Differential Permeabilization Effects of Ca^{2+} and Valinomycin on the Inner and Outer Mitochondrial Membranes as Revealed by Proteomics Analysis of Proteins Released from Mitochondria*[S]

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It is well established that cytochrome *c* is released from mitochondria when the permeability transition (PT) of this organelle is induced by Ca^{2+}. Our previous study showed that valinomycin also caused the release of cytochrome *c* from mitochondria but without inducing this PT (Shinohara, Y., Almofti, M. R., Yamamoto, T., Ishida, T., Kita, F., Kanzaki, H., Ohnishi, M., Yamashita, K., Shimizu, S., and Terada, H. (2002) Permeability transition-independent release of mitochondrial cytochrome c induced by valinomycin. *Eur. J. Biochem.* 269, 5224–5230). These results indicate that cytochrome *c* may be released from mitochondria with or without the induction of PT. In the present study, we examined the protein species released from valinomycin- and Ca^{2+}-treated mitochondria by LC-MS/MS analysis. As a result, the proteins located in the intermembrane space were found to be specifically released from valinomycin-treated mitochondria, whereas those in the intermembrane space and in the matrix were released from Ca^{2+}-treated mitochondria. These results were confirmed by Western analysis. Furthermore to examine how the protein release occurred, we examined the correlation between the species of released proteins and those of the abundant proteins in mitochondria. Consequently most of the proteins released from mitochondria treated with either agent were highly expressed proteins in mitochondria, indicating that the release occurred not selectively but in a manner dependent on the concentration of the proteins. Based on these results, the permeabilization effects of Ca^{2+} and valinomycin on the inner and outer mitochondrial membranes are discussed. ***Molecular & Cellular Proteomics 8:1265–1277, 2009.***

Mitochondria are well known as the organelle for energy conversion in all eukaryotes. This energy conversion, *i.e.* ATP synthesis, is performed by using the electrochemical gradient of H^+ across the inner mitochondrial membrane. To enable effective energy conversion, the mitochondrial inner membrane is highly resistant to the permeation of solutes and ions. However, under certain conditions, such as in the presence of Ca^{2+} and inorganic phosphate, the permeability of this inner membrane is known to be markedly increased. This phenomenon is referred to as the permeability transition (PT)[1] and is believed to result from the formation of a proteinaceous pore, referred to as the PT pore, which makes the inner membrane permeable to various solutes and ions smaller than 1.5 kDa (1–3). The physiological importance of the PT has long been uncertain; however, recent studies have revealed that the changes in the permeability of the inner mitochondrial membrane due to the induction of PT cause the release of cytochrome *c* into the cytosol and that the released cytochrome *c* then triggers subsequent steps of programmed cell death, which is known as apoptosis (4–6). Thus, the PT is considered to be one of the major regulatory steps of apoptosis. However, the questions as to how the PT is induced and how cytochrome *c* is released accompanied by the induction of PT have remained unanswered.

To characterize the features of the mitochondrial PT and to understand the mechanism underlying the release of cytochrome *c* from mitochondria, investigators have studied the effects of various agents on this organelle. As a result, the PT and the release of cytochrome *c* were found to be induced not only by Ca^{2+} but also by other agents (7–9). We also found

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[1] The abbreviations used are: PT, permeability transition; AIF, apoptosis-inducing factor; VDAC, voltage-dependent anion channel; CBB, Coomassie Brilliant Blue; DIABLO, direct IAP-binding protein with low pI.

