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CRYSTALLINE PSEUDOMONAS CYTOCHROME OXIDASE

III. PROPERTIES OF THE PROSTHETIC GROUPS

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SUMMARY

Haem a_2 of Pseudomonas cytochrome oxidase was easily cleaved from the protein moiety by treatment with HCl-acetone. Haematin a_2 was insoluble in acetone and so easily separable from it. Haematin a_2 and haem a_2 had their α-bands at 684 mμ and 626 mμ, respectively. The pyridine-, CN^--, NO- and CO-derivatives of haem a_2 had their α-bands at 620 mμ, 632 mμ, 625 mμ and 644 mμ, respectively. The γ-bands of haematin a_2 and its derivatives were composed of two or three peaks. The ratio of A_γ/A_α for haematin a_2 and its derivatives was very small and not comparable with that of other haems.

The haem bound to the protein moiety and resistant to HCl–acetone treatment was cleaved from the protein by Ag_2SO_4-treatment, and shown to be haematohaem. The absorption spectra of haem a_2 and Pseudomonas cytochrome oxidase are compared.

INTRODUCTION

Although cytochrome a_2, which has haem a_2 as a prothetic group, has not been isolated, haem a_2 itself has been isolated from whole cells of some bacteria and highly purified[1]. Haem a_2 was found to be an iron complex of a chlorin.

As previously mentioned[2-4], Pseudomonas cytochrome oxidase had a complicated spectrum due to a haem a_2 and a haem c present in the same molecule. When the enzyme was treated with acetone containing hydrochloric acid, haem a_2 was extracted into the acetone and the protein moiety which contained the haem c was precipitated. As indicated already, the haem a_2 obtained from Pseudomonas cytochrome oxidase differed in some respects from that extracted from whole bacterial cells[2].

As prosthetic groups of cytochromes, protohaem, haem c, haem a and haem a_2 are known at present[5]. Of these, little is known about haem a_2. Haem a_2 obtained from crystalline Pseudomonas cytochrome oxidase seems to be pure. Therefore, various properties of the haem a_2 from the crystalline enzyme were examined, and those of the haem c bound to the protein moiety and not split off by acid–acetone treatment were also studied.

MATERIALS AND METHODS

Pseudomonas cytochrome oxidase

Crystalline Pseudomonas cytochrome oxidase was prepared by the method previously mentioned[3]. Twice recrystallized enzyme was dissolved in deionized water at a concentration of approx. 10^{-4} M and used for preparation of haem a_2 and haematohaem.

Cleavage of haems from the enzyme protein

Haem a_2 was isolated from the protein moiety by treatment of the enzyme with acetone containing 0.12 N HCl, and haematohaem by the method of PAUL[4] with a slight modification. The details of the procedures used are described below.

Spectrophotometric determinations

Spectrophotometric measurements were performed in a Cary recording spectrophotometer, model 14. To determine the absorption spectra of the NO- and CO-derivatives of haem a_2, Thunberg-type cuvettes were used.

RESULTS

Haem a_2

To 0.5 ml of Pseudomonas cytochrome oxidase solution, were added 4.0 ml of acetone containing 0.12 N HCl, which had been chilled in an ice–NaCl mixture. The mixture was vigorously agitated. After 1 min, it was centrifuged at 2000 × *g* for 2 min, and the reddish green supernatant was separated from the red precipitate. Fig. 1 shows the absorption spectrum of haemin a_2 in acetone. There was an absorption peak at 432 mμ (γ-band) and a broad plateau from 520 mμ to 620 mμ with three slight bumps at 529 mμ, 569 mμ and 606 mμ.

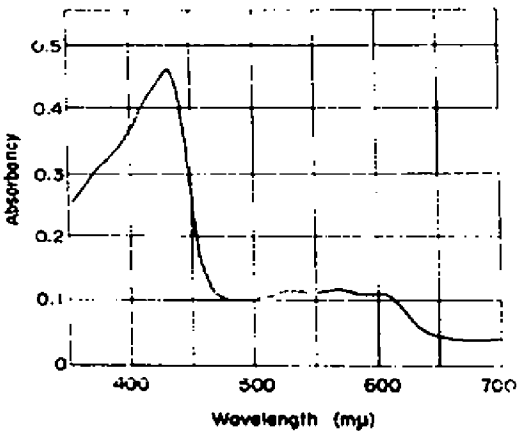

Fig. 1. The absorption spectrum of haemin a_2. Haemin a_2 was dissolved in acetone containing 0.12 N HCl and approx. 11% water.

