Location of the Heme Groups in Cytochrome cd_1 Oxidase from *Pseudomonas aeruginosa*†

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ABSTRACT: The disposition of the heme groups in cytochrome cd_1 oxidase from *Pseudomonas aeruginosa* is studied by emission spectroscopy. This protein of molecular weight 120 000 is composed of two monomers each with a heme *c* and a heme d_1. It has been shown by electron microscopy to be oblong in shape and by preliminary X-ray crystallography to have a twofold axis of rotation. Three electronic energy donors, a singlet tryptophan, a triplet tryptophan, and an attached 8-dimethylamino-1-naphthalenesulfonyl group, all exhibit normal decay lifetimes. It follows that there are parts of the protein at least 80 Å from the nearest heme. The conclusion is that the hemes are all at one end of the molecule.

Cytochrome cd_1 oxidase from *Pseudomonas aeruginosa* is a water-soluble enzyme that can be obtained from the aqueous extract of the bacteria grown anaerobically in the presence of nitrate. This protein consists of two identical subunits and has a twofold symmetry axis. Each subunit of molecular weight 60 000 has a covalently linked heme *c* and a noncovalently linked heme d_1 (Kuronen & Ellfolk, 1972; Gudat et al., 1973; Kuronen et al., 1975). The enzyme can accept electrons from reduced azurin or ferrocytochrome *c*-551 and can convert O_2 to water by a four-electron process or nitrite to NO by a one-electron process. When reduced, hemes d_1 bind CO and CN^- cooperatively (Parr et al., 1975; Barber et al., 1978). Moreover, when the oxidized protein is titrated with a reducing agent, the spectrum does not vary linearly with the fraction reduced, suggesting cooperativity (Blatt & Pecht, 1979). Finally, it is found that while reducing agents initially reduce the heme *c*, the electron is transferred from heme *c* to heme d_1 at a rate of 8 s^{-1} (Wharton et al., 1976). To interpret all these experiments, it would be helpful to know the spatial distribution of the various hemes.

The disposition of hemes within a protein can be inferred qualitatively from the fluorescence intensity of its tryptophans or of its attached fluorescent labels. For example, myoglobin, cytochrome *c*, and hemoglobin exhibit no fluorescence because the hemes are centrally located with respect to the group capable of fluorescing in a relatively small protein or subunit. Electronic energy transfer to the heme is therefore much faster than fluorescence. On the other hand, horseradish peroxidase or hemoglobin with two of its four hemes absent does fluoresce because the heme groups are asymmetrically located or in a larger protein. At least some of the emitting groups are too far from the heme for their emission to be quenched. In this paper, we present the results of a study of cytochrome cd_1 oxidase with three different emitters, the singlet and triplet states of tryptophan and the singlet state of dansyl.[1]

Experimental Section

Materials. A *P. aeruginosa* culture (ATCC 19429 or NCTC 6750) obtained from the American Type Culture Collection was grown on a large scale by the Grain Processing Corp., Muscatine, IA. The medium used was that of Gudat et al. (1973) except that 10 mg/L $CuSO_4$ was added to the medium. The cells were grown at 32 °C under an atmosphere of nitrogen as cover gas. Cytochrome cd_1 oxidase together with the other respiratory-chain proteins cytochrome *c*-551 and azurin were prepared at 4 °C by a procedure described by Gudat et al. (1973) with the following modifications.

(1) A crude extract of soluble proteins was prepared by sonicating a suspension of 450 g of cells in 1.5 L of 0.02 M phosphate buffer, pH 7.0. Pulse sonication with a Branson Model 350 sonifier was performed for 4.5 min with a 30% duty cycle. The suspension was then stirred for 1 h, and the cell debris was removed by centrifugation. This cell debris was resuspended in 1 L of the same buffer, stirred for 0.5 h, and then centrifuged. The supernatants from the two centrifugations were combined.

(2) The $(NH_4)_2SO_4$ fractionation step was performed as in Gudat et al. (1973), but the proteins collected from the 45–95% step were resuspended in 0.01 M Tris,[1] pH 7.5, and dialyzed against several changes of buffer to get rid of salt. The solution was then passed through a DE-52[1] column preequilibrated with the same buffer. The column does not retain the cytochrome cd_1 oxidase, azurin, or cytochrome *c*-551 but helps remove DNA and some unwanted proteins.

