Biochimica et Biophysica Acta, 1020 (1990) 207–212
Elsevier

BBABIO 43296

Monomer-dimer structure of cytochrome-*c* oxidase and cytochrome bc_1 complex from the thermophilic bacterium PS3

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(Received 1 March 1990)
(Revised manuscript received 12 June 1990)

Key words: Cytochrome-*c* oxidase; Cytochrome bc_1 complex; Quarternary structure; Laser light scattering; (Thermophilic bacterium PS3)

Molecular weights of three membrane proteins have been measured in the presence of 0.1% octaethylene glycol *n*-dodecyl ether ($C_{12}E_8$) by the measuring system in which a membrane protein eluted from a gel chromatography column is monitored sequentially for ultraviolet absorption, light scattering and refractive index. The relative molecular mass (M_r) and amount of bound detergent per protein (δ) can be calculated from these data, if instrumental constants are measured using a set of appropriate water-soluble proteins which does not bind nonionic surfactants. The molecular masses of cytochrome *c* oxidase and cytochrome bc_1 complex from the thermophilic bacterium PS3 were determined to be 127 kDa and 185 kDa, respectively, indicating that the oxidase is monomeric, while the bc_1 complex dimeric in the presence of $C_{12}E_8$. The larger apparent molecular mass of about 310 kDa of the PS3 oxidase obtained from the retention time of the gel chromatography (Sone, N., Sekimachi, M. and Kutoh, E. (1987) J. Biol. Chem. 262, 15386–15391) turned out to be due to a high binding ability with the detergent ($\delta = 1.25$ g/g) of this very hydrophobic protein. Analyses of bovine heart cytochrome oxidase, on which monomer/dimer properties have been reported, showed that the enzyme is mainly dimeric ($M_r = 374\,000$), while a small portion is monomeric ($M_r = 191\,000$). Mild alkaline treatment of this enzyme caused monomerization of the enzyme with accompanying aggregate formation. These results, thus, show that this method is suitable to analyze monomer/dimer conversion of membrane protein as well as to estimate structure of membrane proteins.

Introduction

Mitochondrial respiratory complexes such as cytochrome bc_1 complex and cytochrome oxidase are known to be composed of 11–13 subunits [1–3]. In addition, these enzymes are suggested to exist as dimers composed of two sets of individual subunits in mitochondrial membrane [4,5]. In this line, an isolated bovine heart enzyme was reported to be dimeric in nonionic surfactant at neutral pH [6,7]. As to bovine heart cytochrome oxidase, however, several researchers found the presence of monomeric enzyme in the purified sample solubilized by Triton X-100 [8,9] or laurylmaltoside [10–12]. Monomerization of the bovine heart enzyme was also known to occur without inactivation. When the enzyme was incubated at high pH [8,13], in the presence of a low concentration of dodecyl sulfate or guanidine hydrochloride [13], or even by simple dilution [12].

On the other hand bacterial cytochrome oxidases (cytochrome aa_3-type) have a much simpler subunit structure composed of two or three different subunits (see Ref. 14, for review), although we recently found a small fourth subunit (12 kDa), in addition to the original three subunits (56, 38 and 22 kDa) in the thermophilic bacterium PS3 [15,16]. Two-subunit enzymes from *Paracoccus denitrificans* [17,18] and *Nitrobacter agilis* [19] exist as monomers in the presence of Triton X-100. On the other hand the four-subunit enzyme from the thermophilic bacterium PS3 may exist as a dimer, since the apparent molecular mass in the presence of $C_{12}E_8$ was estimated to be 250 kDa in the presence of *N*-lauroylsarcosinate [16] or 310 kDa in Brij 35 [20] by the gel chromatography method, indicating that a dimer is possible if the amounts of the bound surfactant molecules are not large.