Haematin a_2 and haem a_2

To the reddish green solution of haemin a_2 obtained as described above (4.5 ml), 0.6 ml of 2 N NaOH was added with vigorous agitation. The solution became turbid. When it was centrifuged at 2000 × *g* for 2 min, the haematin a_2 settled at the bottom of the tube as a green layer. The colourless acetone supernatant layer was pipetted out. When the green layer was diluted with 0.2 M phosphate buffer (pH 7.0), the pH of the resulting solution was 11. The absorption spectrum of haematin a_2 is shown in Fig. 2. The spectrum of haem a_2, which was made by addition of $Na_2S_2O_4$ to

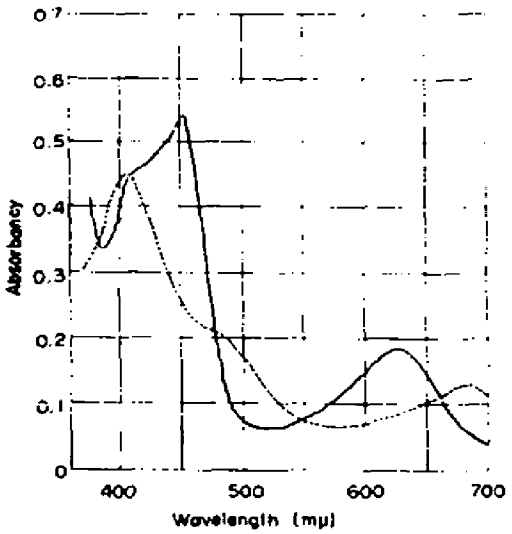

Fig. 2. The absorption spectra of haematin a_2 and haem a_2. Haematin a_2 was dissolved in 0.2 M phosphate buffer, and the pH of the solution was adjusted to 11. The concentration of haematin a_2 was $1.3 \cdot 10^{-5}$ M. Haem a_2 was made by addition of $Na_2S_2O_4$ to haematin a_2. - - - - -, Haematin a_2; ——, haem a_2.

haematin a_2, is also shown. Haematin a_2 had absorption peaks at 405 mμ (γ-band) and 684 mμ (α-band), and a broad shoulder around 480 mμ. When haematin a_2 was reduced to haem a_2 by $Na_2S_2O_4$, a γ-band appeared at 453 mμ with a shoulder around 416 mμ, and the α-band shifted to 626 mμ.

Derivatives of haematin a_2 and haem a_2

Pyridine- and CN^--derivatives of haematin a_2 were obtained by addition of pyridine and KCN to an aqueous solution of haematin a_2. Figs. 3 and 4 show the absorption spectra of the pyridine- and CN^--derivatives of haematin a_2. The absorption spectrum of the pyridine-derivative was not affected by addition of $Na_2S_2O_4$. Thus, the iron atom in the haematin a_2 seems to be reduced by addition of pyridine. In the pyridine haemochromogen of haem a_2, the γ-band seems to consist of three peaks: a main peak at 432 mμ, and two shoulders around 409 mμ and 454 mμ. The α-band was at 620 mμ. The cyanide haemochromogen had an α-band at 632 mμ, γ-

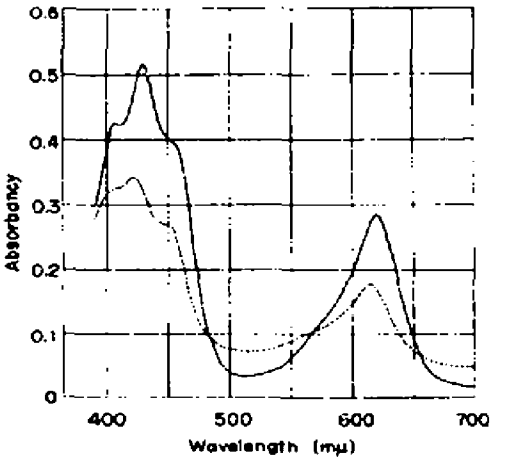

Fig. 3. The absorption spectrum of the pyridine haemochromogen of haem a_2. The concentration of the haemochromogen was $1.17 \cdot 10^{-5}$ M and the pH of the solution was 12 in aqueous solution. The concentration in acetone was $5.6 \cdot 10^{-6}$ M. Addition of $Na_2S_2O_4$ to the solution did not change the absorption spectrum. · · ·—, Aqueous solution; - - - - -, in acetone containing 30% water.

bands at 449 mμ and 480 mμ, and a shoulder around 414 mμ. The γ-band of the cyanide haemochromogen evidently consisted of two peaks.

