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Pseudomonas Cytochrome *c* Peroxidase

III. The Size and Shape of the Enzyme Molecule

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The molecular weight of *Pseudomonas* cytochrome *c* peroxidase was estimated to be 53 500 by sedimentation and diffusion studies. The calculation was based on a sedimentation coefficient of 5.03 S, a diffusion coefficient of 7.47 F, and a partial specific volume of 0.695 ml/g. The frictional ratio equal to 1.12 indicates a symmetrical molecule. An independent value 58 000 of the molecular weight was obtained by thin-layer gel filtration on Sephadex G-100. The iron and heme contents of the enzyme indicate that the molecule contains at least two hemes.

Pseudomonas cytochrome *c* peroxidase (PsCCP) has recently been prepared as a homogeneous protein according to ultracentrifugal and electrophoretic criteria.[1]

This communication reports the determination of sedimentation and diffusion coefficients of the enzyme as well as its partial specific volume. By means of these, the molecular weight of the enzyme was calculated. A confirmative determination of the molecular weight was performed by thin-layer gel filtration on Sephadex G-100.

MATERIALS AND METHODS

Enzymes and proteins. Pseudomonas *cytochrome* c *peroxidase* (PsCCP) was prepared as previously described.[1] The ratios A_{407}/A_{280} of the preparations were 4.10 – 4.20. Horse heart *cytochrome c* (97 %, Type III) and crystalline *ovalbumin* (99 %, Grade V) were commercial preparations from Sigma Chemical Co., crystalline *trypsinogen* from pancreas and *aldolase* from rabbit muscle were purchased from Boehringer & Soehne, and bovine *serum albumin* (Fraction V) was obtained from Armour Pharmaceutical Co.

Ultracentrifugal measurements. The sedimentation velocity of PsCCP was measured at protein concentrations of 1.92 – 5.72 mg/ml in a Beckman-Spinco model E analytical ultracentrifuge at a rotor speed of 59 780 rpm at 20°C. The centrifuge was equipped with a schlieren optical system and a Rotor Temperature Indicator and Control (RTIC) unit. A 12 mm single sector cell (aluminium centerpiece) was used in the sedimentation studies. Twelve exposures were made at intervals of 4 or 8 min in all sedimentation experiments. The sedimentation coefficients expressed in Svedberg units ($S = 10^{-13}$ sec) after correcting

the density and viscosity of the medium to those of pure water at 20°C were calculated as described previously [2] using a partial specific volume of 0.695 ml/g.

Diffusion coefficients were determined in a synthetic boundary cell of the valve type in the ultracentrifuge. The cell was loaded with 0.40 ml of enzyme solution (3.85 – 4.72 mg/ml) and 0.27 ml of buffer solution. The diffusion experiments at 20°C were performed at a rotor speed of 12 590 rpm at which no correction for sedimentation is necessary.[3,4] Boundary spreading was observed with schlieren optics and photographed at 4 min intervals during 48 min. The diffusion coefficients were calculated from data read from magnifications projected onto millimeter graph paper. Areas were measured by mechanical planimetry. Calculations were performed according to the "height-area" method, using a plot according to the relationship

$$(A/H)^2 = 4k^2\pi D(t - t_0) \quad (1)$$

where A is the area between the gradient curve and the base line at the time t from the moment (t_0) when the boundary was formed, H the height of the curve, k the magnification factor along the base line, and D the diffusion coefficient. The diffusion coefficients were corrected for the differences between density and viscosity of the medium and those of water at 20°C. The results are given in Fick units ($F = 10^{-7}$ cm^2 sec^{-1}). Before the sedimentation and diffusion studies, the PsCCP preparations were dialyzed at 4°C against a buffer of pH 7.0 ($\mu = 0.283$), 0.05 M in sodium phosphate and 0.171 M in sodium chloride.