(3) The eluant from the DE-52 column described above was concentrated and then put on a Sephadex G-100 column (5 × 100 cm) to separate the larger molecular weight cytochrome oxidase from the smaller cytochrome *c*-551 and azurin. Some flavoproteins still exist as impurities in the eluants.

(4) After this step, the cytochrome oxidase containing fraction was purified by using a DE-52 column (3.5 × 60 cm) equilibrated in 0.01 M Tris, pH 7.5, with a linear gradient from 0.01 to 0.06 M Tris.

The final product had a purity ratio of $A_{410}/A_{280} > 1.1$ which shows the protein purity to be >95%. The purity was also checked by the appearance of a single band corresponding to M_r 60 000 on a $NaDodSO_4$[1] gel. The ratio $A_{640}/A_{520} > 1.05$ obtained for all batches of enzyme used is an additional check on its purity (Barber et al., 1977). All measurements were made with the fully oxidized protein.

Methods. Fluorescence lifetime measurements were carried out by using a single photon counting technique (Ware, 1971) with an Ortec 9200 photon counting nanosecond fluorometer. Excitation was selected via Corion interference glass filters at 320 nm for dansyls and 295 nm for tryptophans. Emission

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[1] Abbreviations used: Trp, tryptophan; DE-52, diethylaminoethylcellulose; Tris, tris(hydroxymethyl)aminomethane; dansyl, 8-dimethylamino-1-naphthalenesulfonyl; $NaDodSO_4$, sodium dodecyl sulfate.

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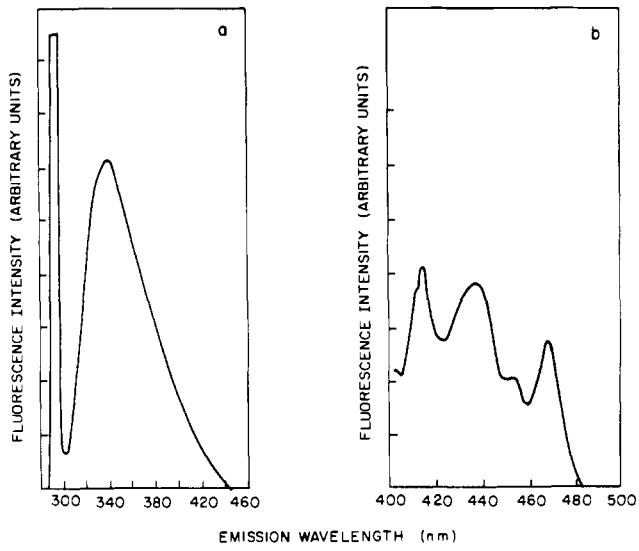

FIGURE 1: (a) Fluorescence emission spectrum (uncorrected) of the *P. aeruginosa* cytochrome cd_1 oxidase. The spectra were recorded at ambient temperature with an excitation wavelength of 295 nm. The OD at 295 nm was below 0.05. (b) Phosphorescence emission spectrum of cytochrome cd_1 oxidase in 1:1 ethylene glycol–water glass at 77 K.

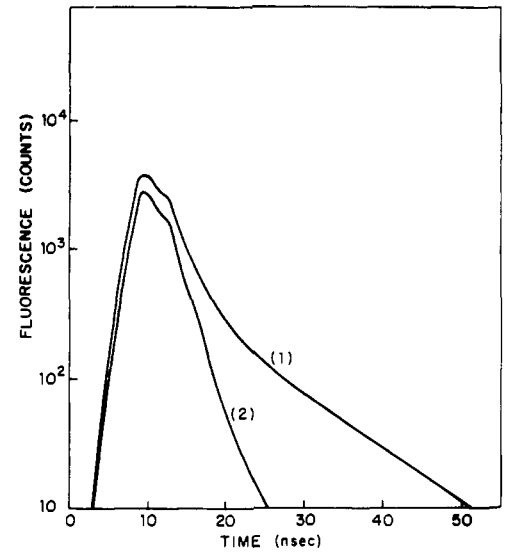

FIGURE 2: Curve 1 represents the nanosecond emission kinetics of tryptophan in cytochrome cd_1 oxidase when excited at 300 nm. The emission is monitored at ambient temperature at 330 nm. Curve 2 is the lamp shape.