During neutralization of the acid acetone solution of haemin a_2, when the concentration of acetone was below approx. 70%, the haematin a_2 formed was not precipitated, but an acetone solution of haematin a_2 was obtained. The absorption spectra of pyridine and cyanide haemochromogens in acetone were similar to those observed in aqueous solution, but the absorption peaks of the pyridine haemochromogen were at shorter wavelengths than in aqueous solution (Figs. 3 and 4). The α-band of the pyridine haemochromogen was at 615 mμ in the acetone solution.

The effects of CO and NO on haematin a_2 and haem a_2 were examined. As

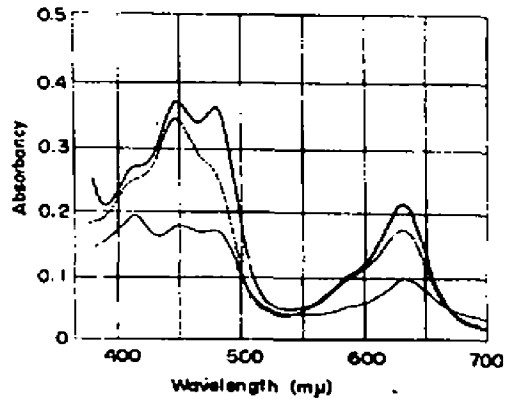

Fig. 4. The absorption spectrum of the cyanide haemochromogen of haem a_2. The concentration of the haemochromogen was $1.07 \cdot 10^{-5}$ M in aqueous solution and $5.6 \cdot 10^{-6}$ M in acetone. The concentration of KCN was 10^{-3} M. - - - - -, KCN added to an aqueous solution of haematin a_2; ——, $Na_2S_2O_4$ added to the above solution; ———, in acetone containing 30% water, after addition of $Na_2S_2O_4$.

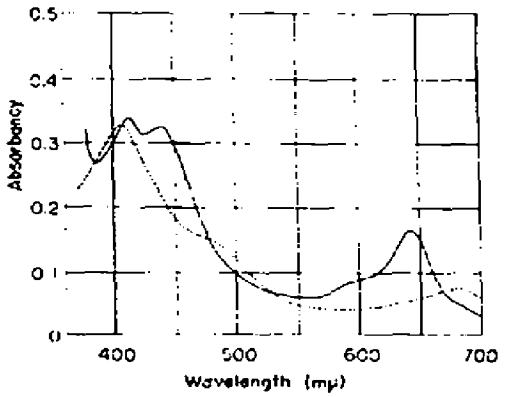

Fig. 5. The absorption spectra of haematin a_2 and haem a_2 under CO. The concentration of haematin a_2 was $8.5 \cdot 10^{-6}$ M and the pH was 11.4. - - - -, Haematin a_2; ———, $Na_2S_2O_4$ added to haematin a_2.

shown in Fig. 5, haematin a_2 was not affected by CO, but the absorption spectrum of haem a_2 under CO was evidently different from that of haem a_2 itself. The α-band was at 644 mμ and the γ-band at 413 mμ and 440 mμ, and there was a shoulder around 600 mμ. In the presence of NO, the absorption spectrum of haematin a_2 was affected (Fig. 6); haematin a_2 had its α-band at 645 mμ and its γ-band at 407 mμ

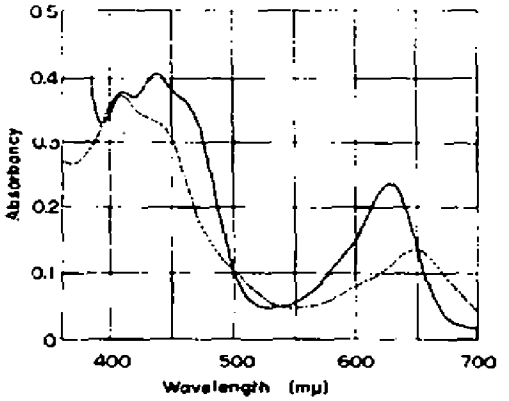

Fig. 6. The absorption spectra of haematin a_2 and haem a_2 under NO. The concentration of haematin a_2 was $1.04 \cdot 10^{-5}$ M and the pH was 11.0. - - - - -, Haematin a_2; ———, $Na_2S_2O_4$ added to haematin a_2.

with a shoulder around 420 mμ. Haem a_2 in NO had its α-band at 625 mμ, and its γ-bands at 408 mμ and 430 mμ with a shoulder around 459 mμ.

