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Molecular Weight of Cytochrome Oxidase

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Abstract

The molecular weight of bovine heart mitochondrial cytochrome oxidase in 2% or 3% deoxycholate was determined by the sedimentation velocity method to be 228,000 daltons from an $S_{20,w} = 8.44 \times 10^{-13}$ sec, a $D_{20,w} = 3.21 \times 10^{-7}$ cm^2 sec^{-1}, and the $\bar{v} = 0.72$ reported by others. The $S_{20,w}$ value was only slightly concentration-dependent. When the deoxycholate in our preparation was replaced with Tween 80 the S value increased to between 16 and 17. When one preparation in Tween 80 was allowed to stand at room temperature, the S value increased in successive determinations to reach 51.5 at the end of approximately 7 h. The minimum molecular weight of the enzyme as calculated from the heme content (determined from the absorbance at 603 nm) and total protein content (determined from total nitrogen) was 110,000. An amino acid analysis when related to the heme content yielded a minimum molecular weight of 85,000.

Introduction

Three determinations of the molecular weight of native, i.e., enzymatically active, bovine heart cytochrome oxidase have been previously reported. Takemori *et al.* [1, 2] calculated a value of 530,000 for the preparation of Okunuki *et al.* [3] in a 0.25% solution of Emasol 1130§ dissolved in 0.1 M

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§ Emasol 1130, polyoxythylene sorbitan monolaurate; Emasol 4130, polyoxyethylene sorbitan monooleate; Tris, tris-(hydroxymethyl)-aminomethane.

phosphate buffer, pH 7.4, from an $S_{20,w} = 21.9 \times 10^{-13}$ sec, a $D_{20,w} = 3.58 \times 10^{-7}$ cm^{-2} sec^{-1} and a $\bar{v} = 0.72$ ml g^{-1}. The minimum molecular weight as obtained from the iron content was approximately 100,000, suggesting that the enzyme was a pentamer. An average value of about 298,000 (or 230,000 when corrected for lipid) was determined by Tzagoloff *et al.* [4] by light scattering for four preparations of cytochrome oxidase (Complex IV) made by the method of Griffiths and Wharton [5]. The enzyme was dissolved in 0.25 M sucrose containing 0.06 M Tris buffer pH 8.2 and 0.02% deoxycholate. The apparent size of the enzyme was lowered to about 100,000 in the presence of 4 or 6 M urea. The average minimum molecular weight based on the heme *a* content as determined from the difference in absorbancy of the reduced spectrum at 605 nm and 630 nm was 124,000, indicating a dimer Recently, Love, Chan and Stotz [6, 7] found that cytochrome oxidase, as prepared by the method of Yonetani [8], had a molecular weight of approximately 200,000, but that at an alkaline pH in a nonionic buffer it dissociated into a monomer having a molecular weight of about 100,000. Most importantly, the monomeric form retained 83-97% of the catalytic activity of the dimer, had a normal absorption spectrum, and reacted with approximately twice as much CO as did a solution of the dimer of equal heme *a* content.

Minimum molecular weights of 72,000 [9], 93,000 [10], 69,300 [11], and 67,000 [12] have also been reported. The first of these was based on an $s/D = 7.7 \times 10^{-7}$ obtained in the presence of 0.002 M sodium dodecyl sulfate, which Criddle and Bock [9] claimed gave a mixture of 85% monomer and 15% dimer. Ambe and Venkataraman [10] analyzed the same preparation as did Criddle and Bock and based their value on the heme *a* content determined as the pyridine hemochrome, while Wainio [11] used the absorbance of the reduced enzyme minus the oxidized enzyme at 605 nm to calculate the heme *a* content. Kagawa [12] irradiated purified cytochrome oxidase with high-energy electrons from a linear accelerator and applied the target theory to deduce the molecular weight.

Orii and Okunuki [13] in 1967 found that treatment of their enzyme (m.w. = 530,000) with sodium dodecyl sulfate resulted in the formation of 16.6 S and 5.7 S species from the original 22.5 S component. Guanidine hydrochloride produced only a 13 S component. Molecular weights of 290,000-330,000 and 67,000 were assigned to the 16.6 S and 5.7 S species, respectively. It was then assumed that the original enzyme was a tetramer, the 16.6 S species a dimer, and the 5.7 S species a half-molecule of the hypothetical monomer which was not identified, but which presumably had a molecular weight approximately equal to that calculated from the heme content, namely, 128,000. Love *et al.*, [6] incubated their 10.5 S dimer at pH 7.4 with 0.2 and 0.4% sodium

dodecyl sulfate for periods of time from 3 h to 3 days and produced 8.6 and 4.6 S species. Both were enzymatically inactive and bore no simple relationship to the 6.0 S monomer obtained at pH 9.5-11.