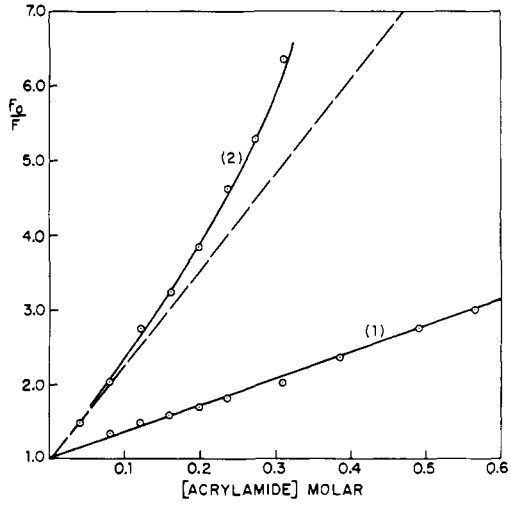

FIGURE 3: Stern–Volmer plots for (1) the cytochrome cd_1 oxidase and (2) *N*-acetyltryptophan amide quenching by acrylamide at 26 °C. The latter shows an upward-rising curve due to static quenching, and the k_Q of 12.8 M^{-1} (see text) is derived from the initial slope shown by the dotted straight line.

was monitored via a monochromator set at 520 nm for dansyls and 340 nm for tryptophans. The tryptophan lifetime was obtained by convoluting the lamp-response curve with a single exponential decay (Ware et al., 1973) and minimizing the difference between the synthesized and experimental decays. The dansyl lifetimes were obtained by fitting the decay curve for times greater than 17 ns after the beginning of the light pulse to a double exponential decay. The phosphorescence lifetime measurements were done in 0.5% glucose glass at pH 7.0 to avoid large background emission observed in ethylene glycol buffer mixtures. A dye laser was used to excite at 293 nm, and emission was sampled with a photomultiplier tube (1P 28) and averaged on a multichannel scaler. Fluorescence spectra were uncorrected for photomultiplier responses because no quantitative use was made of them.

The chemical modification of the proteins by dansyl chloride was done by adding the required amount of dye in ethanol solution to the protein solution in 0.02 M phosphate buffer, pH 8.5, and incubating for 12 h at 4 °C. The excess dye was removed by exhaustive dialysis against several changes of 200-fold excess buffer each time, for 48 h. The specific activity of the radioactive dansyl chloride was determined by using an extinction coefficient of 3.7×10^3 mol^{-1} cm^2 at its peak absorption at 370 nm. All radioactive counting with the protein was done in an aqueous medium.

Results and Discussion

Fluorescence Spectrum and Intensity. The fluorescence spectrum of the protein excited above 295 nm (used to selectively excite tryptophans in the protein) is shown in Figure 1. The fluorescence spectrum peaks around 340 nm, which is typical of proteins whose indole side chains are moderately exposed to the solvent (Burstein et al., 1973). The intensity of the emission is comparable to that of a solution of tryptophan of about four times the molar concentration of the protein. As the latter contains at least 15 Trp residues (Nagata et al., 1976), we conclude that most of the tryptophans do not emit.

Fluorescence Lifetimes. The time decay curve of the fluorescence (Figure 2) excited at 300 nm exhibits a nonexponential decay. After the lamp response was deconvoluted, the decay at longer times could be fitted to a single exponential (4.5 ± 0.5 ns) which is typical of tryptophans with fairly good solvent exposure (Grinvald et al., 1975; Burstein et al., 1973). Analysis of the shorter component was not feasible because of the poor quality of the data; the shorter component did seem to have an intensity comparable to that of the longer component. The complex decay is consistent with a model in which two (or more) tryptophans per subunit emit and have somewhat different microenvironments.

