Small-Angle X-Ray Scattering Study of Cytochrome Oxidase from Beef Heart and Its Complex with Cytochrome *c* Using Contrast Variation Method

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The structure of cytochrome oxidase from beef heart and its complex with cytochrome *c* were investigated in solution by X-ray small-angle scattering. The contrast variation method was applied to the present analysis. The radii of gyration and maximum dimensions were estimated to be 54 and 165 Å for the cytochrome oxidase and 56 and 165 Å for the complex, respectively. A model of the dimer was constructed so as to interpret the X-ray scattering data well. It was concluded that cytochrome *c* is bound to the hydrophilic surface of the enzyme near the edges of the ellipsoidal molecule, and that detergent molecules exist on the surface of the central parts of the enzyme.

Cytochrome oxidase (ferrocytochrome *c*: oxygen oxidoreductase EC 1.9.3.1) is the terminal component of the electron-transfer chain in the mitochondrial inner membrane of eukaryotes. It catalyzes an electron transfer from ferrocytochrome *c* to molecular oxygen and plays an important role as an energy transducer for ATP synthesis. Many biological and physicochemical studies have been undertaken concerning mitochondrial enzymes.[1] In spite of these extensive studies, no structural evidence concerning its precise subunit composition or the locations of the prosthetic groups has been established. For cytochrome oxidase isolated from beef heart, although the number of subunits and the specific heme *a* and copper contents have been reported in many papers, the results show wide variations.

A small-angle X-ray scattering (SAXS) technique followed by a contrast variation method is useful for investigating the protein structure in solution, even though the structure can not be determined accurately. The enzyme is highly hydrophobic and makes a complex with a large number of detergent molecules, forming a micelle in solution. The relative location of each component is detected by the contrast variation method of small-angle X-ray scattering from a solution in which the electron density is close to those the protein or detergent molecules, utilizing the fact that the mean electron density of the detergent micelle is significantly lower than that of the protein.

In the present work, the molecular structures of the cytochrome oxidase from beef heart and its complex with cytochrome *c* was studied using small-angle X-ray scattering. The binding sites of the detergent molecules and cytochrome *c* in the complex are discussed.

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Experimental

Preparation of Samples. Cytochrome oxidase was isolated from beef heart mitochondria according to a method of Fowler et al.,[2] and was refractionated with ammonium sulfate in the presence of 1% cholate by a method of Tzagoloff and MacLennan.[3] The fractionated cytochrome oxidase was further purified by hydrophobic chromatography on phenyl-Sepharose CL-4B[4] and affinity chromatography on cytochrome *c*-Sepharose 4B.[5,6] The purified enzyme was finally applied on Sephacryl S-300 equilibrated with 10 mM Tris-HCl buffer, pH 7.5, containing 0.1% Triton X-100 and 0.1 M NaCl (1 M=1 mol dm^{-3}). An enzyme-cytochrome *c* complex was prepared by adding cytochrome *c* purified by a method of Hagihara et al.[7] to an enzyme solution in heme *c*: a heme *a* ratio of 1 : 2 (1 : 1 mol/mol) and an excess of cytochrome *c* was removed by gel filtration on a Sephadex G-75. The heme *a* concentration was determined from a difference spectrum of oxidase reduced with $Na_2S_2O_4$ using a millimolar extinction coefficient of $\Delta\varepsilon_{605-630}$=16.5 mM^{-1}cm^{-1}.[8] The protein concentration was determined by the biuret method.[9]

The electron densities of the X-ray scatterers were calculated from the number of electrons and the partial specific volumes of the enzyme, bound detergent, and cytochrome *c*. The partial specific volume of the enzyme was calculated to be a weighted mean of those of the enzyme and the bound detergent.[10] The amount of detergent molecules was estimated by gel filtration on Sephacryl S-300.[11] The difference in absorption at 280 nm between an enzyme bound with Triton X-100 and that with cholate resulted in 180 molecules of Triton X-100/heme aa_3. To realize a wide range of electron density of the solvent, sucrose, which exhibits a high electron density, was added to a concentrated protein solution so that the protein concentration could be maintained at 0.5% (w/v). A buffer solution containing the detergent was also prepared under the same conditions as that for the protein solution. Contrast variation measurements were carried out in 0.0, 5.0, 10.0, 18.5, 22.0, 30.7, 36.3, 40.0, 45.0, and 50.0% (w/w) sucrose concentrations, corresponding to averaged electron densities of 0.334, 0.341, 0.348, 0.359, 0.363, 0.377, 0.385, 0.391, 0.400, 0.408 e Å^{-3}.