The protein moiety

The protein moiety, which was separated from the haem a_2 by treatment with HCl–acetone, was not soluble at neutral pH. When the pH of the suspension of the protein moiety was approx. 9, a clear red solution was obtained. It was hard to

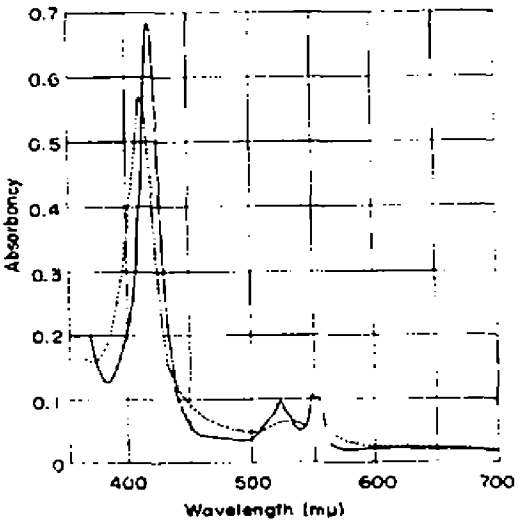

Fig. 7. The absorption spectrum of the protein moiety made by cleavage of haem a_3 from Pseudomonas cytochrome oxidase. The protein moiety was dissolved in 0.2 M phosphate at pH 9.0. - - - - -, Oxidized; ———, reduced with $Na_2S_2O_4$.

dissolve the protein moiety even at pH 9 in the presence of salts. The absorption spectrum of the protein moiety is shown in Fig. 7. There were peaks at 412 mμ and 528 mμ in the oxidized form, and at 418 mμ, 523 mμ, 549 mμ and 554 mμ in the reduced form. The spectrum was very similar to that of typical cytochrome *c* except for the splitting of the α-band in the reduced form. It is noteworthy, however, that the $A_\gamma^{red}/A_\alpha^{red}$ ratio was quite large, and the $A_\alpha^{red}/A_\beta^{red}$ ratio small, and there was a broad absorption band around 620 mμ (Fig. 8). On addition of alkaline pyridine, the twin peaks of the α-band became a single peak at 549 mμ.

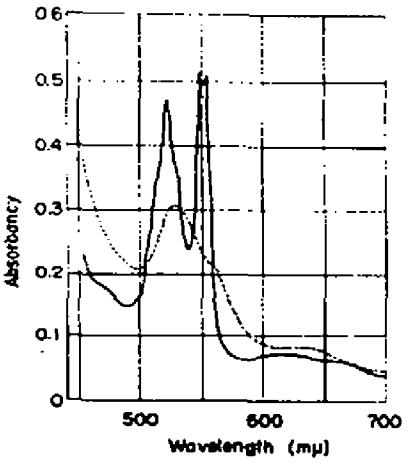

Fig. 8. The absorption spectrum of the protein moiety at 450–700 mμ. Experimental conditions as for Fig. 7. - - - - -, Oxidized; ———, reduced with $Na_2S_2O_4$.

The haem bound to the protein moiety

It seemed very likely that the haem bound to the protein moiety and not split off by acid–acetone treatment was haem *c*, because, as mentioned above, when alkaline pyridine was added to the protein moiety, the two peaks at 549 mμ and 554 mμ fused to a peak at 549 mμ. The haem bound to the protein moiety could be cleaved from the protein by Ag_2SO_4. To about 50 mg of the protein moiety suspended in 4.0 ml of distilled water was added 0.8 ml of glacial acetic acid and 4.0 ml of saturated aqueous Ag_2SO_4. The mixture was heated at 75° in a water bath for 40 min and then extracted 3 times with 30-ml aliquots of glacial acetic acid–ether (1 : 3, v/v). The combined ether layers were washed 6 times with 30-ml aliquots of 5% aqueous NaCl and concentrated to dryness *in vacuo*. The haem adhering to the wall of the vessel was dissolved in dilute NaOH at pH 11. The absorption spectra of the pyridine and cyanide haemochromogens of the haem are shown in Figs. 9 and