Acrylamide Quenching Studies. Acrylamide is a commonly used quencher for the excited singlet state of tryptophans. The quenching mechanism is probably a reversible electron transfer from the excited indole to the conjugated acrylamide π system and back again to the indole cation. The fluorescence quenching data are represented (Figure 3) by the usual Stern–Volmer plot of $F_0/F = 1 + k_Q[Q]$. A given quencher concentration [Q] changes the fluorescence intensity from F_0 to F. We find k_Q is 3.8 M^{-1} for cytochrome cd_1 oxidase and 12.5 M^{-1} for *N*-acetyltryptophan amide. Published values for proteins range from 0.2 M^{-1} for aldolase to 8.6 M^{-1} for pepsin.

Many multitryptophan proteins on denaturation yield a k_Q of 4.8 M^{-1} (Eftink & Ghiron, 1976). Clearly, aldolase and pepsin represent extremes of isolation from and exposure to the solvent. In cytochrome cd_1 oxidase, there is fairly good accessibility of the emitting tryptophans to the acrylamide quencher.

The relationship of k_Q as a function of temperature from 8 to 40 °C is

T (°C)	8	17	26	40
k_Q (M^{-1})	3.3	3.4	3.8	3.8

The fair constancy with temperature of k_Q suggests an absence of conformation changes in the environment of the emitters which would cause differential exposure to the quencher molecules. This is in contrast to the large temperature variation found for k_Q in liver dehydrogenase between 5 and 41 °C (Barboy & Feitelson, 1978).

Phosphorescence Lifetimes. The phosphorescence emission spectrum taken in a 1:1 ethylene glycol–water glass at 77 K is shown in Figure 1b. Most proteins exhibit a 0 → 0 peak around 410–412 nm; the peak of cytochrome cd_1 oxidase is around 414 nm. The phosphorescence decay obtained in 0.5% glucose glass at 77 K (see Methods) can be resolved into two exponentials with lifetimes of 5.4 ± 0.3 and 1.1 ± 0.3 s. As a check, trypsin under the same conditions showed a two-exponential decay with the same two lifetimes. However, *N*-acetyltryptophan amide yields a single exponential decay with a lifetime of 5.7 ± 0.3 s. Most proteins (Longworth, 1971) exhibit a tryptophan phosphorescence lifetime of about 6.0 s. While we do not know the reason for the shorter lifetime, the rather long wavelengths used for excitation (293 nm) and for observation of the emission (440 nm) argue against tyrosine as a source. In any case, the normal longer triplet lifetime shows that at least one tryptophan residue per subunit is very far from any heme to which it could transfer energy while in its triplet state.

Emission from the Dansyl-Modified Protein. Dansyl derivatives of cytochrome cd_1 oxidase and lysozyme were prepared by standard procedures (see Methods). The most likely site of attachment of dansyl is the ϵ-NH_2 group of lysine. Lysozyme has 6 lysines whereas cytochrome cd_1 oxidase has 20 lysines per subunit. However, under identical conditions, when 6 mol of dansyl chloride was added per mol of protein in solution, after extensive dialysis, the fluorescence observed from lysozyme was an order of magnitude larger than that from the oxidase. The fluorescence decay of dansylated lysozyme could be fitted well by a single exponential with a lifetime of 13.5 ± 0.3 ns. On the other hand (Figure 4), dansylated cytochrome cd_1 oxidase exhibits a nonexponential decay. The decay could be fitted to a biexponential decay with lifetimes of 4.2 ± 0.2 and 13.2 ± 0.3 ns. The experiment was repeated with the use of tritiated dansyl chloride. It was found that the fluorescence decays of dansylated lysozyme and cytochrome cd_1 oxidase were identical with the nontritiated solutions and again the fluorescence intensity from dansyl-labeled lysozyme was an order of magnitude larger than that from cytochrome cd_1 oxidase. However, from radioactive counting, it was determined that 2.3 mol of dansyl was bound per mol of cytochrome cd_1 oxidase while only 1.1 mol of dansyl was bound per mol of lysozyme.