Small-Angle X-Ray Scattering. The X-ray source was a

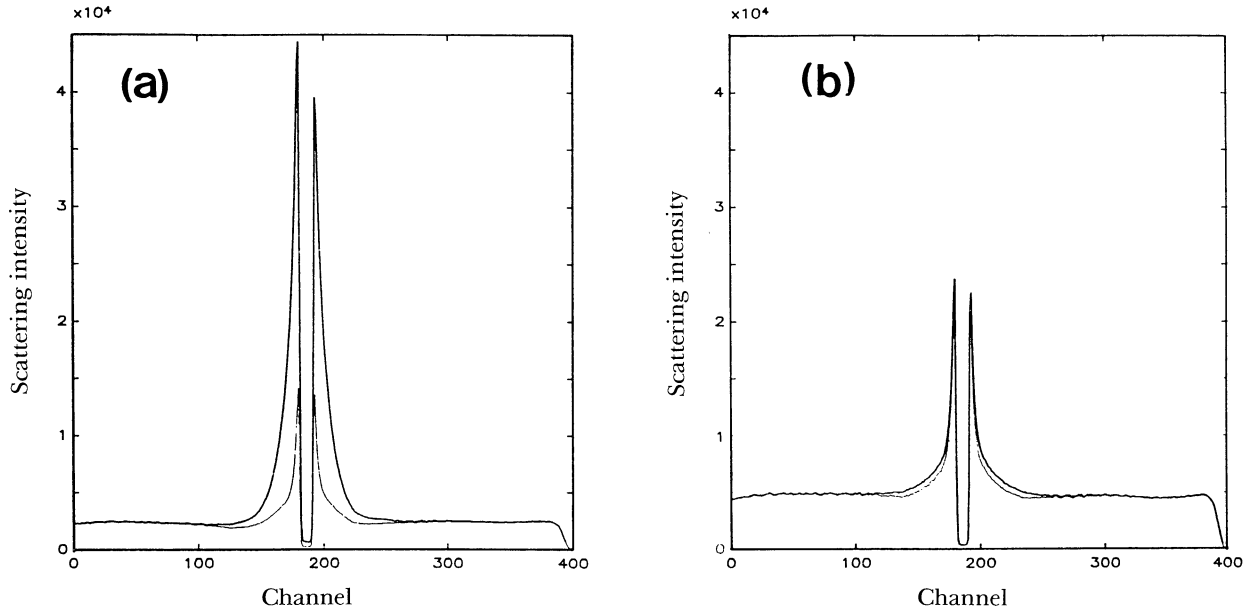


Fig. 1. Typical scattering intensity profiles of cytochrome oxidase solutions containing (a) 0% sucrose and (b) 30.7% sucrose (thick line) and those of the respective cytochrome oxidase free solutions for background intensity subtraction (thin line).

0.4×8 mm spot on the copper anode of a Phillips fine-focus X-ray tube, which was operated at 40 kV-30 mA with a Rigaku Denki D9C X-ray generator. The spot was foreshortened to 0.04×8 mm at a glancing angle of 6°. Ni-filtered X-rays were reflected and focused by a nickel-coated glass mirror (20×60×5 mm), and then introduced to a sample solution through two kinds of limiting slits: the first slit was located behind a mirror holder in order to collimate reflected X-rays; the other one was located in front of a sample holder and was used to reduce parasitic scattering from the edge of the first slit. The scattered X-rays were recorded with a one-dimensional position-sensitive proportional counter (delay-line type) over a scattering range of 4.0×10^{-3} to 7.7×10^{-3} rad. The counter length was 50 mm and was divided into 512 channels on a multichannel analyzer. The sample-to-counter distance was 305 mm. The path between the sample holder and the counter was evacuated in order to eliminate any air scattering. A sample solution was introduced into a 1.0 mϕ thin-walled quartz capillary by an injector incorporated into the sample holder. All X-ray measurements were performed at 5±0.1 °C.

Figure 1 shows typical scattering intensity profiles of cytochrome oxidase and cytochrome oxidase free solutions on a multichannel analyzer. The scattering intensities recorded on both sides of the primary beam were averaged at equivalent points after subtracting background intensities. The center of the primary beam (zero-angle scattering) was adjusted around the center of the quartz capillary, and then refined so as to realize the best coincidence of the scattering intensities at equivalent points. After averaging, an iterative method by O. Glatter[12] was applied in order to deconvolute the slit effects: the beam size, the counter width along the longitudinal axis, and the beam divergent effect. Data smoothing, which was free from random error, was also carried out using the program.