Partial specific volume. The partial specific volume of homogeneous PsCCP was obtained by the density gradient technique of Hvidt *et al.*[5] The gradient was prepared according to Miller and Gasek.[6] Before use, Varsol (Esso, 145/200) and bromobenzene were washed with water, saturated with a 4.5 % solution of potassium bromide in water, and finally filtered through filter paper to remove excess water. The column was kept in a water bath at 20 ± 0.005°C. The gradient was calibrated with 1 μl drops of standard sucrose solutions,[7] the positions of which were measured with a cathetometer. Enzyme solutions of different concentrations were studied in the same buffer as was used in the ultracentrifugation analyses. Each density value represents the mean density of four drops. The partial specific volume $\overline{V}$ was calculated from the slope of the line plotted according to the relationship [8]

$$\overline{V} = \frac{1}{\varrho_0}\left[1 - \left(\frac{\mathrm{d}\varrho}{\mathrm{d}c}\right)_{c \to 0}\right] \quad (2)$$

where ϱ_0 is the density at infinite protein dilution, and c the concentration of protein in g/ml.

Thin-layer gel filtration [9,10] was performed on Sephadex G-100 Superfine (AB Pharmacia). The gel was allowed to swell in an 0.1 M sodium phosphate buffer of pH 7.0, after which the supernatant was decanted off almost completely to get a slurry of the right consistency for spreading. The plates (20 × 36 cm) were coated with an 0.75 mm thick layer of Sephadex by means of a straight rod of stainless steel equipped with collars of electrical tape at both ends. The plates were developed by descending chromatography in a closed chamber. Before the runs, the plates were equilibrated by allowing the solvent to flow through the gel for 8 – 16 h. After equilibration, 1 or 5 μl volumes of the test solutions containing 10 – 50 μg of protein were applied as a series of spots about 2 cm apart on a line 4 cm from the short edge of the plate. To each spot, 10 – 20 μg of cytochrome *c* in 1 μl volume was added as a reference protein. After each run, the components were transferred from the gel layer by adsorption onto a superimposed sheet of Whatman 3 MM filter paper. After the sheet had dried, it was stained with Amidoblack 10 B in methanol-acetic acid and rinsed with 1 % acetic acid. Horse heart cytochrome *c* (mol. wt. 12 400), trypsinogen from pancreas (mol. wt. 23 500), ovalbumin (mol. wt. 45 000), bovine serum albumin (mol. wt. 67 000) and aldolase from rabbit muscle (mol. wt. 149 000) were used as molecular weight markers. The relative mobility, R_{cyt}, of each protein was determined by comparison with the mobility of cytochrome *c*. The linear relationship between R_{cyt} and the logarithm of the molecular weight [11] was used in the estimation of the molecular weight of PsCCP.

Iron analyses were performed by the sulfosalicylic acid method of Lorber [12] as modified by Paul.[13] Before the analyses, the samples were dialyzed against deionized water.

Pyridine hemochrome was prepared according to Paul *et al.*[14]

Protein concentrations of PsCCP solutions were measured spectrophotometrically at 280 nm, using $A_{1cm}^{1\%} = 12.1$, which was calculated on the basis of the dry weight determinations of PsCCP samples.

Dry weights of protein samples were determined by drying to constant weight at 105°C after dialysis against distilled water.

Spectrophotometry was performed on a Beckman DU-2 spectrophotometer.

pH measurements were performed with a Beckman Zeromatic pH meter.

All chemicals were of analytical grade.

RESULTS

Sedimentation analyses. The preparations studied gave only one single sedimentation boundary in the analytical ultracentrifuge at the tested enzyme concentrations. There was no evidence of any aggregates or dissociation products. Sedimentation coefficients determined at different protein concentrations are plotted in Fig. 1. These data are represented by the equation

$$s_{20,w} = (5.03 - 0.018\ c)\ \mathrm{S}$$

where c is the concentration of PsCCP in mg/ml, and $s_{20,w}$ the sedimentation coefficient in pure water at 20°C. By extrapolation to infinite dilution, $s_{20,w}$ was found to be 5.03 S.

Diffusion coefficient. Diffusion coefficients were determined in the analytical ultracentrifuge at protein concentrations of 3.85 – 4.72 mg/ml. Three experiments gave the values 7.18, 7.62 and 7.62 F, the mean of which, 7.47 F, was

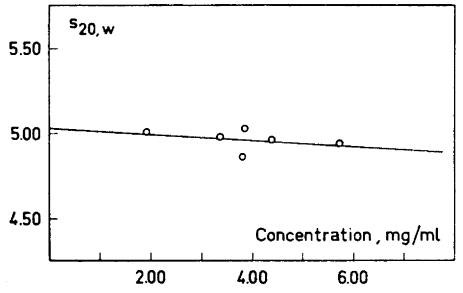

Fig. 1. Dependence of the sedimentation coefficient, $s_{20,w}$, of PsCCP on protein concentration. The line drawn is a least squares fit to the experimental points.

