Vol. 113, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
June 15, 1983 Pages 575-580

PROTON PUMPING AND OXIDASE ACTIVITY OF THERMOPHILIC CYTOCHROME OXIDASE REMAIN AFTER ITS EXTENSIVE PROTEOLYSIS

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Received May 2, 1983

SUMMARY - A proton-pumping heme aa_3-type cytochrome oxidase purified from the thermophilic bacterium PS3 was treated with trypsin, thermolysin, chymotrypsin, subtilisin, or pronase. The cleavage of the oxidase subunits and the effects of their cleavage on the oxidase activity and proton-pumping in reconstituted vesicles were studied. Trypsin and thermolysin cleaved some of the oxidase subunits without affecting the proton-pumping, but subtilisin and pronase cleaved all the subunits resulting in partial decrease in both activities. Chymotrypsin had an intermediate effect. Subunit II of this enzyme contains heme c which is also cleaved by proteases.

During the last few years cytochrome oxidases (E.C. 1.9.3.1) have been purified from a variety of bacteria (1-8). They have a simple subunit composition and are thus suitable for use in analyzing the physiological roles of the subunits. Cytochrome oxidase from the thermophilic bacterium PS3 (PS3 oxidase) seems to be a good model of eucaryotic oxidases, because it consists of three kinds of subunits which correspond to the major subunits of the eucaryotic oxidases (2), it cross reacts immunologically with yeast oxidase (9), and it shows proton-pumping activity coupled to oxidation when reconstituted into phospholipid vesicles. We examined the topology of the subunits and the mechanism of proton-pumping by treating PS3 oxidase with five different proteases, examining the patterns of cleavage, and measuring the activities of the protease-treated oxidase.

MATERIALS AND METHODS

Materials - Cytochrome oxidase (PS3 oxidase) was purified from the thermophilic bacterium PS3 as described previously (1,2). Yeast cytochrome c (Candida krusei) was purchased from Sankyo Co. (Tokyo, Japan). Trypsin was a product of Boeringer (Mannheim, West Germany) and was treated with TPCK[1] by Dr. M. Yoshida. Thermolysin, alpha-chymotrypsin, subtilisin, and pronase were purchased from Nakarai Chemicals Ltd. (Kyoto, Japan), Boeringer (Mannheim, West Germany), Nagase Co. (Amagasaki, Japan), and Kaken Chemical Co. (Tokyo, Japan), respectively.

1. TPCK: L-1-Tosylamide-2-phenylethyl chloromethyl ketone.

0006-291X/83 $1.50

Proteolysis and reconstitution - Treatment with proteases were carried out at 20 °C. PS3 oxidase (80 µg) was solubilized with 40 µl of 0.5 % octylglucoside and 5 mM Na-Tricine (pH 8.0). To this mixture, 800 ng of trypsin, thermolysin (with 1 mM Ca^{2+} (final)), chymotrypsin, subtilisin, or pronase was added, and after incubation for one hour, the same amount of protease was added again and the reaction mixture was incubated for 16 hours. Then 10 µl of the reaction mixture was taken and mixed with formic acid (1 µl of 10 % solution) and PMSF[2](1 µl of 122 mM ethanolic solution) for SDS-PAGE[3] . For thermolysin treatment 1 µl of 100 mM CDTA[4] was used in place of PMSF. The rest of the reaction mixture was reconstituted into proteoliposomes with 300 µl of soybean phospholipid vesicles by the freeze-thawing method (11). The freeze-thawing-sonication cycle was repeated three times.

Measurement of H^+-translocation and oxidation of ferrocytochrome c - The pH change in the suspension of reconstituted vesicles caused by oxidation of ferrocytochrome c was measured as described in (10) with a Beckmann No. 39505 microelectrode. H^+-translocating activity was defined as the maximum acidification after addition of ferrocytochrome c in the absence of FCCP[5]. Oxidation activity was defined as the initial velocity of alkalinization after addition of ferrocytochrome c in the presence of 300 ng/ml FCCP.

Other methods - SDS-PAGE was carried out as described in (12) except that the acrylamide concentration of the separating gel was 12 % instead of 16 %. Protein was determined by the method of Schaffner and Weissmann (13).

RESULTS

Cleavage of PS3 oxidase by proteases - Solubilized PS3 oxidase can be digested partially or totally depending on the protease used. Figure 1 shows the SDS-PAGE patterns of oxidase samples digested with five different proteases in the presence of 0.5 % octylglucoside. Trypsin digested subunits II and III, thermolysin and chymotrypsin digested subunit III mainly, and subtilisin and pronase digested all three subunits (Plate A). Some proteolytic fragments derived from subunit II could be detected by staining the gel for heme (Plate B) with o-tolidine (14), because PS3 oxidase contains cytochrome c on subunit II (2). Proteolytic fragments with molecular weights exceeding 20,000 can be seen in tracks of even number.