The radius of gyration, R_g, was calculated from both Guinier plots[13] of the scattering intensity data, $I(\boldsymbol{h})$, in a smaller-angle scattering region, and from an integration of the distance distribution function, $P(\boldsymbol{r})$, which (multiplied by 4π) represents for homologous particles (X-ray scatterers) the number of distances within the particle and is obtained from a Fourier transformation of $I(\boldsymbol{h})$ using the following equation:

$$P(\boldsymbol{r}) = 1/2\pi^2 \left(\int_0^\infty I(\boldsymbol{h}) \cdot \boldsymbol{hr} \cdot \sin(\boldsymbol{hr})\, d\boldsymbol{h}\right) \quad (1)$$

$h = 4\pi \sin\theta/\lambda$, 2θ; scattering angle
λ; wavelength

The $P(\boldsymbol{r})$ function was also utilized in order to estimate the maximum dimension of the X-ray scatterer from a distance r, where $P(\boldsymbol{r})$ decreases to zero. The model was constructed by assemblies of spheres to fit the calculated $P(\boldsymbol{r})$ functions to the observed ones.

Results

Table 1 summarizes the R_g and D_{max} obtained in various sucrose concentrations. The R_gs of the enzyme are smaller than that of the complex with cytochrome *c* in all sucrose concentrations, though the D_{max}s are the same as each other in cases (a), (b), and (c). The increase of R_g in the complex indicates that cytochrome *c* is not buried inside but, rather, far from the center of the enzyme. However, cytochrome *c* should not be outside of the enzyme, judging from the equivalent D_{max}. The dimeric enzyme is assumed to bind two molecules of cytochrome *c* symmetrically, and the radius of gyration of the complex is calculated using the radius and molecular weight (R_{g1} and m_1 for oxidase and R_{g2} and m_2 for cytochrome *c*), as well as the distance (*X*) between the two molecules, as

Table 1. Radius of Gyration(R_g) and Maximum Dimension(D_{max}) of the Cytochrome Oxidase and its Complex with Cytochrome c[a)]

Sample	Sucrose concn	Complex with cytochrome c			Cytochrome oxidase		
		R_g[b)]	R_{g_A}[c)]	D_{max}[d)]	R_g	R_{g_A}	D_{max}
	%	Å	Å	Å	Å	Å	Å
(a)	0.0	55.5(1)	55(1)	165(8)	53.8(1)	54(1)	165(8)
(b)	5.0	55.4(1)	54	160	53.5(2)	54	161
(c)	10.0	55.7(2)	55	156	52.3(4)	52	152
(d)	18.5	55.2(2)	54	156	49.7(2)	49	141
(e)	22.0	54(2)	53(2)	147(6)	48.2(2)	49(2)	149(6)
(f)	30.7	41.4(3)	44	140	35.2(2)	36	135
(g)	36.3	18.3(1)	29(2)		—	—	
(h)	40.0	—[e)]	—		—	—	
(i)	45.0	17.6(1)	13		—	—	
(j)	50.0	28.7(1)	28(2)		—	—	

a) Standard deviations are in parenthesis. b) Radius of gyration calculated from Guinier plot. c) Radius of gyration calculated from distance distribution function. d) Maximum dimension. e) Not detected.

follows:

$$R_g^2(\text{complex}) = (m_1 R_{g1}^2 + 2m_2 R_{g2}^2 + 2m_2 X^2)/(m_1 + 2m_2). \quad (2)$$

By using m_1=260000, m_2=13000, R_{g1}=53.5 Å, R_{g2}=12 Å, R_g(complex)=55.1 Å, X was calculated to be 68 Å, which is less than half of D_{max} by 12 Å. This also indicates that cytochrome c is not deeply buried in the enzyme, but clings to it, since half of the D_{max} of cytochrome c has been estimated to be about 17 Å from a crystal-structure analysis.