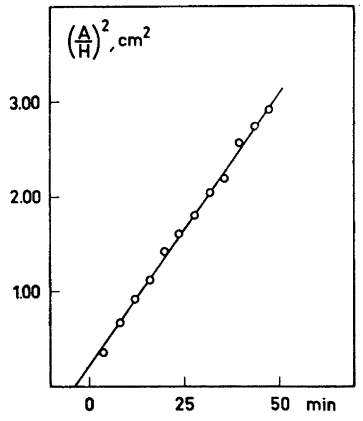

Fig. 2. A plot according to the equation of the "height-area" method for the determination of the diffusion coefficient of PsCCP. The concentration of PsCCP was 4.72 mg/ml. Time was measured from the moment when the boundary was formed between the buffer and the protein solution in the same buffer. The drawn line corresponds to $D_{20,w} = 7.62$ F. The extrapolated t_0 was equal to 4 min.

taken as $D_{20,w}$. The extrapolated t_0-values were between 1 and 5 min. A representative plot of the data from one experiment is shown in Fig. 2.

Partial specific volume. The concentration range employed in the determination of the partial specific volume of PsCCP was between 1 and 4 mg/ml. The partial specific volumes obtained in different experiments for the protein dissolved in 0.05 M sodium phosphate buffer of pH 7.0, 0.171 M in sodium chloride, at 20°C were 0.686, 0.694, 0.704, and 0.697 ml/g. The mean value 0.695 ml/g was used in the calculation of the molecular weight. A plot of density against enzyme concentration is shown in Fig. 3.

Molecular weight. The molecular weight of PsCCP calculated from the sedimentation coefficient, diffusion coefficient and partial specific volume, using Svedberg's formula [15]

$$M = \frac{RTs}{D\,(1 - \bar{V}\varrho)} \tag{3}$$

was found to be 53 500.

Molecular shape. A frictional ratio, f/f_0, equal to 1.12 was found for PsCCP, using the formula [15]

$$f/f_0 = \left(\frac{1 - \bar{V}\varrho}{D_{20,w}^{\,2} \cdot s_{20,w} \cdot \bar{V}}\right)^{1/3} \times 10^{-8} \tag{4}$$

The frictional ratio can be represented as a product of two factors, f/f_e and f_e/f_0, the first one representing the effect of hydration, and the second one that of asymmetry of the molecule.[16] Kraemer has presented for the hydration factor the relationship [16]

$$f/f_e = \left(1 + \frac{w}{\bar{V}\varrho}\right)^{1/3} \tag{5}$$

in which w is the hydration in grams of water, bound by one gram of protein, and ϱ the density of water solvating one gram of pure solute of partial specific volume $\bar{V}$. The maximum possible degree of hydration for PsCCP would make f/f_e equal to 1.12, and would lead to a value of 0.28 for w. If the frictional ratio were due only to asymmetry, the axial ratios would be equal to about 3 for both prolate and oblate ellipsoids.[15] The water content of 28 % obtained on the basis of w is so high that the frictional coefficient of PsCCP can hardly be explained only by hydration.

Estimation of the molecular weight by Sephadex thin-layer gel filtration. In order to corroborate the value of 53 500 for the molecular weight of PsCCP, an independent value of the molecular weight was obtained by the technique of thin-layer gel filtration on superfine Sephadex G-100 beads. A typical plot of R_{cyt} values against the logarithm of the known molecular weights of the marker proteins is shown in Fig. 4. Seven separate determinations gave the values 57 000, 55 000, 56 000, 60 000, 60 000, 60 000, and 58 000 for PsCCP. The mean of these values is 58 000, which is in agreement with the value obtained in the ultracentrifugation experiments.

