Molecular Weight of Two States of Cytochrome c Oxidase*

BRUCE LOVE, SAMUEL H. P. CHAN,† and ELMER STOTZ

From the Department of Biochemistry, School of Medicine and Dentistry, The University of Rochester, Rochester, New York 14620

SUMMARY

Cytochrome oxidase, as prepared by the Yonetani method, is shown to have a molecular weight of 200,000 (±5%). At alkaline pH (from pH 9.5 to 11, 0.01 M phosphate, 0.1% nonionic detergent) the enzyme dissociates to a monomer species with a molecular weight of 100,000 (±5%). Qualitative results are presented to show that the rate of conversion of dimer to monomer is slow but strongly pH dependent. Between pH 9.5 and 10.5 a mixture of monomer and dimer is obtained when incubation at 4°C is relatively short (<10 hours), but the conversion is essentially complete upon standing at pH 10.5 for 24 hours. Although monomer preparations can be obtained which appear to be monodisperse from Archibald studies, the dimer isolated at neutral pH contains a small and probably variable amount of the monomer.

Molecular weight studies directed toward understanding the possible polymeric forms of cytochrome oxidase have been carried out by Okunuki and his collaborators (1-4), who reported a molecular weight of 550,000 for the enzyme in Emulsol solution. The minimum molecular weight (termed monomer weight in this paper) was calculated to be about 100,000 based upon heme A and amino acid analysis, or 93,000 on a lipid free basis (1). The 530,000 molecular weight species was interpreted therefore as a pentamer but was later considered to be a tetramer (5). The monomer form of the enzyme was not observed in studies of the enzyme in Emulsol solution. Cridle and Bock (6), with the use of the preparation of Ambe and Venkataraman (7), reported a monomeric form of the enzyme with a molecular weight of 72,000. This preparation involved a thioglycolate treatment, and the ultracentrifugal analysis was carried out in a medium containing sodium dodecyl sulfate. Ambe and Venkataraman (7) reported their enzyme to be inactive, but it could be partially reactivated by the addition of phospholipids. Tzagoloff et al. (8), in light-scattering studies of Complex IV, as prepared by Griffiths and Wharton (9), found molecular weights from 220,000 to 234,000. These values were obtained when the specific refractive increment was corrected for the contribution by lipid. Without this correction molecular weights from 266,000 to 312,000 were reported.

In the present work ultracentrifuge studies are described which demonstrate that the enzyme can be obtained in two states, one corresponding to a 100,000 molecular weight monomer and the other to a dimer. These results, in conjunction with activity studies and spectroscopic properties of the monomer reported in the following paper, have an important bearing on the cytochrome a₃ question.

MATERIALS AND METHODS

Enzyme Isolation—Cytochrome oxidase was prepared from bovine heart by the method of Yonetani (10, 11) with 10% sodium cholate (Nutritional Biochemicals). The preparation of the oxidase met the authors' criteria of purity. The final dispersion was made in 0.1% Emulsol (12) rather than Tween 80. As an additional step, the preparation in 0.1% Emulsol containing 0.01 M potassium phosphate buffer, pH 7.4, was centrifuged at 104,000 × g for 120 min, as suggested in the method of Horie and Morrison (12), for better removal of lipids and other impurities. Three layers resulted, a colorless top layer containing lipid, a green layer containing the oxidase, and a white pellet containing modified oxidase and other contaminants. After careful removal of the top layer by aspiration, the second layer was removed and filtered with Whatman No. 50 filter paper. The clear solution of the purified oxidase thus obtained was used for the studies reported in this paper. The lipid content of a typical preparation was found to be 14% according to the method of Vorbeck and Marinetti (13). Based on extinction coefficients for protein and for heme A (10, 11), our typical purified preparation contained 0.0072 mg of heme A per mg of protein.