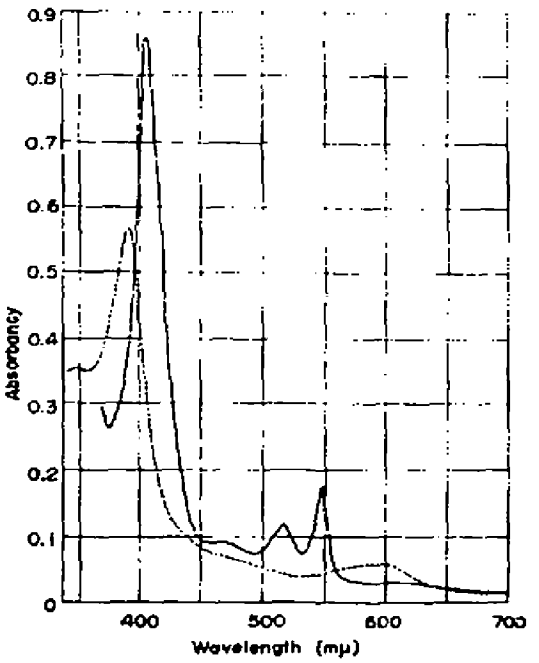

Fig. 9. The absorption spectrum of the pyridine haemochromogen of haematohaem cleaved from the protein moiety of Pseudomonas cytochrome oxidase by Ag_2SO_4-treatment. pH 11.0. - - - - -, Oxidized, ——, reduced with $Na_2S_2O_4$.

10. The pyridine haemochromogen of the haem had peaks at 392 mμ and 593 mμ in the oxidized form, and at 405 mμ, 464 mμ, 516 mμ and 546 mμ in the reduced form. The cyanide haemochromogen of the haem had peaks at 415 mμ and 536 mμ in the oxidized form, and at 426 mμ, 532 mμ and 558 mμ in the reduced form. From the absorption spectra, the haem isolated seems to be haematohaem. Therefore, it is evident that the haem bound to the protein moeity and not split off by acid–acetone treatment was haem *c*.

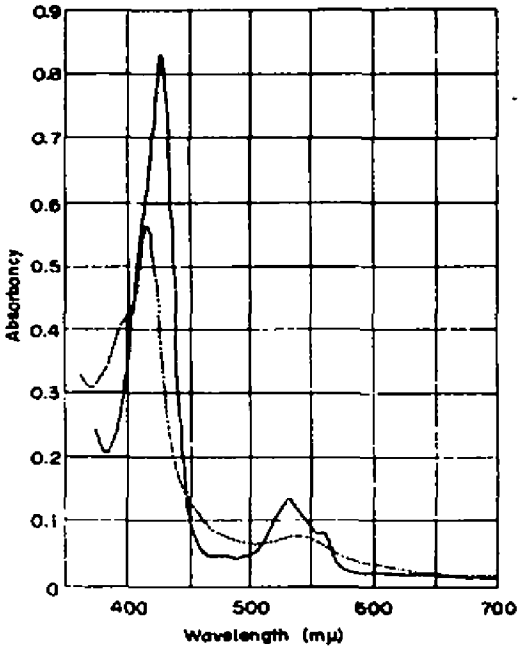

Fig. 10. The absorption spectrum of the cyanide haemochromogen of haematohaem cleaved from the protein moiety of Pseudomonas cytochrome oxidase by Ag_2SO_4-treatment. pH 11.0. - - - -, Oxidized; ——, reduced with $Na_2S_2O_4$.

DISCUSSION

BARRETT[1] extracted haem a_2 from whole cells of *Aerobacter aerogenes* and highly purified it. This haem a_2 was lipophilic and its pyridine-, CN^-- and CO-derivatives had their α-bands at 613 mμ, 618 mμ and 618–620 mμ, respectively. On the other hand, our haem a_2 isolated from a crystalline Pseudomonas cytochrome oxidase was hydrophilic, and its pyridine-, CN^-- and CO-derivatives had their α-bands at 620 mμ, 632 mμ and 644 mμ, respectively. Therefore, the haem a_2 prepared from our crystalline enzyme was apparently different from the haem a_2 obtained by BARRETT. However, it is noteworthy that the latter haem a_2 preparation was contaminated with lipids. It may be that some lipophilic contaminations made the haem a_2 obtained from *A. aerogenes* lipophilic and caused the shifting of the absorption peaks of the haem a_2 derivatives.

