peak was seen as a bump in the absolute spectrum, and perhaps is the γ-band

of haem a_2, as already mentioned. Therefore, this may be the γ-band of cytochrome a_2. Thus, the γ-band of cytochrome a_2 had very low absorbancy compared with the α-band of other cytochromes, as reported by other workers[8,17]. However it can be concluded that cytochrome a_2 has a distinctive γ-band.

In the presence of CN^-, a peak appeared at 627 mμ when the enzyme was reduced with $Na_2S_2O_4$ or ascorbate. In this case, a bump appeared at 443 mμ and a shoulder around 472 mμ. These spectral properties were quite similar to those observed in the crude preparation of the enzyme in the absence of CN^-. Although the activity of Pseudomonas cytochrome oxidase was inhibited by CN^-, the crude preparation of the enzyme was highly active. Thus, the crude preparation of the enzyme seemed to be combined with some substance which did not inhibit the enzyme. The difference spectrum in the presence of CN^- showed two peaks at 443 mμ and 472 mμ. Therefore, it seemed very likely that the γ-band of the normal enzyme at 460 mμ was split into two parts.

The presence of CO considerably depressed the absorbancy of the reduced enzyme in the red region of the spectrum, and the peak of the normal enzyme at 460 mμ disappeared. It is very probable that as the α-band due to haem a_2 was depressed, the corresponding γ-band was also depressed. However, as shown in Fig. 8, it is very curious that when an enzyme solution which had been in contact with CO, was evacuated and then air was introduced, the γ-band disappeared, whereas the α-band was scarcely depressed. In the presence of NO, a peak at 665 mμ appeared and the corresponding γ-band disappeared as in the presence of CO. As mentioned above, in the spectrum of the normal enzyme, the α-band due to haem a_2 varied both in absorbancy and position with pH and with the reducing agent used, while the corresponding γ-band was invariable at least in position. The facts described above show that the relation between the α-band and the γ-band is very complicated, although it is sure that the two bands are due to haem a_2 (see ref. 18).

It was shown previously[12] that Pseudomonas cytochrome oxidase which had been reduced with ascorbate or hydroquinone showed a bump around 570 mμ on addition of NO_2^- under anaerobic conditions or in the presence of NO. This finding was confirmed with the crystalline preparation. It is very curious that when the bump had once appeared, the peaks at 549 mμ and 554 mμ, which were seen in the reduced form of the enzyme, did not appear by addition of $Na_2S_2O_4$, although in the red region of the spectrum a peak appeared at 665 mμ. The bump at 570 mμ was not seen when NO_2^- or NO was added to enzyme which had been reduced with $Na_2S_2O_4$ or with a yeast lactate dehydrogenase system. It was also not seen when NO_2^- was added to enzyme reduced with ascorbate in CO. It is very likely that the bump at 570 mμ is due to the reaction of NO with the free ascorbate radical, monodehydroascorbate, formed during the reaction. The facts mentioned above, also confirmed with the crystalline preparation of the enzyme, that the product of reduction of NO_2^- by the enzyme was NO.

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