PS3 oxidase solubilized with other detergents can also be cleaved: essentially the same results were obtained with sodium deoxycholate, sodium N-lauroylsarcosinate, Triton X-100, and Emasol 1130, but the products were not suitable for reconstitution studies (data not shown). Proteolysis in

2. PMSF: Phenylmethylsulfonyl fluoride.
3. SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
4. CDTA: trans-1,2-Diaminocyclohexane-N,N,N',N',-tetra-acetic acid.
5. FCCP: Carbonyl cyanide p-trifluoromethoxyphenyl hydrazone.

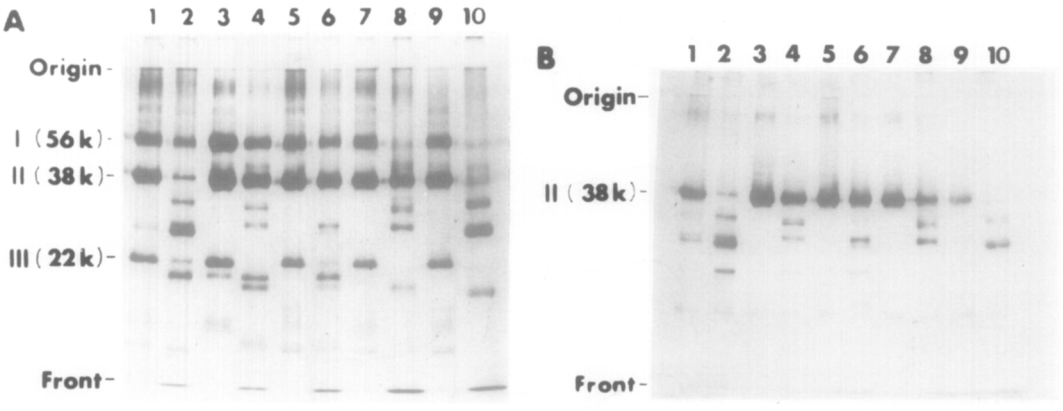

Figure 1. SDS-gel electrophoresis of protease-treated PS3 oxidase - Protease-treated oxidases were prepared and subjected to electrophoresis as described in "Materials and Methods". The gel was stained first for heme with o-tolidine (Plate B), and then the same gel was stained again for protein with Coomassie brilliant blue R250 (Plate A). The tracks of even numbers were protease-treated oxidases and the tracks of odd numbers were zero time controls. The treatments were as follows: tracks 1 and 2, trypsin; tracks 3 and 4, thermolysin; tracks 5 and 6, chymotrypsin; tracks 7 and 8, subtilisin; tracks 9 and 10, pronase. The amount of protein in each track was 15 μg.

the presence of 1 mM dithiothreitol gave the same result as with the oxidized enzyme (in the absence of reductants).

Effects of proteases on the oxidation of ferrocytochrome c and translocation of H^+ by PS3 oxidase - Protease-treated PS3 oxidase was reconstituted to determine the effects of the proteases on two of its activities, oxidation of ferrocytochrome c and translocation of H^+. Figure 2 shows the pH change in the suspension of oxidase vesicles after a ferrocytochrome c pulse. Both H^+-pumping activity and oxidation activity was detected in all vesicles, but the extents of these activities differed from sample to sample (Table 1). After proteolysis with trypsin or thermolysin the two activities were almost intact (Fig. 2, traces B and C), but treatment with subtilisin or pronase lowered the activities significantly (traces E and F). Chymotrypsin had an intermediate effect (trace D). The two activities decreased in parallel, and the initial velocity of acidification decreased after treatment with subtilisin or pronase.