A further complication is revealed by asking the question whether a subunit with a molecular weight much less than 100,000 is the true enzyme. It remains to be determined whether cytochrome oxidase has two subunits [14-17], four subunits [18-19], or six subunits [20-21]. Among the several possibilities are: (1) that two subunits are cytochromes *a* and a_3; (2) that two subunits are cytochrome oxidase and a core protein; (3) that the subunits are catalytic and regulatory proteins and that the latter are components of the energy transducing system at site III; (4) that one or more of the subunits are impurities. Unfortunately, the present study sheds no light on this problem because our interest has been to delineate the upper limit of the size of the cytochrome oxidase monomer.

The amino acid composition of cytochrome oxidase as determined in two laboratories differs widely with respect to the content of several amino acids. The particular difference is in cysteine which was determined by Matsubara *et al.* [22] to be 7 per heme or per molecular weight of about 93,000, and 16 per 100,000 g by Shmukler [23]. Other marked differences are in the content of histidine [29-30 vs 19], aspartate [58-61 vs 77], glycine [58-59 vs 73], methionine [35 vs 24] and tryptophan [30 vs 8]. It is to be noted that the cytochrome oxidase of Shmukler was prepared from rat liver by a method which has never been published (Polis and Wyeth), and that the equivalent weight of the lipid-free enzyme based on the iron content was 80,000.

The molecular weight of freshly prepared beef heart mitochondrial cytochrome oxidase in deoxycholate has been determined by us to be 228,000 daltons by the sedimentation velocity method, 110,000 from the total protein and heme content and 85,000 when calculated from the amino acid and heme content. These results indicate that the enzyme as isolated into deoxycholate is a dimer. When the deoxycholate of the preparation was replaced with Tween 80, the enzyme polymerized further.

Methods

Cytochrome oxidase was prepared by the method of Wainio [11] and was usually stored frozen in 3 ml aliquots at the temperature of solid CO_2. When the sedimentation coefficient and the diffusion constant were to be determined the method of preparation was changed so that the first deoxycholate extraction was made at 22.5 mg/ml and the second deoxycholate extraction at 30 mg/ml. No attempt was made to

concentrate the protein because it was felt that the treatment might have led to polymerization. *Activity* of the enzyme was determined spectrophotometrically by following the oxidation of ferrocytochrome *c* (Sigma Chemical Co., Type IV, horse heart) at 550 nm in 0.1 M phosphate buffer, pH 6.0 [24]. *Heme* concentration was calculated from the absorbance difference at 605 nm (reduced enzyme minus oxidized enzyme) by applying the factor $\Delta\epsilon = 7.6 \times 10^3$ cm^2 (g atom Fe)$^{-1}$ [25]. *Protein* was determined by the micro-Kjeldahl method. The factor 6.25 was used to convert total nitrogen to protein. *Deoxycholate* was determined by the method of Szalkowski and Mader [26] as adapted by Kremzner and Wainio [27]. *Sedimentation coefficients* were determined at 20° with a Spinco Model E ultracentrifuge equipped with a phase plate schlieren diaphragm. *Diffusion constants* were determined in the Model E ultracentrifuge with a double sector synthetic boundary cell after dialysis against 3% of deoxycholate in 0.1 M phosphate buffer, pH 8.0. *Density* was measured with a 25 ml pycnometer, and *viscosity* with an Oswald viscosimeter. *Spectrophotometric measurements* were made with a Cary Model 11 recording instrument. *Amino acid analyses* were performed with a Beckman Model 120B analyzer. When deoxycholate, lipids and heme were removed prior to the analysis, this was accomplished by homogenizing the sample twice with four volumes of absolute ethanol and allowing the mixture to stand each time at 45° for 30 min. The precipitate (after centrifugation) was treated twice with chloroform-methanol (2 : 1, v/v) for 15 min each time. The precipitate was then homogenized with acetone : concentrated HCl (99 : 1, v/v) to remove the heme *a*, and was finally washed with ethyl ether and dried *in vacuo*. All preparations were hydrolyzed in 6 N HCl in sealed tubes under high vacuum for 72 h. *Tryptophan* was determined by the method of Opieńska-Blauth *et al.* [28].