One of the authors (T.T.) and his colleagues have

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developed a novel method to estimate a molecular weight of a protein and an amount of bound lipid including surfactant to protein [21–23]; the elution of a high performance gel chromatography is directly measured using a tandem array of a low angle laser light scattering photometer (LS meter), a spectrophotometer (UV meter) and a differential refractometer (RI meter). The method has been successfully applied on Na,K-ATPase [24,25] and *E. coli* porin and λ receptor [26]. The method is rapid, convenient and accurate, and needs only small amount of the sample.

Recent advance of usage of monochromatic light at 660 nm for the refractometer has made the measurement successful for colored proteins such as cytochromes including bovine cytochrome oxidase as shown in the present paper. This paper reports the measurement of M_r and δ of PS3 cytochrome oxidase and cytochrome bc_1 (b_6f) complex. The conclusion that the former is monomeric, while the latter dimeric, will also be discussed in relation to the target sizes of PS3 cytochrome oxidase obtained by radiation-inactivation experiments, which showed 80–100 kDa for oxidase activity and 190–230 kDa for H^+ pumping activity [27].

Experimental procedures

Materials

PS3 cytochrome oxidase [16] and cytochrome bc_1 (b_6f) complex [28] were purified as described previously. Bovine heart cytochrome oxidase [29] was kindly given by Dr. Y. Orii of Kyoto University. Octaethylene glycol *n*-dodecyl ether ($C_{12}E_8$), nonanoyl-*N*-methyl glucamide (MEGA-9) and *n*-octyl D-glucoside were the products of Nikko Chemicals Co. (Tokyo), Dojin Chemicals Co. (Kumamoto), and Sigma Chemicals (St Louis), respectively. Glutamate dehydrogenase (yeast), lactate dehydrogenase (pig heart), and enolase (yeast) were kindly supplied by Oriental Yeast Co. (Tokyo).

Extinction coefficients

The determination of the molecular weight of a protein using the present technique requires its specific extinction coefficient [23]. In order to know the amount of protein, the hydrolyzate of PS3 cytochrome oxidase in 6 M HCl was analyzed by reverse-phase chromatography after the addition of *o*-phthalaldehyde. Only the amounts of Gly, Ala, Val, Phe and Leu were compared with the values already reported for the amino acid composition of PS3 cytochrome oxidase [16]. The amounts of sample used were 10–30 μg. For the measurement of absorbance at 280 nm the oxidase samples containing Triton X-100 were first substituted by $C_{12}E_8$ or MEGA-9 by gel chromatography with a column of TSK gel G-4000SW equilibrated with 0.4% $C_{12}E_8$ or MEGA-9. Three independent measurements of A_{280} and protein contents of the samples according to determination of the five amino acids in PS3 cytochrome oxidase gave a mean absorption coefficient at 280 nm (A) of 2.2 $mg^{-1} \cdot ml \cdot cm^{-1}$. The value for PS3 cytochrome bc_1 complex was 2.3, which was obtained by measuring the absorbance at 280 nm and the total amount of amino acids in 1 ml determined by an amino acid analyzer system, ABI 420A/130A/920A (Applied Biosystems, Foster City, CA).

High-performance gel chromatography

An aliquot of 5–50 μl of the purified enzyme was injected into a TSK-Gel G3000SWXL (Tosoh Co., Tokyo) equipped with a guard-column of TSK-GEL G3000SWXL (Tosoh Co., Tokyo) equipped with a guard-column of TSK-GEL GSWP (Tosoh) through a liquid chromatography system of HLC 803A (Tosoh). The column had been equilibrated and was eluted with a buffer solution containing 1 mg/ml $C_{12}E_8$, 0.1 M NaCl and 20 mM sodium phosphate (pH 6.8) at a flow rate of 0.3 ml/min and at 23 ± 2° C, unless otherwise described. The eluate from the column was monitored successively with a spectrophotometer model UV-8000 (Tosoh) at 280 nm and a low-angle laser light scattering photometer model LS-8000 (Tosoh) and a precision differential refractometer RI-8011 (Tosoh). A sample injector with a 100 μl loop was used and a degasser model ERC-3310 (Erma Optical Works, Japan) was introduced between solvent reservoir and pump. LS-8000 used He-Ne laser light and RI-8011 used a monochromatic light at 660 nm.