The scattering of an X-ray from a particle depends on the difference in the electron density between the particle and the solvent, as follows:[14)]

$$I(\boldsymbol{h}) = \Delta\rho^2 I_v(\boldsymbol{h}) + \Delta\rho I_{vf}(\boldsymbol{h}) + I_f(\boldsymbol{h}), \quad (3)$$

where $\Delta\rho$ is the difference between the averaged electron density between the particle and solvent ($\Delta\rho = \rho_{scatterer} - \rho_{solvent}$), I_v is a shape function of the particle, I_f is from the internal structure of the particle, and I_{vf} is a correlation term between the shape and internal structure of the particle, respectively. The I_v mainly contributes to $I(\boldsymbol{h})$, and the latter two term are quite weak, except for $\Delta\rho$=0. In the present case, a large number of detergent molecules are bound to the hydrophobic surface of the enzyme as a micelle. The averaged electron density of the micelle of Triton X-100 (0.363 e Å^{-3}) was lower than that of the protein, though both were higher than that of 0.1% Triton X-100 solution (pH 7.5, 10 mM Tris-HCl). The square root of the zero-angle scattering intensity, $\sqrt{I(0)}$, was plotted against the electron density of the solvent. In the equation, the I_{vf} and I_f terms are zero at $\boldsymbol{h}$=0, since the average of the internal variation of the structure is zero, and $I(0)$ against the electron density plot may be linear if I_v is not changed; that is, a invariant volume hypothesis[15)] is valid. In Fig. 2, both plots of the enzyme and enzyme-cytochrome c complex revealed excellent linearity to ascertain that the enzyme does not react with sucrose. From this plot, the averaged electron density of the particle was derived to be 0.38 e Å^{-3} at $I(0)$=0, which is in good agreement with the calculated value of the detergent-bound enzyme.

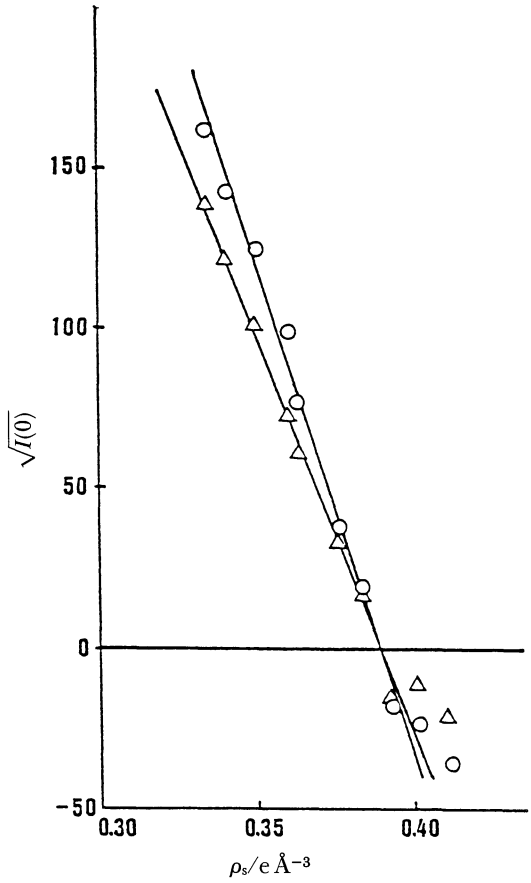


Fig. 2. Relationship between $\sqrt{I(0)}$ and the electron density of a solvent, ρ_s; (△): cytochrome oxidase; (○): cytochrome oxidase -cytochrome c complex.

The distance distribution function, $P(\boldsymbol{r})$, was utilized in the present structural study. As shown in