Results

The sedimentation boundaries yielded an average $S_{20,w}$ value of 8.44 for two preparations (three determinations each) when corrected for density and viscosity. A more rapidly sedimenting boundary which was sometimes seen may have been a small amount of cytochrome *b*, or cytochrome $b + c_1$, or even of polymeric cytochrome oxidase. See Fig. 1 for a representative determination.

When three different preparations were centrifuged, each at a different protein concentration, it was found that the $S_{20,w}$ value was only slightly dependent on concentration (Fig. 2). The extrapolated value is 8.5×10^{-13} sec.

Incubation in the presence of 1.38×10^{-3} M (0.04%) sodium dodecyl sulfate + 1×10^{-3} M EDTA or 3.45×10^{-3} M (0.1%) SDS + 1×10^{-3} M

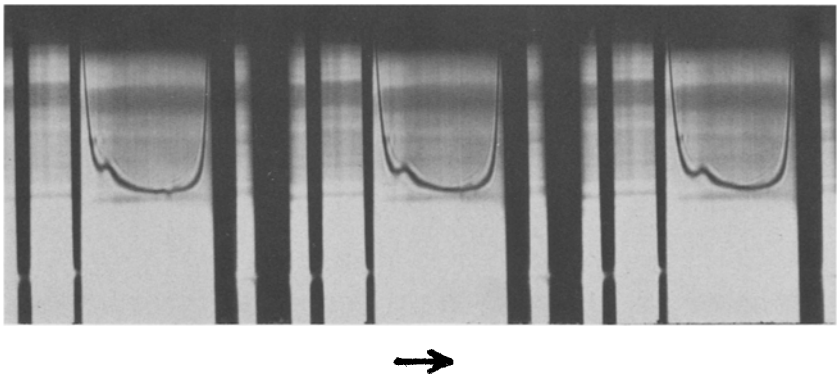

Figure 1. Sedimentation of cytochrome *c* oxidase at 59,780 rpm. Temperature, 24°; 1.94 mg/ml protein; 1.76×10^{-5} M heme based on $\Delta\epsilon$ at 605 nm = 7.6×10^{3} cm^2 $(g\ atom\ Fe)^{-1}$; 30 mg/ml deoxycholate; phosphate buffer, pH 8.0; photographs at 8, 12 and 16 m. The $S_{20,w}$ value was 8.24.

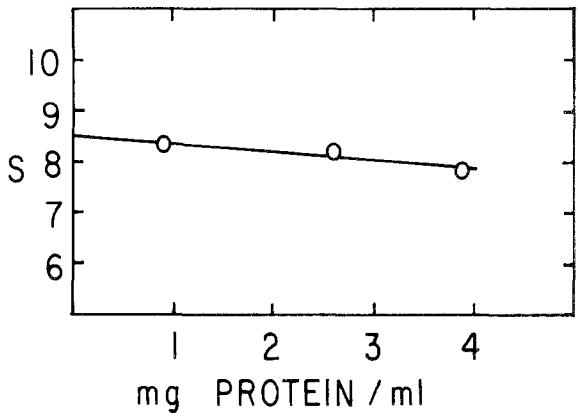

Figure 2. Concentration dependence of the $S_{20,w}$ value for cytochrome *c* oxidase. Each of the three points was obtained with a different preparation.

EDTA for 2 h at room temperature was without effect on the S value. No change in the S value was observed after the enzyme had been in contact with 0.1% dithiothreitol for 2 h at room temperature. Although treatment with 1×10^{-2} M KCN appeared to lower the $S_{20,w}$ value by about 10%, the calculated molecular weight was unchanged when the diffusion coefficient was determined in the presence of 1×10^{-2} M KCN.