Calculation of molecular weight and lipid binding

The molecular weight of a protein can be calculated from the peak heights of the outputs of the spectrophotometer (UV meter) at 280 nm, of the scattering photometer (LS meter) and the refractometer (RI meter), using the following equation [22]:

$$M_r = \frac{k(\text{Output})_{UV}}{(\text{Output})_{UV}\, dn/dc_p} = \frac{k(\text{Output})_{LS} \cdot (\text{Output})_{UV}}{k'A(\text{Output})_{RI}(\text{Output})_{RI}} \quad (1)$$

where dn/dc_p is the specific refractive increment index of a protein with surfactant/phospholipid, A is the absorbance at 280 nm of the protein at 1 mg/ml, and k and k' are the constants for the instrument. $(\text{Output})_{UV}$, $(\text{Output})_{LS}$ and $(\text{Output})_{RI}$ are the outputs of the UV meter, LS meter and RI meter, respectively. When a water-soluble protein which usually does not bind nonionic detergent is used, $dn/dc_p = (dn/dc)_{prot} = 0.187$ [23], and thus k and k' were determined using a set of water soluble proteins, such as glutamate dehydrogenase, lactate dehydrogenase and enolase [25].

When a membrane protein is used, $dn/dc_p =$

$(dn/dc)_{prot} + \delta(dn/dc)_s$. Thus δ (g/g) can be obtained as follows:

$$\delta = (k'A(\text{Output})_{RI}/(\text{Output})_{UV} - (dn/dc)_{prot})/(dn/dc)_s \quad (2)$$

where $(dn/dc)_s$ is known to be 0.138 [26].

Results

Cytochrome oxidase from the thermophilic bacterium PS3 (10–125 μg) was applied to a TSK-gel G-3000SW column. Typical elution patterns monitored by an ultraviolet meter, an RI meter and an LS meter are shown in Fig. 1. Instrumental constants, k and k' were determined to be 11 220 and 0.318 using glutamate dehydrogenase, lactate dehydrogenase and enolase [25] under the setting condition. By using these values and $A_{280} = 2.2$, the molecular weight of PS3 cytochrome oxidase was determined to be 130 000 for the main peak at 24 min. The sum of four subunits (56–60, 38, 22, 12 kDa) of PS3 cytochrome oxidase was 128–132 kDa [15,16,30], indicating that the enzyme in $C_{12}E_8$ is monomeric. The shoulder at 21.5 min seemed to be due to oligomers of the enzyme, while the second peak, showing a very low peak in the ultraviolet and relatively intense in the RI and LS at 30 min, should be due to mixed micelles of $C_{12}E_8$ and Triton X-100 which contaminated the enzyme preparation according to the purification procedure [16]. The injection of 0.1% solution of authentic Triton X-100 resulted in the appearance of the peaks at the same position (not shown). The other peaks beyond this point were due to the presence of low-molecular-weight substances, because the output of the LS meter was very low, if present at all.

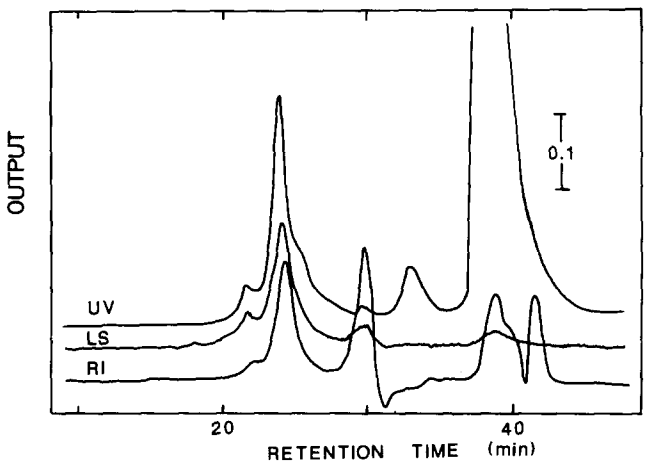

Fig. 1. Chromatograms of PS3 cytochrome oxidase. A small aliquot of PS3 cytochrome oxidase (36 μg protein) was applied to a TSK-gel G3000SWXL column. Elution was monitored by a spectrophotometer (UV), a scattering photometer (LS) and a refractometer (RI). Gain settings were 0.32 for ultraviolet, 16 for LS and 8 for RI. The elution buffer contained 1 mg/ml $C_{12}E_8$, 0.1 M NaCl and 20 mM sodium phosphate buffer (pH 6.8). Flow rate was 0.3 ml/min.