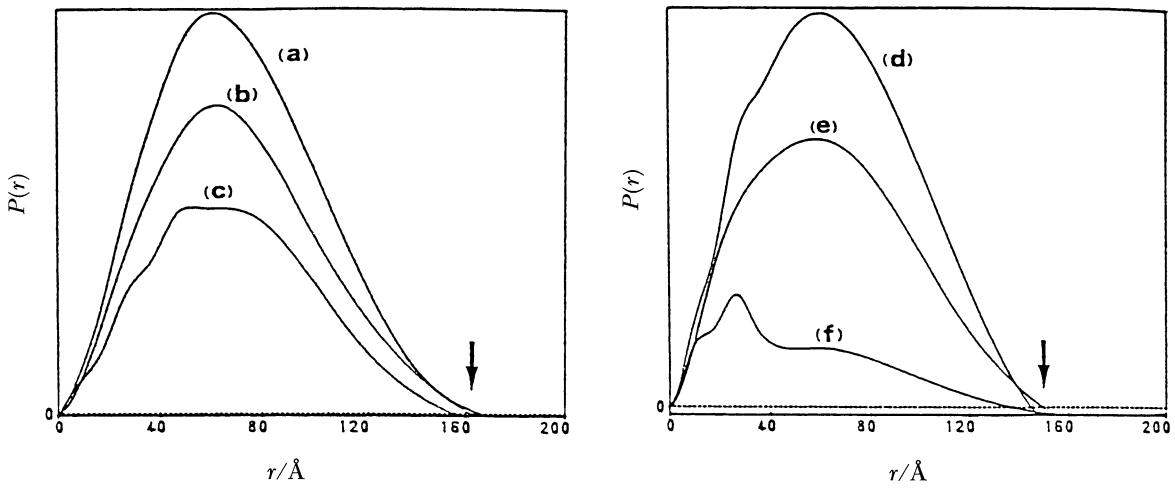


Fig. 3. Distance distribution functions $P(r)$'s for cytochrome oxidase in various sucrose concentrations; ((a) to (f)) correspond to those of Table 1. Arrows indicate the maximum dimension of the enzyme particle.

Fig. 3, the single broad profile of the $P(r)$'s trended to express an undulating character with an increase in the electron density of the solvent. However, the function at the highest sucrose concentration ((f) in Fig. 3) represented a single profile similar to those in the lower sucrose concentration (a)—(c). This behavior suggests that the enzyme comprises different electron-density regions (detergent and protein) separately. When the averaged electron density of the solvent was matched with the detergent, the protein was visible and the detergent invisible ((e) in Fig. 3). The I_f and I_{vf} terms of the protein moiety could be neglected, since the data observed in the present analysis was within a 20 Å resolution, which was too low to show any fine variations of the electron density in one molecule. The electron density of the solvent matched that of the protein erases the part of the protein. The intermediate gives a positive protein and a negative detergent to the $P(r)$ function ((f) in Fig.3).

The SAXS profile was compared with some theoretical intensity curves for various eccentricities. As drawn in Fig. 4, the curve of an ellipsoid with an eccentricity of 2.0 expressed good coincidence with the observed profile in the small-angle region ($2\theta < 14 \times 10^{-3}$ rad). Disagreement of the observed profile at slightly high angles was caused by deviations from a uniform electron density within a prolate ellipsoid model. A molecular model was simulated as assemblies of spheres with a radius of 12 Å, half of the resolution of the observed data, where the electron density of the polypeptide moiety was 0.408 e Å^{-3}, and that of the detergent was 0.363 e Å^{-3}. At first, 56 spheres were gathered in a prolate ellipsoid whose axes were 30, 30, and 50 Å. Spheres as detergent molecules were located around the side surface of the model. The model was refined so as to relate the

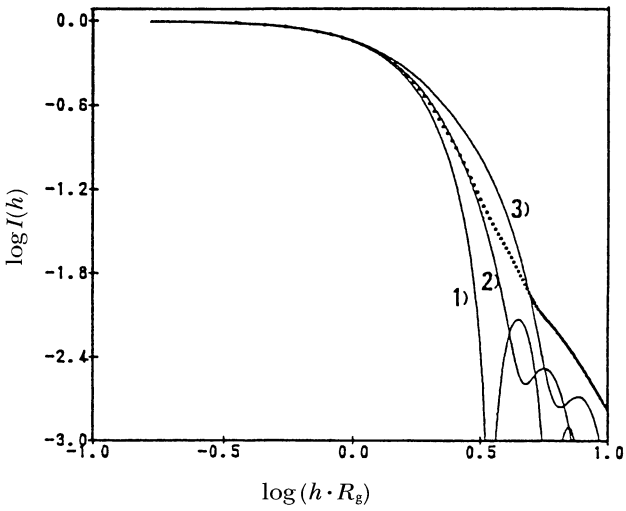


Fig. 4. Log I-log (hR_g) plot of scattering intensities of the cytochrome oxidase in 0% sucrose concentration. Superimposed are the logarithms of the theoretical scattering intensity function (solid line) of the prolate ellipsoids with eccentricities, ω of 1) ω=1.0 (sphere), 2) ω=2.0, and 3) ω=3.0.

calculated scattering data with the observed data, step by step, by the addition of a new sphere and/or the removal of a sphere. The final model comprised 74 spheres, in which 30 were designated as bound-detergent molecules. It should be noted that the relative orientation of individual spheres is not biochemically significant, that is, they do not represent the position of enzyme subunits, but only express a domain having a certain value of electron density. Spheres used as detergent molecules were located around the side surface of the model. Two spheres of cytochrome c were also combined so as to satisfy the distance derived from the complex experiments. The