Ultracentrifuge Methods—Sedimentation studies were carried out at 10°C with a Spincor model E analytical ultracentrifuge equipped with electronic speed control. The RTIC unit was calibrated with a National Bureau of Standards thermometer, and all sedimentation coefficients were corrected to s_{20,w} values, with relative viscosities measured with a Beckman-Spinco concentric cylinder viscometer. All ultracentrifuge plate measurements were made with a Nikon (Nippon Kogaku, K. K., Tokyo, Japan) two-dimensional microcomparator, with micrometers reading directly to 0.0002 cm. Computer processing of sedimentation data was carried out on an IBM 360/44 computing system. Our program calculated sedimentation rate from linear...
least square slopes, and by Trautman's quadratic least square procedure which has a lower internal standard error (14). In the latter method the internal sedimentation rate is calculated from the slope of the tangent to the least square quadratic line at the mean of the corrected times. Our computer program for processing data from the Archibald approach to equilibrium method is essentially like that of O'Connor, Bakerman, and Hersh (15) with linear least square extrapolation used to locate the gradient at the meniscus or bottom. The program also included calculation of weight average sedimentation coefficients from Baldwin's transport equation (16). The total solute concentration was determined from an artificial boundary experiment at 12,000 rpm in a valve-type cell.

**Diffusion Coefficients**—Diffusion coefficients were measured at 20° from the spreading of an artificial boundary. The solvent layer was the dialysate after the oxidase was dialyzed against the gradient at the meniscus or bottom. The program also included calculation of weight average sedimentation coefficients from Baldwin's transport equation (16). The total solute concentration was determined from an artificial boundary experiment at 12,000 rpm in a valve-type cell.

**Densities and Apparent Specific Volume**—The densities of the buffers used were measured by the density gradient column method of Linderstrom-Lang and Lanz (17). Chlorobenzene was used as the lower phase and n-chlorobutane as the upper phase. KCl solutions were used to calibrate the column, which was held at 20° with a Tampon (Neslab Institute, Portsmouth, New Hampshire) model T9 constant temperature bath and PBC4 cooling unit. The density of the unknown is calculated from

\[ 4\pi Dt = A^2/H^2 \]

where \( D \) is the diffusion coefficient (cm²/sec²), \( A \) is the area under the boundary (cm²), \( H \) is the maximum ordinate (cm), and \( t \) is the time in seconds.

**Sedimentation Velocity**—Fig. 1 shows the typical sedimentation behavior of a purified cytochrome oxidase preparation at two different dilutions at pH 7.4. At both dilutions there is a sharp major boundary, with an indication of a small amount of a slower component trailing the major species. This slower component was generally present in small amounts in all preparations of the enzyme. The boundary at the meniscus, which does not migrate appreciably during the run, was shown to be due to the detergent micelle (Emasol) by performing a sedimentation velocity run on the solvent without the enzyme.

The sedimentation pattern presented in Fig. 1 is for Preparation D; other data on this preparation are recorded in Table I. It will be noted that this preparation showed an internal sedimentation rate of 10.5 S, and that there is no demonstrable concentration dependence of the sedimentation rate over a 3-fold dilution range. Table I also includes sedimentation velocity data for several different enzyme preparations labeled A to F. These data are presented to indicate the range of values that can occur depending upon the variability of product obtained during routine isolation. As seen in Table I, the range in internal sedimentation rate observed was 10.2 to 11.0 S for samples run at pH 7.4.

As also illustrated in Table I, samples stored at 4° for periods of a week or longer show higher and atypical values of up to 14 S (see Sample C). Similarly, abnormal sedimentation rates were observed with preparations resulting from insufficient ammonium sulfate fractionation and therefore not meeting the criteria of purity for the Yonetani preparation (see Sample F). Similar studies were conducted with oxidase preparations when the pH had been raised to a range of 9.5 to 11.0. Fig. 2 shows the results of an experiment in which the pH of Preparation D had been raised to 10.1 with 1 N NaOH and then stored at 4° for 10 hours. It is noted that there are now two components. Sedimentation velocity measurements show that one has an internal sedimentation rate of 10.5 S as observed at pH 7.4, and a second has an apparent sedimentation rate of 5.7 S. When the sample is maintained at pH 10.1 for 24 hours at 4°, the patterns obtained are shown in Fig. 3. Now a single component is observed with a considerably lower sedimentation velocity. It would appear that standing at an elevated pH has resulted in a conversion of the 10.5 S species to a lighter component, with Fig. 2 illustrating the mixture.