Heme content of PsCCP. The iron content of PsCCP was found to be 0.259 %, which corresponds to a minimum molecular weight of 21 600. The

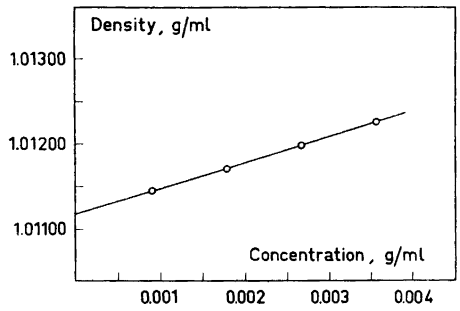

Fig. 3. A plot of density against protein concentration for PsCCP in 0.05 M sodium phosphate buffer, pH 7.0, 0.171 M in sodium chloride, for the determination of the partial specific volume of PsCCP. From this plot, the value 0.694 ml/mg is obtained.

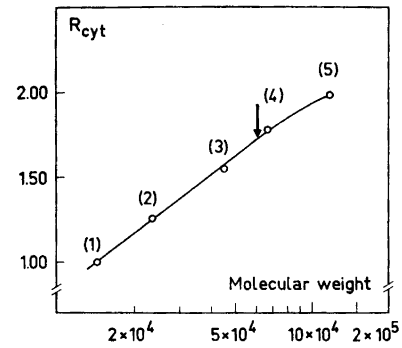

Fig. 4. Determination of molecular weight of PsCCP by thin-layer gel filtration on Sephadex G-100. Experimental details are given in the text. The mobilities of proteins relative to that of cytochrome *c*, R_{cyt}, are plotted against the logarithm of the molecular weight. The marker proteins are horse heart cytochrome *c* (mol. wt. 12 400) (1), trypsinogen from pancreas (mol. wt. 23 500) (2), ovalbumin (mol. wt. 45 000) (3), bovine serum albumine (mol. wt. 67 000) (4), and aldolase from rabbit muscle (mol. wt. 149 000) (5). The arrow indicates the relative mobility of PsCCP.

pyridine hemochrome of PsCCP was earlier found to be very similar to that of animal cytochrome *c*.[1] Using the millimolar absorptivity of pyridine ferrohemochrome of heme *c* at the α maximum, $\varepsilon = 29.1$ mM^{-1} cm^{-1},[17] the heme content of PsCCP was found to be 41 μmol/g of protein, which corresponds to a minimum molecular weight of 24 400.

DISCUSSION

The molecular weight 53 500 of PsCCP calculated from sedimentation and diffusion data agrees with the value 58 000 obtained by gel filtration on Sephadex G-100. However, the molecular weights calculated for two iron atoms or two heme groups per enzyme molecule are 43 200 and 48 800, respectively, both of which deviate from the above two values. This discrepancy cannot be satisfactorily explained at present. However, a molecular weight of 43 200 is definitely too low for PsCCP, since ovalbumin (mol. wt. 45 000), used as standard in the gel filtration experiments, in all cases showed a markedly smaller mobility than PsCCP, which indicates a larger size of the latter. In addition, it seems improbable that a symmetric molecule of weight 43 200 would have a sedimentation constant of 5.03 S. The high iron content of the preparation might be due to some external iron that was not removed even by exhaustive dialysis against deionized water. The heme content was also somewhat high, possibly because it was calculated on the basis of the millimolar

absorptivity of the pyridine ferrohemochrome of heme *c* of animal cytochrome *c*. It is, however, evident from these analyses that the molecule of PsCCP contains at least two heme groups.

The molecular weight of PsCCP deviates considerably from that of yeast cytochrome *c* peroxidase (YCCP), which was found to be 34 000.[2] In contrast to PsCCP, the yeast enzyme contains only one heme group per molecule. The two enzymes differ also slightly in shape, the molecule of YCCP being highly symmetric with the frictional ratio, f/f_0, equal to 1.03.[2] It seems that PsCCP is an exception among the low-molecular-weight peroxidases in having more than one heme per molecule. The only peroxidase, that to our knowledge contains two hemes per molecule, is myeloperoxidase, the molecular weight of which is 149 000 [18] and therefore considerably higher than that of PsCCP.

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Note added in proof. The bacterial strain used in this series of studies was originally reported to be *Pseudomonas fluorescens;* it has now been indentified as *P. aeruginosa* (Dr. N. O. Kaplan, personal communication to N. E.).

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