The data above are explained by postulating three classes of excited-state dansyl groups. The most abundant class consists of those which transfer their electronic energy to the hemes quantitatively and therefore do not emit. A second class consists of those whose energy transfer and fluorescence rates are comparable so that they emit at reduced intensity and reduced lifetime. The faster decay rate of 4.2 ± 0.2 ns may be regarded as an average of all the decay rates of the partially quenched dansyl groups. However, in any case, we have made no use of the above lifetime. The third class, the smallest of all, exhibits normal fluorescence properties because these dansyl groups are remote from the hemes.

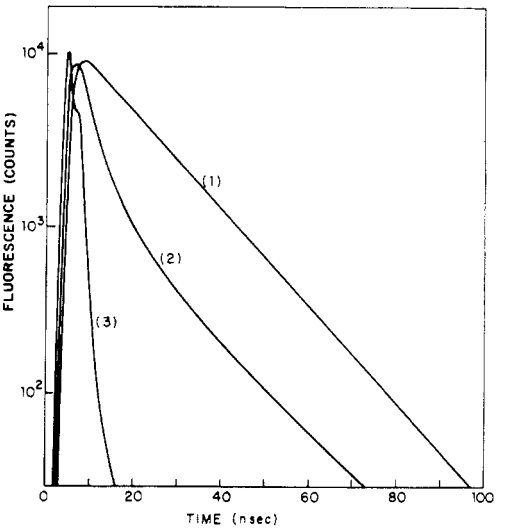

FIGURE 4: Fluorescence decay curves of (1) dansyl-labeled lysozyme and (2) dansyl-labeled cytochrome cd_1 oxidase. The excitation wavelength used is 320 nm, and the emission was observed at 520 nm. Curve 3 shows the lamp shape.

Distances between Hemes and the Fluorophores. Forster's theory of energy transfer is expressed in terms of a characteristic distance

$$R_0 = (9.7 \times 10^3)(J\kappa^2 Q_0 n^{-4})^{1/6}\ \text{Å}$$

where n is the refractive index of the medium (1.4 for proteins), Q_0 is the quantum yield for emission of the donor in the absence of the acceptor, and κ^2 is an angular factor which could vary between 0 and 4 but whose average value over all orientations of donor and acceptor is $^2/_3$. One justification for adopting this value is that the dansyl is most likely to label the ϵ-NH_2 groups of the most exposed of the 40 lysines which are present in the protein. Considering the flexibility of the long lysine side chain, the transition dipole should be effectively isotropic over the relatively long fluorescence lifetime of the dansyl label. Confirming this view is the low polarization, $(I_{VV} - GI_{VH})/(I_{VV} + GI_{VH})$, which is 0.067 at 520 nm at 25 °C with 330-nm excitation for the dansyl label. I_{JK} above refers to the fluorescence intensity observed, and the subscripts J and K refer to the horizontal or vertical positions of the excitation and emission polarizers, respectively. The correction factor G was calculated as $G = I_{HV}/I_{HH}$. In addition, the heme absorption dipole moment is degenerate in its own plane, further limiting the probable range of κ^2. J is an overlap integral of the normalized emission spectrum $F_0(\lambda)$ and the absorption spectrum of the acceptor $\epsilon_A(\lambda)$ (λ in Å).

$$J = \int_0^\infty F_D(\lambda)\epsilon_A(\lambda)\lambda^4\, d\lambda / \int_0^\infty F_D(\lambda)\, d\lambda$$

In the present case, $\epsilon_A(\lambda)$ is the molar extinction coefficient of the protein. Using 0.14 as the fluorescence quantum yield for Trp, we find R_0 for transfer from singlet Trp to the heme is 34.5 Å. For the triplet state of Trp, the overlap integral J is much larger, but because the phosphorescence quantum yield, taken to be that of the most proteins, is only 0.035, the R_0 for transfer from the triplet state of Trp to the hemes is

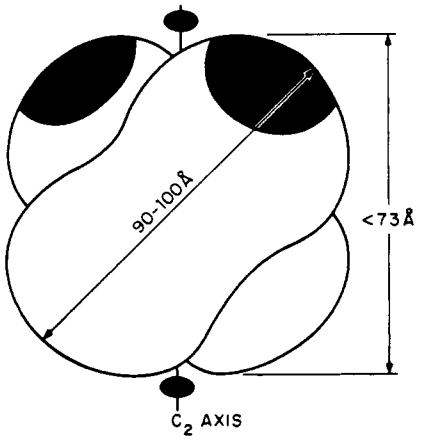

FIGURE 5: The shape, dimension, and symmetry of *P. aeruginosa* cytochrome cd_1 oxidase as obtained from electron microscopy and preliminary X-ray investigation (see text). The shaded portion at the end of the molecule shows roughly the location of hemes as inferred from emission studies.