**Table I**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>pH</th>
<th>Concentration</th>
<th>Internal sedimentation rate</th>
<th>Molecular weight (Svedberg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>7.4</td>
<td>0.6</td>
<td>10.7</td>
<td>184,500 0.72</td>
</tr>
<tr>
<td>B</td>
<td>7.4</td>
<td>0.6</td>
<td>11.5</td>
<td>190,100 0.72</td>
</tr>
<tr>
<td>C</td>
<td>7.4</td>
<td>0.6</td>
<td>10.2</td>
<td>188,700 0.72</td>
</tr>
<tr>
<td>D</td>
<td>7.4</td>
<td>0.9</td>
<td>10.4</td>
<td>188,500 0.72</td>
</tr>
<tr>
<td>E</td>
<td>7.4</td>
<td>0.3</td>
<td>10.8</td>
<td>186,700 0.72</td>
</tr>
<tr>
<td>F</td>
<td>7.4</td>
<td>0.6</td>
<td>11.1</td>
<td>185,700 0.72</td>
</tr>
<tr>
<td>A</td>
<td>10.5</td>
<td>0.6</td>
<td>11.1</td>
<td>102,200 0.73</td>
</tr>
<tr>
<td>B</td>
<td>11.0</td>
<td>0.6</td>
<td>6.2</td>
<td>106,700 0.73</td>
</tr>
<tr>
<td>C</td>
<td>11.0</td>
<td>0.6</td>
<td>6.4</td>
<td>101,800 0.72</td>
</tr>
<tr>
<td>D</td>
<td>11.0</td>
<td>0.6</td>
<td>6.0</td>
<td>100,100 0.73</td>
</tr>
<tr>
<td>E</td>
<td>10.1</td>
<td>0.6</td>
<td>6.1</td>
<td>11.1</td>
</tr>
<tr>
<td>F</td>
<td>7.4</td>
<td>0.6</td>
<td>14.1</td>
<td>14.2</td>
</tr>
</tbody>
</table>

* Internal sedimentation rate is used to calculate the molecular weight from the Svedberg equation.
* Maintained at the stated pH for 3 hours.
* Treated for 10 hours at pH 11.0.
* Stored for 3 days at pH 10.1.
* Sample showing aggregation after 2 weeks storage at 4°.
* This sample resulted from only two fractionations between 26 and 33% saturation of ammonium sulfate and did not meet the spectral criteria designated (10, 11) as the highest purity material.

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Molecular Weight of Cytochrome Oxidase

FIG. 1. Sedimentation velocity pattern of cytochrome oxidase. Protein concentration 0.9% in lower pattern, 0.3% in upper pattern, pH 7.4. Photographed at 28 and 36 min after reaching 60,000 rpm. The boundaries at the menisci are Kerosol, and sedimentation is from left to right.

FIG. 2. Sedimentation velocity pattern of cytochrome oxidase. Sample kept at pH 10.1 for 10 hours at 4°C. Protein concentration is 0.3% in the upper pattern and 0.6% in the lower pattern. Photographed at 18 and 26 min after reaching 60,000 rpm. Sedimentation is from left to right.

FIG. 3. Sedimentation velocity pattern of cytochrome oxidase. Sample kept at pH 10.1 for 24 hours at 4°C. Protein concentration is 0.3% in the upper pattern and 0.9% in the lower pattern. Photographed at 19 and 23 min after reaching 60,000 rpm. Sedimentation is from left to right.

FIG. 4. Sedimentation velocity pattern of cytochrome oxidase. In the upper pattern the sample was kept at pH 10.5 for 3 hours at 4°C. Protein concentration was 0.6%. Photographed at 32 and 40 min after reaching 60,000 rpm. Sedimentation is from left to right.