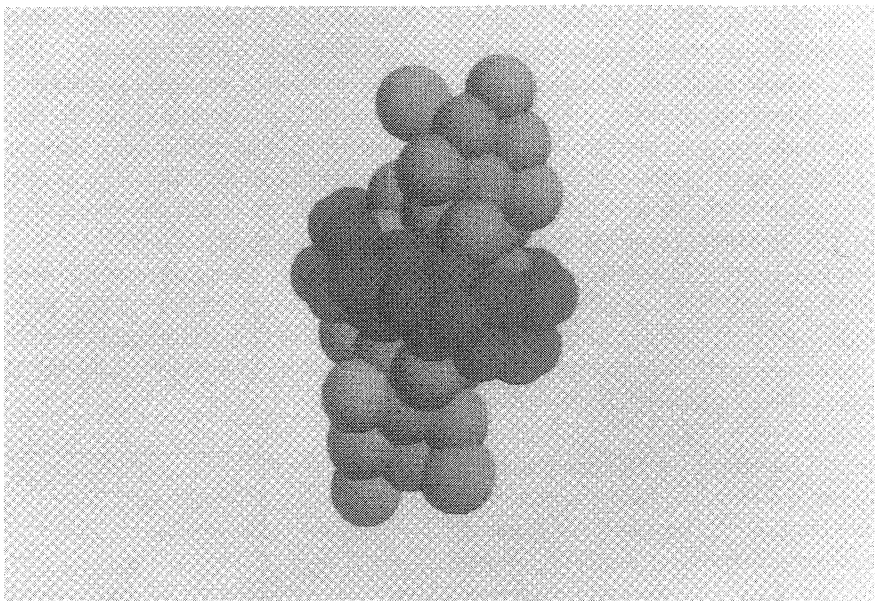


Fig. 5. Proposed dimeric structure of cytochrome oxidase complexed with cytochrome *c* drawn as a sphere model; central dark spheres, Triton X-100; slightly dark two spheres, cytochrome *c*; others, cytochrome oxidase. The size of spheres for proteins or detergents is not real one, but the spheres are used to depict the positions of their components.

consensus model shown in Fig. 5 shows two monomers in contact with each other by a relation of twofold symmetry and reproduced the structural parameters obtained in the present study, qualitatively. Hydrophobic part of the enzyme was protected by detergents, which seems to be likely in the lipid-bilayer in mitochondrial membrane, while hydrophilic part or cytochrome *c* binding site is far from each other.

Discussion

The maximum dimension of the enzyme under the highest contrast was clarified to be 165 Å, and its volume 650000 Å^3. From an electron microscopic study,[16] the span of the Y-shaped cytochrome oxidase monomer was about 70—80 Å in the mitochondrial membrane. These facts suggest that the enzyme exists as a dimer in a solution containing 0.1% Triton X-100. The enzyme, therefore, as the prolate ellipsoidal form derived from Fig. 4, comprises the connection of two identical monomers so as to contact with each Y-head. The force of this connection is considered to be a hydrophobic interaction of each subunit. This dimeric form is consistent with an ultracentrifugation study in the presence of 0.1% Triton X-100.

The values of maximum dimensions at various electron-density levels reveal an aspect of the binding

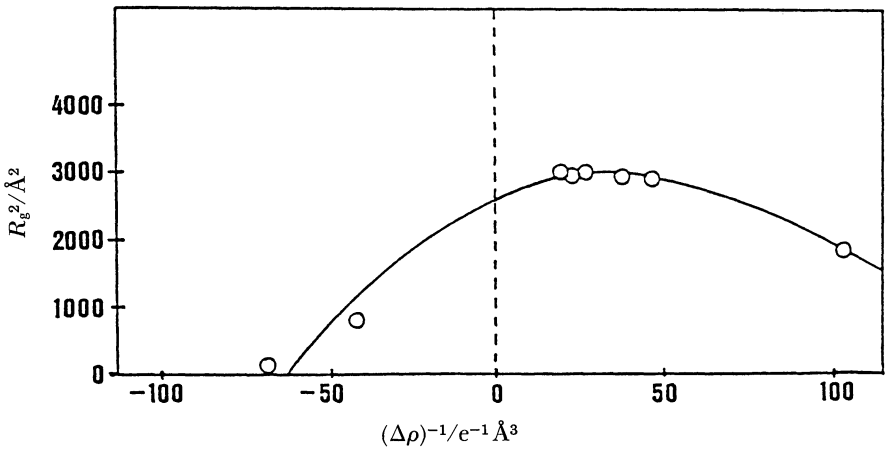


Fig. 6. Relationship between R_g^2 and $1/\Delta\rho$ (Stuhrmann plot) of cytochrome oxidase-cytochrome *c* complex.