When a preparation of cytochrome oxidase which contained a large amount of cytochrome *b* was subjected to ultracentrifugation (Fig. 3), the slowest moving boundary had an uncorrected S value of 7.82. It was calculated from the absorption spectrum of the reduced components by the use of simultaneous equations [29] that the ratio of the concentration of cytochrome oxidase to cytochrome *b* was 6.5 : 1; The next most prominent boundary in Fig. 3 which was probably due to cytochrome *b* had an S value of 12.3. Thus even in a mixture of proteins cytochrome oxidase had the expected sedimentation coefficient.

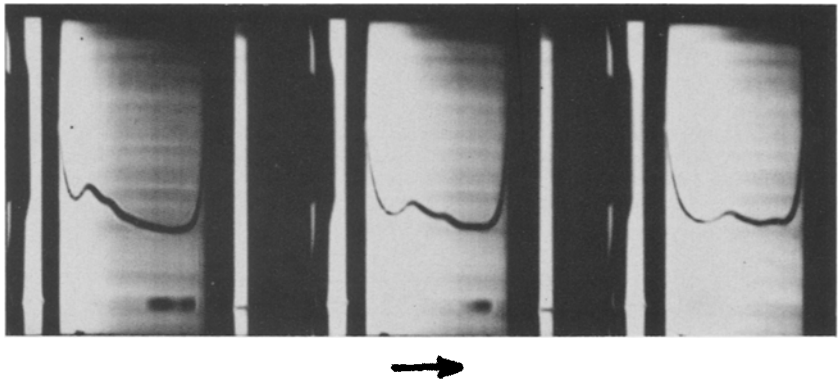

Figure 3. Sedimentation of cytochrome *c* oxidase in the presence of other heme proteins. Speed, 50,880 rpm; 24.5°; 2.6 mg/ml protein; 2.0×10^{-5} M cytochrome oxidase; 0.31×10^{-5} M cytochrome *b*; phosphate buffer, pH 8.0; photographs at 32, 48 and 64 min; S value for cytochrome oxidase, 7.82; S value for cytochrome *b*, 12.3.

In order to determine whether the large S value reported in the literature by others [2] might have been due to aggregation of an active enzyme of smaller size, the deoxycholate in our preparation was replaced with Tween 80 (Atlas Powder Co., Wilmington, Del.; same as Emasol 1130). Tween 80 was added to the enzyme to a final concentration of 0.1%. After 30 min at room temperature the mixture was placed onto a column of Sephadex G-25 which had been previously equilibrated with 0.1% Tween 80 in 0.1 M phosphate, pH 7.5. The Tween-phosphate was also used to elute the enzyme. The enzyme was finally dialyzed overnight at 4° against 5% Carbowax (polyethylene glycol compound 20 M; Union Carbide Corp., South Charleston, W. Va)+ 0.1% Tween 80 in 0.1 M phosphate, pH 7.5, in order to concentrate it. A slight precipitate was removed by centrifuging for 15 min at 39,100 x g. The supernatant fluid contained 3.08 mg protein/ml and was subjected to sedimentation in the analytical ultracentrifuge (Fig. 4). The S value obtained was 17.0 which indicated aggregation.

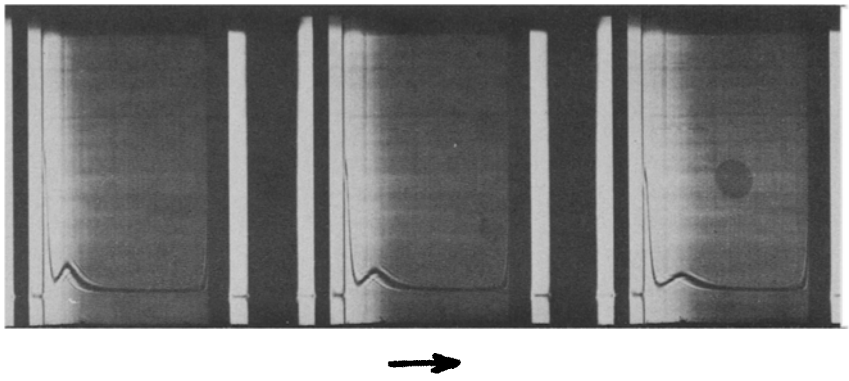

Figure 4. Sedimentation of cytochrome *c* oxidase after replacement of deoxycholate with Tween 80. Speed, 19,160 rpm; 25°; 3.1 mg/ml protein; 0.1% Tween 80 in 0.1 M phosphate buffer, pH 7.4; photographs at 4, 6 and 8 min. The S value was 16.95.