The rate at which the 10.5 S species of oxidase is converted to the 6.0 S component is strongly pH dependent. In Fig. 4 two samples are compared after 3 hours of storage at 4°C, one at pH 10.5 and the other at pH 11.0. It is evident that at pH 11.0 the 10.5 S oxidase has been completely converted to the 6.0 S species, while at pH 10.5 the sample is still a mixture of the two components. The lower pH at which dissociation appears to occur is 9.6, and at this pH only a small amount of the 6.0 S form is detectable in 3 hours.

The molecular weights calculated from the sedimentation velocity experiments are reported in Table I along with the diffusion coefficients measured in synthetic boundary experiments. At neutral pH the results indicate that the molecule has a molecular weight from 185,000 to 200,000. After exposure to pH 0.5 to 1.1 it is converted to a lighter species of molecular weight from 100,000 to 107,000. If an average value of the diffusion coefficient of $4.82 \times 10^{-10}$ (cm² see⁻¹) is used along with the value of 10.5 for the internal sedimentation rate, the molecular weight of the heavier component would be 189,300. This is about 5% below the 200,000 value one would expect for a dimer if 100,000 is accepted as the minimum monomer molecular weight. A discrepancy of 5% is within the precision with which the diffusion coefficient was determined in these experiments, particularly since we are dealing with a monomer-dimer mixture.

Since other laboratories (5) have obtained species of the enzyme with low sedimentation values by the addition of sodium dodecyl sulfate, a short study was made of the effect of this reagent on the dimer. The 10.5 S enzyme was incubated at pH 7.4 with 0.2 and 0.4% sodium dodecyl sulfate for periods of time from 3 hours to 3 days. It was found that both concentrations of sodium dodecyl sulfate caused dissociation of the 10.5 S enzyme into two components, one 8.5 to 8.7 S and the other 4.5 to 4.7 S. The dissociation was observed to occur within 3 hours, and the amounts of the two species did not change appreciably when the incubation was extended to 3 days. However, the products of sodium dodecyl sulfate dissociation were not enzymatically active (4) and do not bear any simple relationship to the 6.0 S monomer studied here.

Archibald Method—To lend further support to the molecular weight results just presented, both the monomer and dimer were studied by the Archibald method. A typical Archibald run is shown in Fig. 5, and the molecular weight obtained from each frame is given in Table II. It is apparent from Table II that as time increases the meniscus is progressively depleted of dimer and the weight average molecular weight at the meniscus approaches the monomer value. This result confirms the supposition made earlier (Fig. 1) that in a typical preparation of the enzyme there is a small amount of monomer present. The meniscus molecular weight is given preference as far as precision is concerned because of the problem of deviated light at the cell bottom (18). Lack of agreement between molecular weights

1 In the following paper it will be shown that the monomer and dimer forms exhibit comparable biological activity.
FIG. 5. Archibald experiment. Upper patterns are the artificial boundary run at 12,000 rpm, protein concentration 0.6%, pH 7.4. Lower set of patterns were run at 6000 rpm, protein concentration 0.6%, pH 7.4. Upper set photographed at 8-min intervals and the lower set at 128-min intervals, phase plate 70°. Sequence is left to right.

<table>
<thead>
<tr>
<th>Time (%)</th>
<th>0.6% concentration</th>
<th>0.3% concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>128</td>
<td>207,000</td>
<td>208,000</td>
</tr>
<tr>
<td>256</td>
<td>167,000</td>
<td>165,000</td>
</tr>
<tr>
<td>384</td>
<td>148,000</td>
<td>150,000</td>
</tr>
<tr>
<td>512</td>
<td>135,000</td>
<td>136,000</td>
</tr>
<tr>
<td>640</td>
<td>121,000</td>
<td>121,000</td>
</tr>
</tbody>
</table>

TABLE III
Weight average molecular weights obtained by Archibald method.
The sample was kept at pH 11.0 for 5 hours at 4° prior to starting the run. The times are given in minutes at the speed indicated. One hour was allowed for solute redistribution after each change in speed. The concentration was 0.6%.