site of detergents. A D_{max} of 165 Å under highest contrast exhibits no significant change below sucrose concentrations of 22%, whose electron density is close to that of the bound detergent micelle. This indicates that the enzyme binds the detergent molecules at the sites where its longest span is not varied. Judging from a negative quadratic character of the R_g^2—$1/\Delta\rho$ plot depicted in Fig. 6, the enzyme comprises an electron-dense region in its core and an electron-sparse region at its surface.[14] From these results it may be concluded that the detergent molecules are bound to the side surface of the ellipsoidal dimer, where the Y-heads of two monomers interact with each other by the hydrophobic characters.

A model construction based on the obtained molecular parameters and other structural information[1,16,17] definitely express the characteristics of the enzyme in solution. A preliminary structure analysis shows that the overall structure of the enzyme can be approximated by a prolate ellipsoid with an eccentricity of 2.0. An ellipsoidal model comprising 74 spheres shows that two monomers contact each other through a twofold symmetry. The hydrophobic part of the enzyme is protected by detergents, which seems to be likely in the lipid-bilayer in a mitochondrial membrane, while the hydrophilic part or the cytochrome *c* binding site is far from each other. From these results, the monomer model is consistent with the fact that the molecule passes through the mitochondrial membrane perpendicularly.[16]

The enzyme involves a variety of dense electron regions which are beyond the resolution of the data. Although in the small-angle region the scattering profile calculated according to the rigid body of uniform electron density agreed well with that observed, the consistency between that observed and calculated was worse at the higher-angle region, as is shown in Fig.4. The depicted model, which has two kinds of electron density levels in the ellipsoidal body, decreased the discrepancy, as shown in Fig.7. This feature of the enzyme is also enforced by the effects of contrast on the $P(r)$ function. The calculated $P(r)$s in various contrasts are qualitatively consistent with those of experiments (Fig. 8), from which it may be concluded that the model was constructed correctly. However, the fact that the model is the one which reasonably describes the results from the present study should be noted.

Although there were some errors in X-ray experiments, within the error the present model qualitatively expresses the character of the enzyme in solution, and describes not only the shape of the enzyme

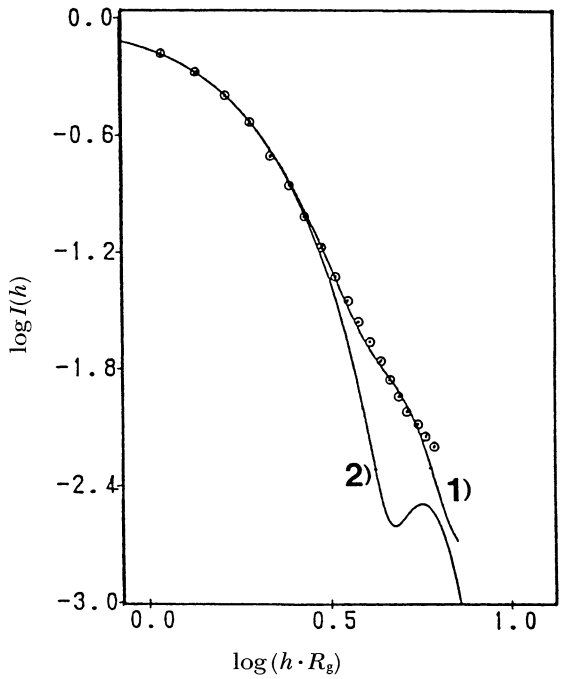
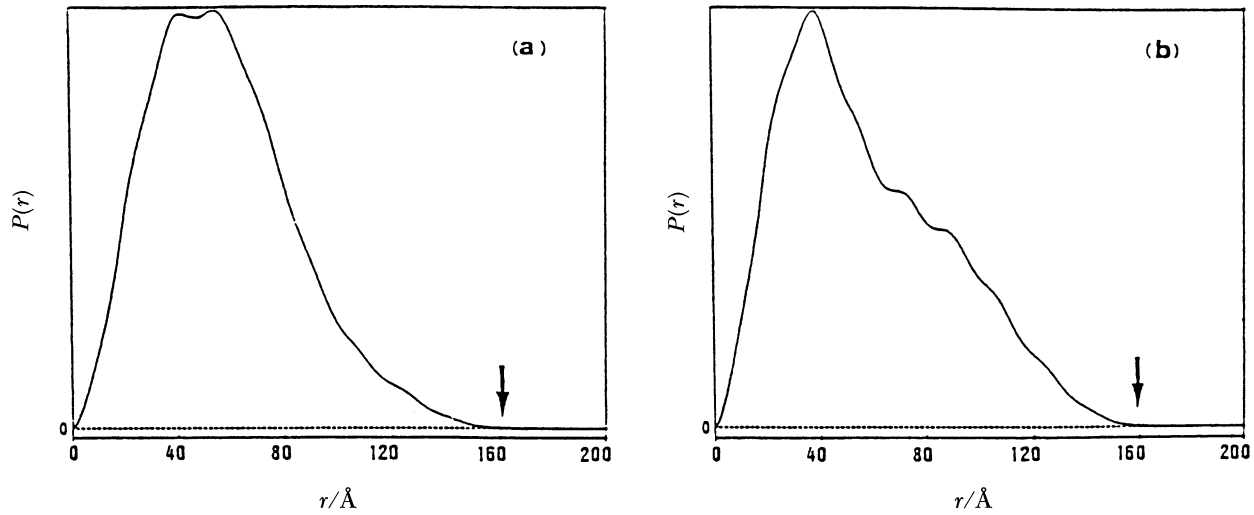