TABLE I
Amino acid sequences of the peptides used for the preparation of antibodies used in this study

Antibody against	Accession[a]	Loc[b]	Amino acid sequence[c]	Region[d]
Adenylate kinase 2	P29410	IMS	TVKQAEMLDDLMDKRKEKLDC	104–123
Adenine nucleotide translocase	Q09073	IM	VQHASKQISAEKQYKGIIDC	38–57
Apoptosis-inducing factor	Q9JM53	IMS	NRMPIARKIIKDGEQHEDLC	582–600
Cytochrome *c*	P62898	IMS	HTVEKGGKHKTGPNLHGLFC	19–37
3-Hydroxymethylglutaryl-CoA synthase 2	P22791	M	NQREQFYHKVNFSPPGDTSNC	465–484
Ornithine carbamoyltransferase	P00481	M	KGYEPDPNIVKLAEQYAKENC	221–240
Sulfite oxidase	Q07116	IMS	SEESYSHWQRRDYKGFSPSVC	389–408
Voltage-dependent anion channel	Q9Z2L0	OM	FQLHTNVNDGTEFGGSIYQKVC	178–198

[a] Accession number used in UniProt.
[b] Location of the protein in mitochondria is shown by the following abbreviations: IM, inner membrane; IMS, intermembrane space; M, matrix; and OM, outer membrane.
[c] Except for adenine nucleotide translocase, C-terminal cysteine residues were artificially introduced to enable conjugation with maleimide-activated hemocyanin.
[d] Regions of individual peptides in the entire proteins are shown.

that copper-*o*-phenanthroline (10), metal ions (11), and cyanine dyes (12, 13) induced this PT and the release of cytochrome *c* from mitochondria. Furthermore we reported that valinomycin, known as a potassium-selective ionophore, also induces the release of cytochrome *c* from mitochondria but without the induction of PT (14). This finding indicated that cytochrome *c* could be released from mitochondria in two different manners: one with the induction of PT and the other without it. To understand how cytochrome *c* is released from mitochondria, it is very important to know what protein species are released from mitochondria concomitant with the release of cytochrome *c*. To address these questions, in the present study we used a mass spectrometry (LC-MS/MS system)-based proteome analysis approach, which allowed us to identify the protein species present in a limited amount of protein samples. Using proteomics techniques, we examined the protein species released from mitochondria treated with valinomycin or with Ca^{2+}, and we discuss our findings on the status of inner and outer mitochondrial membranes treated with these agents.

EXPERIMENTAL PROCEDURES

Materials—Valinomycin (code V-0627) was purchased from Sigma. Maleimide-activated keyhole limpet hemocyanin (code 77606) was obtained from Pierce. The ECL kit (code RPN2106), Pharmalyte (code 17-0456-01), and anti-rabbit IgG conjugated with peroxidase (code NA934) were obtained from GE Healthcare. Polyclonal antibody against Smac/DIABLO (code SA-219) came from BIOMOL Research Laboratories, Inc.

Preparation of Mitochondria from Rat Liver—Liver mitochondria were isolated as described previously (15) with the following modification to purify them. Briefly the liver obtained from normal male Wistar rat was minced in +EDTA medium (250 mM sucrose, 2 mM Tris-Cl, 1 mM EDTA, pH 7.4) and then homogenized at low speed in a chilled Potter-Elvehjem homogenizer. This homogenate was subsequently centrifuged for 5 min at 800 × *g* at 4 °C to remove nuclei, erythrocytes, unbroken liver cells, and debris after which approximately three-quarters of the supernatant was transferred to new tubes and centrifuged under the same conditions. The resulting supernatant was centrifuged for 10 min at about 6800 × *g*. The obtained crude mitochondrial pellet thus obtained was resuspended in +EDTA medium and centrifuged under the same conditions. The resulting pellet was resuspended and centrifuged at 17,400 × *g* for 10 min, and the subsequent pellet was resuspended and centrifuged under the same conditions except that −EDTA medium (250 mM sucrose, 2 mM Tris-Cl, pH 7.4) was used. The protein concentration of the final mitochondrial suspension was determined by the Biuret method with bovine serum albumin used as the standard.

Measurements of Turbidity of Mitochondrial Suspension—The turbidity of mitochondrial suspensions was measured by monitoring the absorbance at 540 nm with a Shimadzu spectrophotometer, model UV-3000.