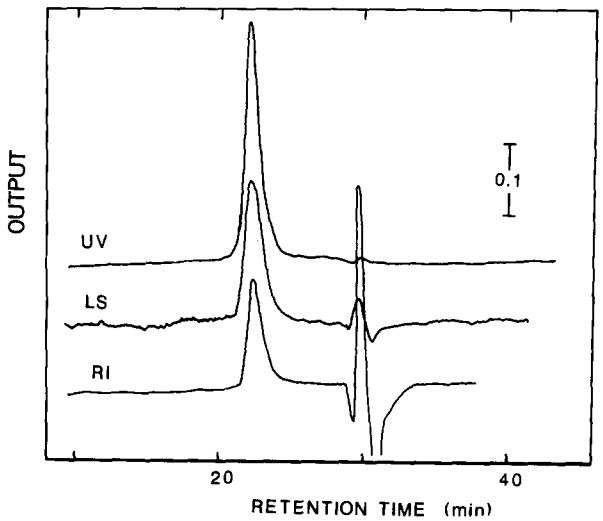

Fig. 2. Chromatograms of PS3 cytochrome bc_1 complex. PS3 bc_1 complex (25 μg) was used. Other conditions were the same as in Fig. 1, except that the gain setting for LS was 32.

Cytochrome bc_1 complex from PS3 (15–50 μg) was also applied to the column, and Fig. 2 shows the elution patterns of a typical result. The first peak at 22.5 min, almost the only peak absorbing ultraviolet light, should be due to the bc_1 complex, while the second peak at 30 min, intense in the output of RI, is due to micelles of $C_{12}E_8$ with MEGA-9. The PS3 bc_1 complex was purified in the presence of MEGA-9 [28]. Since the A value of PS3 cytochrome bc_1 complex was 2.3, the molecular weight of PS3 cytochrome bc_1 complex was determined to be 185 kDa. This molecular mass indicated that the PS3 bc_1 complex is dimeric, just as the mitochondrial enzyme is, since the molecular mass of monomer (a sum of subunit masses 28, 23, 22 and 14 kDa) was 87 kDa [28].

Using Eqn. 2, δ can be calculated. The values for PS3 cytochrome oxidase and bc_1 complex were 1.25 and 0.99, respectively.

Fig. 3 shows the effect of protein concentration on the apparent molecular weight obtained. The plots for cytochrome oxidase was weakly but apparently concentration-dependent, and M_r at the zero protein concentration was determined to be 127 000, although the average value of them was 135 000. On the other hand, no concentration-dependence was observed for the bc_1 complex. The reason for this concentration-dependence of the oxidase is not known at present. Since the $(\text{Output})_{UV}/(\text{Output})_{RI}$ was constant, the possibility that absorption of light at 633 nm due to cytochrome oxidase may interfere the optical measurement can be ruled out. The ratios of $(\text{Output})_{LS}/(\text{Output})_{RI}$ did not vary in the main peaks due to cytochrome oxidase and the bc_1 complex. Thus it is likely that the peaks are composed of the same molecular species, respectively (no micro-