Fig. 7. Log*I*-log (hR_g) plot of the observed scattering intensity. Superimposed are the logarithms of the theoretical scattering intensity functions of a final sphere model: 1) and a preliminary ellipsoidal model with eccentricity of 2.0 : 2).



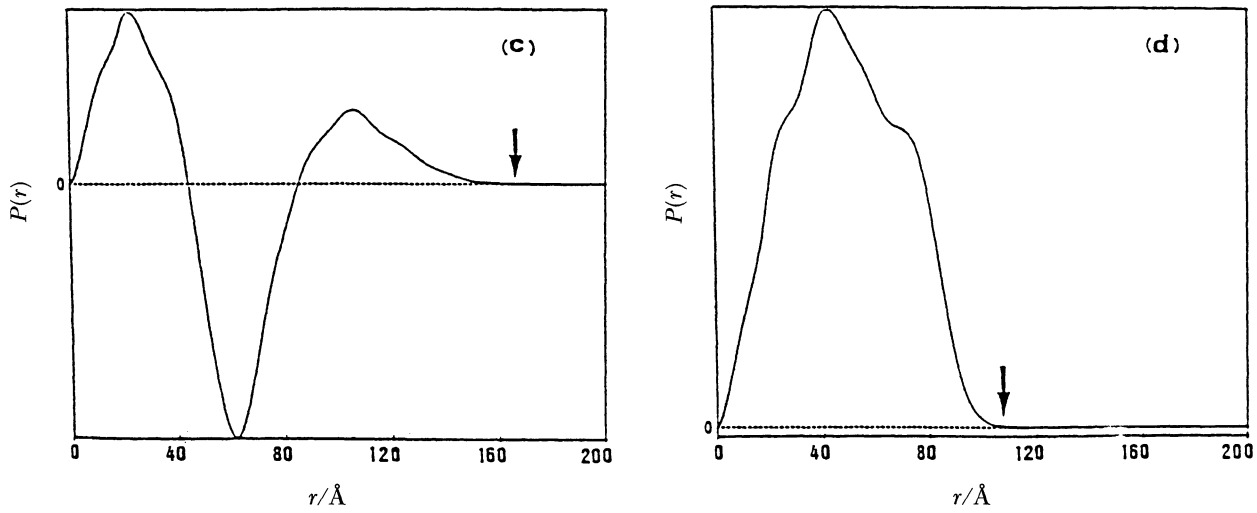


Fig. 8. Distance distribution functions $P(r)$'s for the final sphere model; (a), 0% sucrose; (b), 22% sucrose; (c), 36.3% sucrose; (d), 50% sucrose. Arrows indicate the maximum dimensions.

but also the location of the bound detergent and cytochrome *c*. Judging from only the proposed model, the molecule comprises a hydrophobic central core with detergent molecules and hydrophilic edge regions. Hydrophilic cytochrome *c* is expected to bind to the edge of the oxidase due to the electrostatic character, as discussed and established in our previous paper.[17] The limits of resolution and simulation, however, restrain any detailed modeling of the enzyme. To overcome this problem, high-angle scattering measurements or a crystal structure analysis is indispensable.

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