In another experiment, freeze-dried oxidase was dissolved in 0.1 M Tris buffer, pH 8.0, containing 0.1% Tween 80 and was dialyzed against Tris-Tween for 8 h at 4°. After the suspension had stood overnight at 4°, the white precipitate of deoxycholate containing some Tween 80 was removed by centrifugation. The supernatant fluid was twice treated at room temperature with Amberlite IRA-40 (Rohm and Haas, Philadelphia; OH^- form) to remove more of the deoxycholate, first for 1.5 h and then for 0.5 h. For both exchanges the resin : protein ratio was 15 : 1 (w/w). After removal of the resin, the solution was concentrated by dialysis at 4° against 30% Carbowax 20 M in Tris-Tween. The final solution which contained 3.9 mg protein/ml was placed in a 10 ml graduate cylinder at room temperature. The first $S_{20,w}$ value was 16.3. Approximately 2 h later a second determination on an aliquot taken by pipette from near the top of the undisturbed graduate cylinder gave a value of 21.9. Approximately 2 h later, or 5 h after having been placed in the graduate, the enzyme had an $S_{20,w}$ = 43.8, while at 7 h the $S_{20,w}$ had risen to 51.5. There was virtually no evidence of heterogeneity in any of the samples centrifuged.

Another difference between the deoxycholate-solubilized and Tween-solubilized enzyme was the response to sodium dodecyl sulfate. As seen in Fig. 5, the activity of the Tween-solubilized enzyme was stimulated by SDS before it was inhibited. This is the same result that was reported by Orii and Okunuki [13], who also found that their enzyme was depolymerized by SDS. In contrast, the activity of the deoxycholate-solubilized enzyme was not stimulated by SDS (Fig. 5) and, as mentioned above, there was no change in the S value when 0.1% SDS was added.

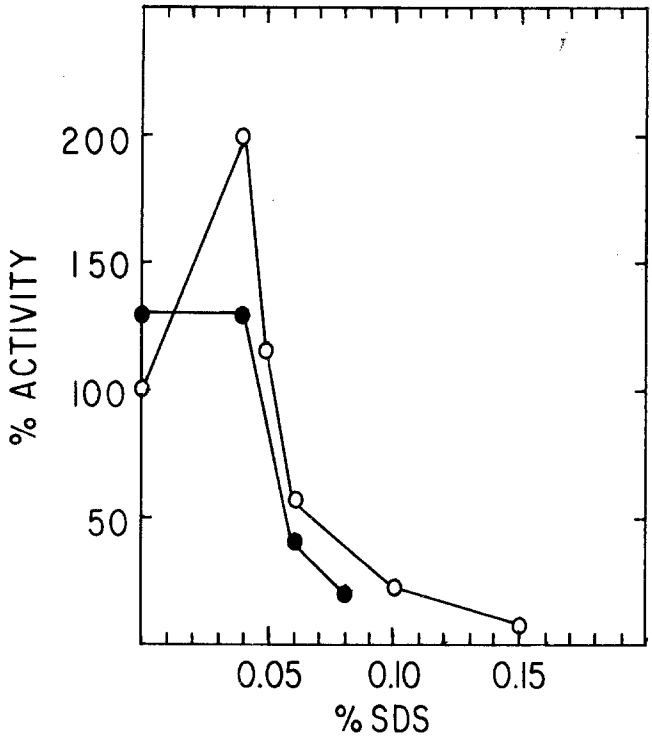

Figure 5. The effect of sodium dodecyl sulfate (SDS) on the activity of cytochrome *c* oxidase in Tween 80 (○) and in deoxycholate (●). The activity of the preparation in Tween 80 is taken as 100%.

A diffusion constant was determined for the same preparations of cytochrome oxidase which had an average sedimentation coefficient of $S_{20,w} = 8.44 \times 10^{-13}$ sec. The $D_{20,w}$ value obtained was 3.21×10^{-7} cm^2 sec^{-7}. See Fig. 6 for a representative determination. A molecular weight of 228,000 was calculated by employing the partial specific volume of 0.72 ml g^{-1} determined by Takemori *et al.* [2] and Love *et al.* [6].