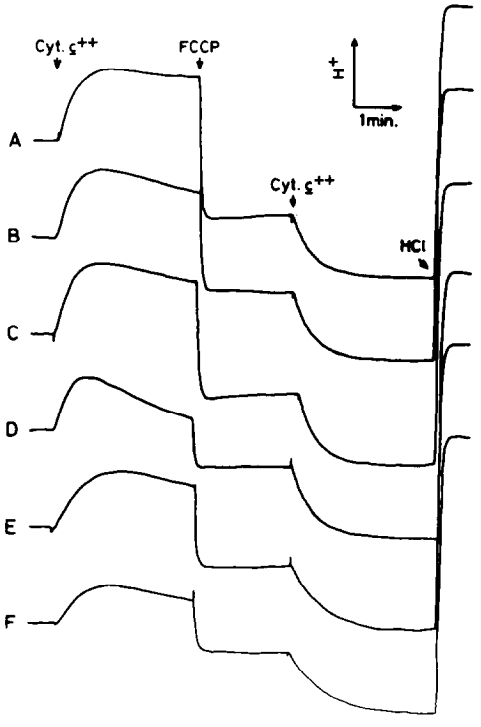

Figure 2. Proton-pumping of protease-treated PS3 oxidase reconstituted into vesicles - Vesicles (50 µl) prepared as described in "Materials and Methods" were suspended in 2 ml of assay medium (0.1 mM K-MOPS* (pH 6.6), 25 mM K_2SO_4, 2.5 mM $MgSO_4$, degassed by boiling). The suspension was mixed with 75 ng of valinomycin (1.5 µl of 50 µg/ml methanolic solution), and incubated for 40 minutes at 30°C under bubbling with argon. Then the pH was measured. The additions indicated by arrows were as follows: cyt c++, ferrocytochrome c (6.29 n mol in 3.5 µl of pH-adjusted assay medium); FCCP, 600 ng (3 µl of 2 ug/ml methanolic solution); HCl, 20 n mol (10 µl of 2mM solution). The treatments were as follows: trace A, none (as control); trace B, trypsin; trace C, thermolysin; trace D, chymotrypsin; trace E, subtilisin; trace F, pronase.
*MOPS: 3-[N-Morpholino]propanesulfonic acid.

DISCUSSION

As shown in this study, proteolysis of PS3 oxidase can be controlled by selection of a suitable protease. Proteases with strict amino acid specificity yield large proteolytic fragments of definite sizes (Fig. 1), and the three subunits of the PS3 oxidase have different susceptibilities to different proteases. Subunit III was digested by all the proteases tested, whereas subunit I was stable against trypsin and subunit II was almost stable against thermolysin and chymotrypsin. These results suggest that subunit III is located on the surface of the PS3 oxidase.

Table 1. - Effect of proteolysis on the oxidation and proton-pumping of PS3 oxidase.

Treatment	Oxidation of cyt.c++ $\frac{\mu \text{ mol cyt.c}^{2+}}{\text{min·mg ox.}^{c}}$	(%)	Ejection of H+ n mol	(%)	H+/e ratio[a,b]	(%)
None[d]	1.50	100.0	5.23	100.0	0.83	100.0
Trypsin	1.47	97.8	4.97	95.0	0.79	95.2
Thermolysin	1.54	102.3	5.02	96.0	0.80	96.4
Chymotrypsin	1.33	88.5	3.99	76.3	0.63	75.9
Subtilsin	0.92	61.4	3.89	74.4	0.62	74.7
Pronase	0.89	59.0	2.78	53.2	0.44	53.0

The values in this table were calculated from Figure 2.
a: Defined as the ratio of the amount of ejected protons to the amount of added ferrocytochrome c.
b: The amount of ferrocytochrome c was calculated from spectroscopic values using $\Delta\varepsilon_{549}$(Red-Ox) = 24.33.
c: PS3 cytochrome oxidase.
d: Control without protease.

Subunit II of this enzyme is unique in containing heme c and in having a molecular weight of 38,000 (2), which is much larger than that of subunit II of human mitochondrial cytochrome oxidase (molecular weight 25,500, 227 residues) (15). Subunit II of PS3 oxidase cross reacts with subunit II of yeast mitochondrial cytochrome oxidase (9). Thus, it may contain some cytochrome c portion that can be cleaved by proteolysis.

PS3 oxidase still acts as a proton pump after treatment with proteases: more than half the proton-pumping activity remained after treatment with subtilisin or pronase, though no intact subunits could be detected on gel (Fig. 1). Comparison of Table 1 with Figure 1 shows that inactivation occurs when all the subunits have been attacked, suggesting that the presence of any intact subunit can maintain the essential quaternary structure in the products of proteolysis.

The parallel between decrease in oxidation rate and decrease in proton-pumping is very interesting and suggests that the active sites for the two activities are close to each other. This is in contrast to the finding that after DCCD-treatment of this enzyme (10) oxidative activity remained

after loss of proton-pumping. This discrepancy shows that DCCD inhibits proton-pumping more specifically than any of the proteases.

These present results show that protease can be used as a probe in studies on the assembly or topology of the subunits of PS3 oxidase. Further extensive studies are still needed on problems, such as the hydrodynamic properties and nature of the protease-resistant sites of the protease-treated oxidase.

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