Transmission Electron Microscopic Analysis of Mitochondrial Configuration—Mitochondrial configurations were analyzed by transmission electron microscopy according to the method reported previously (14).

Preparation of the Protein Samples Released from Mitochondria—Mitochondria were suspended in 22 ml of $+P_i$ medium (200 mM sucrose, 10 mM potassium phosphate buffer, pH 7.4) supplemented with 10 mM succinate (plus 0.5 μg of rotenone/mg of protein) as a respiratory substrate to make their final protein concentration 0.7 mg of protein/ml. After the addition of 2.5 μM valinomycin or 100 μM Ca^{2+}, they were incubated at 25 °C for 4 min and then centrifuged at 17,400 × *g* for 10 min. The obtained supernatants were centrifuged once more. The resulting supernatants were further centrifuged at 230,000 × *g* for 60 min at 4 °C. The proteins present in the supernatants were precipitated by the addition of trichloroacetic acid or acetone. The obtained precipitates were solubilized with 160 μl of 1% SDS.

Preparation of Antibodies—Polyclonal antibodies were raised by injection of synthetic peptides corresponding to parts of various mitochondrial proteins into adult New Zealand White rabbits as described in our previous study (14). The amino acid sequences of the peptides used as immunogens are summarized in Table I. These peptides were conjugated with keyhole limpet hemocyanin, then emulsified with Freund's adjuvant, and injected into the rabbits. Total blood was obtained 7 days after the final booster shot and was kept at room temperature for 1 h and then overnight at 4 °C. The blood clot was removed by centrifugation at 3,000 × *g* for 10 min at 4 °C, and the resulting supernatant was used as antiserum without further purification.

Protein Detection by Western Blotting—Proteins obtained as described above were solubilized in extraction buffer (12.5 mM Tris, pH 6.8, containing 1% (w/v) SDS, 10% (w/v) glycerol, 1% (w/v) dithiothreitol, and 0.05% (w/v) bromphenol blue). SDS-PAGE was performed in 10% (w/v) and 20% (w/v) acrylamide gels essentially as described previously (14). After transfer of proteins in the gel to

nitrocellulose membranes, the membranes were soaked for 1 h in TS buffer (20 mM sodium phosphate buffer, pH 7.4, containing 0.05% (w/v) Tween 20, 150 mM NaCl, and 5% (w/v) skim milk). The blocked membranes were then incubated with antibodies against individual mitochondrial proteins for 1 h. Antibodies against adenylate kinase 2, adenine nucleotide translocase, cytochrome *c*, 3-hydroxymethylglutaryl-CoA synthase 2, ornithine carbamoyltransferase, and sulfite oxidase were used at a 1000-fold dilution, and those against Smac/DIABLO and voltage-dependent anion channel were used at an 800- and 5000-fold dilution, respectively, with TS buffer containing 5% (w/v) skim milk as the diluent. After washing with TS buffer, the membranes were incubated with secondary antibody (anti-rabbit IgG conjugated with peroxidase; 2000-fold diluted with TS buffer) for 1 h. Specific binding of antibodies was visualized by the use of ECL reagents and subsequent exposure to x-ray film (Fuji Photo Film).