Numerous attempts to determine the diffusion constant by free boundary diffusion in a Perkin-Elmer electrophoresis apparatus failed because the deoxycholate in phosphate buffer gelled and gave such skewed patterns that no meaningful calculations could be made. More than a dozen attempts were also made to determine the diffusion constant in a valve-type synthetic boundary cell in the analytical ultracentrifuge. The average of seven determinations was 4.33×10^{-7} cm^2 sec^{-1}, but the range was from 1.99 to 6.58×10^{-7}. It is likely that the variability was due to the deoxycholate which the preparations contained.

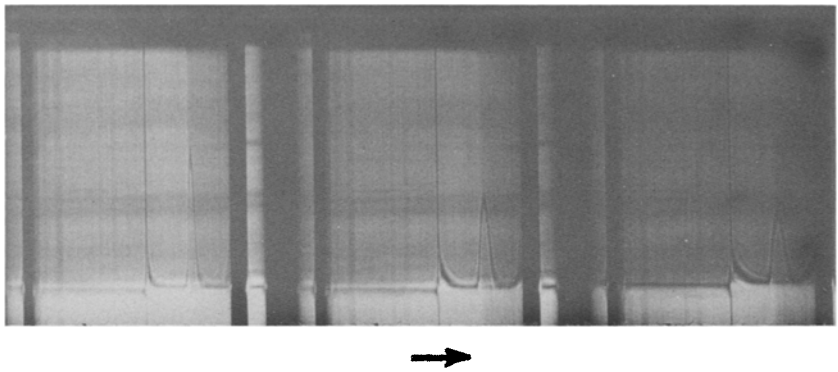

Figure 6. Diffusion of cytochrome *c* oxidase at 20°. The preparation was dialyzed against 1 liter of a solution of 30 mg deoxycholate/ml 0.1 M phosphate buffer, pH 8.0, for 2 h at room temperature. Photographs were taken at 16, 32 and 48 min. The $D_{20,w}$ value was 3.32×10^{-7} cm^2 sec^{-1}.

The minimum molecular weight found for the protein alone of one preparation by calculating the heme concentration (2.37×10^{-5} M) from $\Delta\epsilon = 7.6 \times 10^3$ cm^2 $(g\ atom\ Fe)^{-1}$ and the protein concentration (2.609 mg/ml) from the total nitrogen was 110,000.

Amino acid analyses of one preparation gave a minimum molecular weight for the protein of about 85,000. These data are presented in Table I where the analysis of a sample from which the deoxycholate, lipid and heme were removed before hydrolysis and where the analyses of Matsubara *et al.* [22] and Shmukler [23] are given for comparison. There is reasonably good agreement for our preparation as analyzed before and after removal of the deoxycholate, lipid and heme, except for the glutamic acid and ammonia. When comparing our preparation to those of Matsubara *et al.* and Shmukler, it is apparent that our preparation differs from both with respect to threonine, serine, proline, leucine and tryptophan, and is more like the preparation of Matsubara *et al.* with respect to aspartic acid and glycine and more like the preparation of Shmukler with respect to histidine, methionine, tyrosine and ammonia.

Discussion

Our sedimentation coefficient of 8.44 S is to be compared with the value of 21.9 reported by Takemori *et al.* [2] from which they calculated a molecular weight of 530,000, with the value of 12 S obtained by Tzagoloff *et al.* [4], and with the value of approximately 10.6 reported

TABLE I. Amino acid composition of cytochrome oxidase

Amino Acid	Residues per protein molecule			
	This paper		Matsubara [22]	Shmukler [23]
	Before RDLH[a]	After RDLH[a]		
Lys	42	37	39	37
His	20	19	30	19
Arg	27	28	31	32
Asp	55	55	60	77
Thr	42	39	53	59
Ser	35	36	54	57
Glu	53	63	60	65
Pro	32	30	46	42
Gly	53	58	59	73
Ala	56	61	62	66
Cys	5	5	(6)7[b]	16
Val	41	45	51	56
Met	21	22	(32)35[b]	24
Ile	37	42	43	46
Leu	76	78	87	88
Tyr	27	27	33	24
Phe	43	41	47	42
Trp	44[b]	44[b]	30[b]	8
NH_3	111	78	59	74
Molecular weight	84,022[c]	85,657[c]	93,378[d]	92,078

[a] Removal of deoxycholate, lipid and heme.
[b] From other analyses.
[c] Based on the heme content.
[d] Based on the seven cysteine residues.