<table>
<thead>
<tr>
<th>Time</th>
<th>Speed</th>
<th>Molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Meniscus</td>
</tr>
<tr>
<td>120</td>
<td>6000</td>
<td>104,000</td>
</tr>
<tr>
<td>240</td>
<td>7200</td>
<td>100,000</td>
</tr>
<tr>
<td>300</td>
<td>8000</td>
<td>98,000</td>
</tr>
</tbody>
</table>

In the present work both sedimentation velocity and Archibald studies show that treatment of cytochrome oxidase at pH 9.5 to 11.0 will produce a species of oxidase with a molecular weight of 100,000. This species has been termed the enzyme monomer based on a minimum molecular weight of 100,000 calculated from heme A and iron analysis (2). This does not imply that the monomer could not be further dissociated; since, however, the monomer (mol wt 100,000) has one heme A, it is clear that any subfractions of the monomer could not be identical and therefore for clarity and in relation to the existing literature, the term monomer should be retained for the 100,000 molecular weight material. The sedimentation velocity studies at neutral pH show the enzyme to be a dimer with a molecular weight of 189,300, while the Archibald results when extrapolated to zero time give a molecular weight of 200,000. It must be pointed out that these results do not take into account the amount of detergent bound or exchanged for lipid, and, therefore, the molecular weights reported here may be refined in future sedimentation equilibrium studies.

The dimer molecular weights reported here are not in agreement with the results of Takemori, Sekuzu, and Okunuki (3) who reported a 530,000 molecular weight when the enzyme was studied in Emasol solution, later interpreted to be a tetramer.
Since our initial results were obtained in a Tween 80 solvent system it was thought that solvent differences might explain the discrepancy. However, all data reported in this paper were obtained after changing to the Emasol-phosphate buffer system used by Okunuki and his collaborators. Although the detergent system used is the same, it appears that the Yonetani type preparation used here is not identical with that employed by Takemori et al. (2, 3) in reporting their molecular weight data. A difference in lipid content between the two preparations could be responsible for the difference in the apparent state of aggregation. The final high speed centrifugation used in the isolation of the enzyme here reduced the lipid content of our preparation to 14%. We have of course observed higher sedimentation coefficients when the enzyme was allowed to stand at 4° for 2 weeks, or when ammonium sulfate fractions of lower purity were examined (see Table I).

Our molecular weights are in somewhat better agreement with Tzagoloff et al. (8), who reported a molecular weight of 230,000 from light-scattering studies of Complex IV. It was pointed out in the introduction, however, that this value applies only after correction for a lipid content of about 25%, a value substantially greater than that obtained in the present preparation.

The monomer studied by Criddle and Bock (6), which has a molecular weight of 72,000 when measured in sodium dodecyl sulfate solution, was isolated under conditions which were not comparable to those employed in the present work. The Ambe and Venkataraman isolation method employed by Criddle and Bock includes a 2- to 4-hour incubation with 0.3 M sodium thioglycolate and results in a product of low activity unless reconstituted by the addition of phospholipid. The difference in monomer molecular weight obtained by Criddle and Bock and the value reported here cannot be accounted for by a difference in lipid content. The most probable explanation is that different subunits were obtained after disulfide exchange with the thioglycolate.

Lemberg and Pilger (20) found that in cholate buffer systems alkaline pH led to a modified cytochrome oxidase which was, in many ways, similar to a protein hemochromogen and was not enzymatically active. It has also been shown by Takemori and King (21), and Lemberg (22), that above pH 11.5 an intramolecular Schiff base is formed. The spectroscopic and activity studies on the monomer reported in the following paper show that these reactions had not occurred in the experiments reported here. With the information presently available it would be premature to discuss possible mechanisms for alkaline dissociation.

REFERENCES
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