31 Å. The dansyl–ferriheme R_0 has been calculated (Weber & Teale, 1959) to be 58 Å.

R_0 is the distance at which the rate of transfer from an excited donor to an acceptor is equal to the rate of emission. At a distance $1.5R_0$, 8% of the emission is quenched. Inasmuch as we find at least one tryptophan whose singlet and triplet lifetimes exhibit virtually no quenching and can attach preferably to a lysine, a dansyl label which is also not quenched, parts of the protein must be further than 80 Å from the nearest heme.

Conclusions

Electron microscopy studies (Saraste et al., 1977) show that the subunits of cytochrome cd_1 oxidase have a generally oblong shape (Figure 5) with a length of 90–100 Å. Preliminary X-ray crystallography shows the molecule to have a twofold axis of symmetry (Takano et al., 1979). The structural and the fluorescence data lead to the conclusion that all four hemes are located at one end of the molecule as shown in Figure 5. This result is of great interest in connection with the functioning of the enzyme. The cooperativity of the hemes as well as facile interheme electron transfer is reasonable if the hemes are located close to each other.

References

Barber, D., Parr, S. R., & Greenwood, C. (1977) *Biochem. J. 163*, 629–632.

Barber, D., Parr, S. R., & Greenwood, C. (1978) *Biochem. J. 175*, 239–249.

Barboy, N., & Feitelson, J. (1978) *Biochemistry 17*, 4923–4926.

Blatt, Y., & Pecht, I. (1979) *Biochemistry 18*, 2917–2922.

Burstein, E. A., Vedenkina, N. S., & Ivkova, M. N. (1973) *Photochem. Photobiol. 18*, 263–279.

Eftink, M. R., & Ghiron, C. A. (1976) *Biochemistry 15*, 672–680.

Grinvald, A., Schlessinger, J., Pecht, I., & Steinberg. I. F. (1975) *Biochemistry 14*, 1921–1928.

Gudat, J. C., Singh, J., & Wharton, D. C. (1973) *Biochim. Biophys. Acta 292*, 376–390.

Kuronen, T., & Ellfolk, N. (1972) *Biochim. Biophys. Acta 275*, 308–318.

Kuronen, T., Saraste, M., & Ellfolk, N. (1975) *Biochim. Biophys. Acta 393*, 48–54.

Longworth, J. W. (1971) in *Excited States of Proteins and Nucleic Acids* (Steiner, R. F., & Weinryb, I., Eds.) Plenum Press, New York.

Nagata, Y., Yamanaka, T., & Okumuki, K. (1976) *Biochim. Biophys. Acta 421*, 668–671.

Parr, S. R., Wilson, M. T., & Greenwood, C. (1975) *Biochem. J. 151*, 51–59.

Saraste, M., Virtanen, I., & Kuronen, T. (1977) *Biochim. Biophys. Acta. 492*, 156–162.

Takano, T., Dickerson, R. E., Schichman, S. A., & Meyer, T. E. (1979) *J. Mol. Biol. 133*, 185–188.

Ware, W. R. (1971) in *Creation & Detection of Excited States* (Lamola, A. A., Ed.) Marcel Dekker, New York.

Ware, W. R., Daemeng, L. J., & Nemzek, T. L. (1973) *J. Phys. Chem. 77*, 2038–2048.

Weber, G., & Teale, F. J. W. (1959) *Discuss. Faraday Soc. 27*, 134–157.

Wharton, D. C., & Gibson, Q. H. (1976) *Biochim. Biophys. Acta 430*, 445–453.