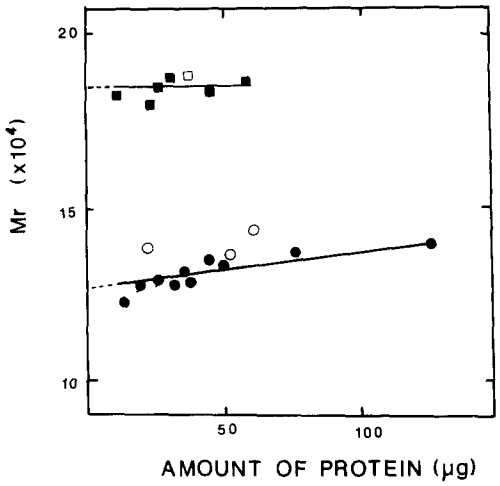

Fig. 3. Effects of amounts of protein added on molecular weights of PS3 cytochrome oxidase (○, ●) and cytochrome bc_1 complex (□, ■). The filled circles and squares showed that the elution was carried out in the standard medium containing 0.1 M NaCl, and the open circles and squares in the medium containing 0.6 M NaCl.

heterogeneity). In Fig. 3 the data obtained in the presence of 0.6 M NaCl are also plotted. Virtually no difference was observed.

The present method was also applied to the bovine heart enzyme, of which conversion of dimer to monomer and M_r and δ values have been reported. Fig. 4A shows elution patterns of bovine heart cytochrome oxidase prepared according to the method of Orii et al. [29]. The main peak at 22 min gave $M_r = 374000$ and $\delta = 0.62$, while the shoulder at 24.5 min gave $M_r = 191000$ and $\delta = 0.70$, when an A value of 2.2 was assumed. The small shoulder in the outputs of ultraviolet or RI, but a prominent peak in the LS output, at the void volume (20 min) showed the presence of a small amount of aggregate. It is known that monomeric enzymes of bovine heart cytochrome oxidase can be induced by putting dimeric enzymes at pH 8.5–9.5 [8,13]. Fig. 4B shows elution patterns of high pH-treated bovine cytochrome oxidase. The main peak was observed at 24.5 min, and practically no peak was seen at the 22 min-dimer region. The peak at 20 min was fairly high in comparison with those of the traces in Fig. 4A, indicating that aggregates were also formed by the treatment. The data of the main peak gave $M_r = 159000$ and $\delta = 0.50$.

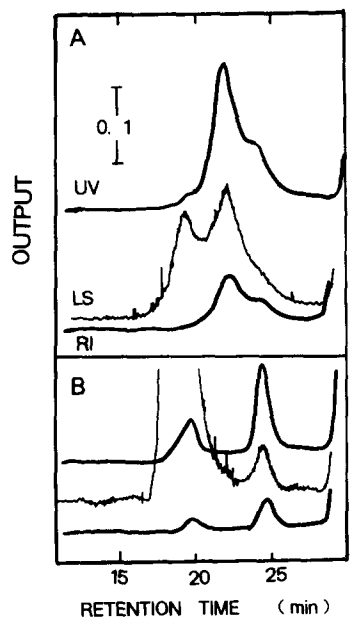

Fig. 4. Chromatograms of bovine heart cytochrome oxidase. The elution conditions were the same as those in Fig. 1. (A) The enzyme [29] was solubilized in 2% $C_{12}E_8$ at 120 min before the injection. (B) pH of the enzyme solution in 2% $C_{12}E_8$ was adjusted to 9.5, and after 30 min the solution was neutralized, and then injected. The flow rate was 0.31 ml/min.

These data clearly indicate that bovine heart cytochrome oxidase (Orii's preparation) is mainly composed of dimers, although small portion of it is monomeric. It was also observed that the enzyme preparation was aged or kept in the presence of Triton X-100, the monomer became gradually dominant (data not shown).

Discussion

Measurements of M_r and δ values of bovine heart cytochrome oxidase have been reported by several research groups using equilibrium centrifugation and/or gel chromatography techniques [6,8–0,12,31]. In the latter method, the amount of bound detergent δ must be monitored independently in addition to a set of suitable marker proteins, whose M_r values are known. As to the bovine enzyme in Triton X-100, $M_r = 385000$ and $\delta = 0.60$ for the dimer enzyme [31], and $M_r = 200000$ and $\delta = 0.63$ [9] were reported, while with the enzyme in laurylmaltoside, $M_r = 400000$ and $\delta = 0.64$ for the dimer [12], and $M_r = 194000$ and $\delta = 0.55$ for the monomer [10], respectively. Our present data of $C_{12}E_8$ coincide well with these data. The conversion phenomenon from dimeric enzyme to monomers reported previously was also confirmed in the present work. Thus the present novel method seems useful for analysis of monomer/dimer conversion of mitochondrial cytochrome oxidase.