by Love *et al.* [6]. Tzagoloff *et al.* did not calculate a molecular weight based on their S value, but chose instead to report a molecular weight of 290,000 determined from light scattering data. It may be assumed that they were not able to determine a diffusion constant. Our sedimentation coefficient of 8.44 S is of the size that Takemori *et al.* said they could not find in their depolymerization with sodium dodecyl sulfate, that is, between their presumed 16.6 S dimer and their 5.7 S half-molecule. Our S value may differ from that of Love *et al.* [6] because their enzyme was in 0.1% Emasol 4130 at pH 7.4 while our enzyme was in 1.9% sodium deoxycholate at pH 8.0. We conclude that the 8.44 S species is dimeric cytochrome oxidase.

Our value of 3.21×10^{-7} cm^2 sec^{-1} for the diffusion coefficient is to be compared with the two other values in the literature, i.e., that of Takemori *et al.* [2], who determined a coefficient of 3.58×10^{-7} cm^2 sec^{-1}, and that of Love *et al.* [6], who reported a value of about

4.83×10^{-7} cm^2 sec^{-1} for their dimer. The difference between our $D_{20,w}$ value and that of Love *et al.* [6] may be due to a difference in the conformation of the monomer in the two surface active agents, i.e., in deoxycholate and Emasol 4130, respectively. Criddle and Bock [9] calculated s/D values from approach to sedimentation equilibrium data. In order for us to have gotten their value of 7.7×10^{-7} for s/D, our $D_{20,w}$ value would have to have been 10.5×10^{-7} cm^2 sec^{-1} or our $S_{20,w}$ value would have to have been 5.3×10^{-13} sec.

The molecular weight of 228,000 which we calculated from $S_{20,w} = 8.44 \times 10^{-7}$ sec, $D_{20,w} = 3.21 \times 10^{-7}$ cm^2 sec^{-1} and $\bar{v} = 0.72$ g ml^{-1} of Takemori *et al.* [2], is smaller than two values in the literature and agrees with the third. Takemori *et al.* [2] found a value of 530,000 (a pentamer or a tetramer) from sedimentation and diffusion data, and Tzagoloff *et al.* [4] reported a value of 290,000 based on light scattering. Our enzyme is of the same size as was determined by Love *et al.* [6] for their dimer. Our value is approximately twice that that has been reported by ourselves in this publication and by several other investigators [2, 4, 10] for the minimum molecular weight.

The effect of Tween 80 which was to cause aggregation is in keeping with the high molecular weight of cytochrome oxidase in Emasols [2] and with the observations of Tzagoloff *et al.* [4], who found ready aggregation when the enzyme was diluted with 0.33% taurocholate at concentrations below 5 mg protein/ml, but especially when the dilution was made without bile salt in the diluting medium. Takemori *et al.* [2] depolymerized their enzyme with sodium dodecyl sulfate, whereas it had no effect on our preparation. Criddle and Bock [9] stated that the preparation which they used [10] for the determination of the molecular weight in the presence of sodium dodecyl sulfate showed five to six sedimenting components in the absence of the surface active agent. Furthermore, when the protein solution was allowed to stand for a week or two, there was a slow increase in the heavier aggregates at the expense of the lighter ones.

A detailed comparison of our analyses of the amino acid composition of cytochrome oxidase with those of Matsubara *et al.* and Shmukler is without importance until it is known which preparation is the purest one. In general, however, the analyses are the same, except that our preparation differs from the others with respect to threonine, serine, proline and leucine which are lower, and with respect to tryptophan which is higher. We verify the conclusion of Matsubara *et al.* that there is a high content of lipophilic residues. The accuracy of the molecular weight as calculated from the amino acid content depends largely on the accuracy of the method used to calculate the heme content. In our experiments, the heme was calculated from the absorbance difference at 605 nm (reduced enzyme minus oxidized enzyme) by employing the factor $\Delta\epsilon = 7.6 \times 10^3$ cm^2 (g atom Fe)$^{-1}$ [25] which had been calcu-

lated from the reduction at equilibrium of cytochrome oxidase by ferrocytochrome *c* under anaerobic conditions. The use of other methods for calculating the heme or the iron content would undoubtedly change the molecular weight somewhat, although we have shown that a determination of the heme content as the pyridine hemochrome would give the same value.*

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