The γ-band of haem a_2 was very different from that of protohaem, haematohaem and haem a, in that it consisted of several peaks. This is due to the chemical structure of haem a_2. Thus, according to BARRETT[1], haem a_2 is an iron complex of a chlorin instead of porphyrin.

The absorption peaks of haematin a_2 and haem a_2 compounds are summarized in Table I. As shown in Table I, there was no relation between the positions of the α- and γ-bands. The ratio of A_γ/A_α in haem a_2 was very small and not comparable with that observed in protohaem, haematohaem and haem a (see ref. 5).

As the oxidized form of Pseudomonas cytochrome oxidase had its α-band at 635 mμ (see ref. 4), it is obvious that the peak of haematin a_2 at 684 mμ shifted to

TABLE I

SUMMARY OF THE POSITIONS OF THE ABSORPTION BANDS OF HAEM a_2

Compound	Solvent	λ_{max} (mμ) γ-band	α-band	ε^*_{α} (M^{-1}cm)	A_γ/A_α**
Haemin a_2	Acetone	432	—	—	—
Haematin a_2	Water	405	584	$2.7 \cdot 10^3$	3.5
Haem a_2	Water	453	626	$14 \cdot 10^3$	3.0
Pyridine haem a_2	Water	432	620	$24 \cdot 10^3$	1.8
CN^--haem a_2	Water	449 480	632	$20 \cdot 10^3$	1.7
NO–haematin a_2	Water	407	645	$13 \cdot 10^3$	2.8
NO–haem a_2	Water	408 436	625	$20 \cdot 10^3$	2.0
CO–haem a_2	Water	413 440	644	$20 \cdot 10^3$	2.0
Pyridine–haem a_2	Acetone	421	615	$32 \cdot 10^3$	1.9
CN^- haem a_2	Acetone	414 450	635	$18 \cdot 10^3$	1.9

* Determined from the concentration of the enzyme from which haem a_2 was isolated.
** The highest peak was selected when two or more peaks were present.

shorter wavelengths on combination of the haem with the protein moiety[7]. The bump observed at 460 mμ in the reduced form of the enzyme seemed to be the γ-band of haem a_2 at 453 mμ. Thus, the conclusion made previously that the bump was the γ-band of the haem a_3 was correct[4].

In good accordance with the two distinct peaks of the CN^--compound of haem a_2 at 449 mμ and 480 mμ, two shoulders were observed at 443 mμ and 472 mμ in the reduced form of the enzyme[4].

There were some differences between the absorption spectra of the enzyme and those of haem a_2. (a) In the presence of CO, the absorbancy of the enzyme in the red region of the spectrum was very depressed in the reduced form, whereas the CO-compound of haem a_2 had a strong α-band. (b) The reduced enzyme had absorption peaks at 625 mμ and 660 mμ in the presence of CN^- and NO, respectively, whereas haem a_2 had its α-band at 632 mμ and 625 mμ in the presence of CN^- and NO, respectively. Thus, the absorption spectrum of haem a_2 was probably greatly modified by combination with the protein moiety.

The haem still bound to the protein moiety after treatment of the enzyme with HCl–acetone was isolated by Ag_2SO_4-treatment, and was shown to be haematohaem. Therefore the haem bound to the protein moiety was shown to be haem *c*. It is very curious that the α-band of haem *c* had two peaks in the bound state.

The ratio of $A^{red}_{\gamma}/A^{red}_{\alpha}$ in the protein moiety obtained by HCl–acetone treatment of the Pseudomonas cytochrome oxidase was 6.6, and that of $A^{red}_{\alpha}/A^{red}_{\beta}$ 1.08. These ratios are very large and very small, respectively, for a *c*-type cytochrome. A similar small value for the $A^{red}_{\alpha}/A^{red}_{\beta}$ ratio is found with an alcohol dehydrogenase of *Acetobacter sp.*[8]. The protein moiety of Pseudomonas cytochrome oxidase had a broad absorption band around 620 mμ. The properties of this band will be the subject of further investigation.

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