The present results have also shown that PS3 cytochrome oxidase exists as a monomer in the detergent solution, while PS3 cytochrome bc_1 complex is dimeric. The optical absorbance in the visible region by these cytochromes did not disturb the measurements when laser-light source at 633 nm and monochromatic light at 660 nm for the LS and RI meters were used, respectively. The apparent molecular weight of 310000 for PS3 cytochrome oxidase, obtained by comparing the retention time with standard proteins [20], is due to the

very hydrophobic nature of the (monomer) enzyme which binds 1.25 times as much detergent ($\delta = 1.25$). Much smaller δ values of 0.55–0.64 g/g and 0.87 g/g have been reported for mitochondrial [10,12,32] and *P. denitrificans* [17] enzymes, respectively. In PS3 bc_1 complex, dimer enzyme ($M_r = 185\,000$) bound the same amount of the surfactant ($\delta = 0.99$).

PS3 cytochrome oxidase showed a good H^+-pumping activity [16,32], and the target size of H^+ pumping activity of the enzyme by high-energy electron radiation was 220–240 kDa, while that of the oxidase activity was 80–100 kDa [27]. This result was interpreted that the monomer enzyme can oxidize cytochrome *c* without H^+ pumping, while dimeric structure is necessary for H^+ pumping [27]. Recently, Finel and Wikström reported that monomeric mitochondrial cytochrome oxidase from bovine heart fractionated by density gradient centrifugation in the presence of cholate, formed a dimer after reconstitution into liposomes with phospholipids, even after resolubilizing with cholate [33,34]. This work, thus, suggests that the monomeric PS3 enzymes solubilized with a surfactant may form dimers in phospholipid bilayer. If so, the target size for H^+ pump activity may be in between the size of the monomer and the dimer, not the value of the dimer itself even if the dimeric enzyme is necessary for H^+-pumping. However, since the irradiation was carried out with the enzyme in highly-oligomeric or aggregated state with small amounts of cholate and Triton X-100 [16], the reassembly of intact monomer enzyme during the reconstitution of proteoliposomes by a freeze-thaw-sonication procedure [27], seemed difficult. This fact may be the reason for the target size of H^+ pump activity to be 220–240 kDa, suggesting that the dimer is necessary for H^+-pumping and re-assembly of monomer enzyme, forming intact dimer and leaving radiation-inactivated monomer behind, does not take place.

Recently Moody et al. showed that partial inhibition of cytochrome oxidase by cyanide does not decrease the H^+/O ratio [35]. As they pointed out, this result does not exclude the possibility that H^+ pumping can only occur in dimers in which even one monomer may be liganded by the inhibitor.

The present study has shown that cytochrome oxidase from PS3 composed of four subunits is monomeric in the presence of $C_{12}E_8$. All bacterial cytochrome oxidases hitherto studied are thus found to be monomeric with a surfactant. At present it is not known what is the cause of this difference between bacterial enzymes and mostly dimeric mitochondrial enzymes. The present technique to obtain the molecular weight and the amount of lipid/detergent binding accurately will be useful for future works. Hayashi et al. have pointed out that conversion of $\alpha\beta$ protomer of Na,K-ATPase to $\alpha_2\beta_2$ dimer may play an important role for energy coupling [24,25].

Acknowledgements

We thank Dr. Orii (Kyoto University) for his kind donation of bovine heart cytochrome *c* oxidase and valuable discussion. This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan to N.S. (61480468 and 62617514). Most parts of this study were carried out during the stay of N.S. at the Institute for Protein Research as a visiting research fellow.

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