Mass Spectrometry and Protein Identification—After SDS-PAGE, discrete protein bands were excised from the CBB-stained gel. In-gel digestion with trypsin was carried out as described previously (16). Then to facilitate the extraction of digested peptides from the gel pieces, the gel pieces were first swelled by the addition of 70 μl of 25 mM NH_4HCO_3 and then shrunk by addition of 210 μl of acetonitrile. Subsequently a similar course of the extraction of digested peptides by swelling/shrinkage of gel pieces was performed in 5% (v/v) formic acid and acetonitrile, and the obtained supernatants were collected. After the tryptic peptides had been air-dried, they were resuspended in 5% acetonitrile, 0.1% formic acid and injected into a CapLC system (Waters). They were first preconcentrated on a 300-μm $\times$ 5-mm C_{18} PepMap100 precolumn (LC Packings) and then separated for 40 min at a flow rate of 200 nl/min on a 75-μm $\times$ 15-cm C_{18} PepMap100 column (LC Packings) with a linear gradient of 5–60% acetonitrile containing 0.1% formic acid. The eluted peptides were subjected to mass spectrum analysis using a Q-TOF Ultima instrument (MicroMass) directly coupled to a nano-LC system. Spectrum analysis was performed by using the survey scanning mode of MassLynx 4.0 (Waters). In this mode, each spectrum was scanned for less than 5 s. For the selection condition of the precursors in the MS/MS analysis, a mode enabling repeated selection of certain precursor ions was used. Other parameters were set at default values. The obtained peak lists were subjected to a database search using the MASCOT server 1.905 (Matrix Science, London, UK) and a UniProt database (release date, January 9, 2007; 252,616 sequences and 92,372,123 residues). Search parameters were as follow: *Rattus* species, trypsin specificity, one allowed missed cleavage, carbamidomethylation fixed modification, methionine oxidation variable modification, precursor ion mass tolerance of 1.0 Da, and fragment ion mass tolerance of $\pm$0.67 Da.

In these processes, if a certain protein was identified by more than two peptides showing ion scores higher than 23, which is a statistically significant ion score (expectation value, $p < 0.05$), this result was cited without further manual validation. However, if a protein was identified by only one peptide showing a score higher than 23, the MASCOT suggestion was manually inspected, and at least three consecutive y- or b-ions with a significant signal-to-background ratio were required for the identification. The intracellular locations of individual proteins were determined from their annotations in UniProt or based on the published literature.

RESULTS

Preparation of Protein Samples Released from Mitochondria—For preparation of samples of proteins released from mitochondria, the use of highly purified and functionally intact mitochondria is essential. Thus, we adopted the more stringent conditions of differential centrifugation for isolation of mitochondria from rat liver as described under "Experimental Procedures." Our previous study using mitochondria prepared by the general protocol showed that valinomycin and Ca^{2+} caused a decrease in the turbidity of mitochondrial suspensions, but the membrane structures of the treated mitochondria were markedly different between the two treatments (14). Thus, we first examined whether the mitochondria prepared under the above higher stringency condition of centrifugation showed the reported response to valinomycin and Ca^{2+}. As shown in Fig. 1, when 2.5 μM valinomycin was added to mitochondria, the turbidity of the mitochondrial suspension decreased. The addition of 100 μM Ca^{2+} also caused a decrease in the turbidity of mitochondrial suspensions that was more substantial than that caused by 2.5 μM valinomycin. The decrease in turbidity induced by Ca^{2+} was suppressed by 1 μM cyclosporin A, which is known to be a specific inhibitor of the PT (data not shown). When the configurations of mitochondria treated with valinomycin or with Ca^{2+} were examined by transmission electron microscopy (Fig. 2), no massive swelling or complete disappearance of the inner membrane structure was observed with valinomycin-treated mitochondria, whereas Ca^{2+} caused massive swelling and disappearance of the inner membrane structure, which are generally observed in PT-induced mitochondria (7, 10–14). Thus, we concluded that the mitochondria prepared in the present study showed essentially the same properties as those used in the previous study (14).

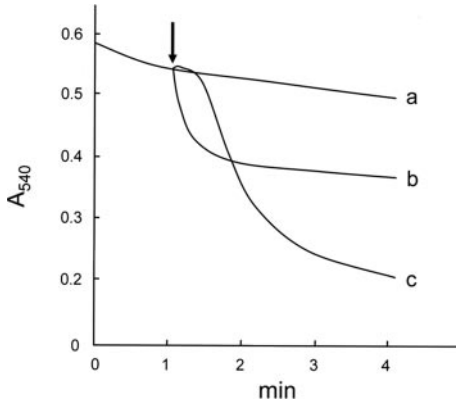

FIG. 1. **Effects of valinomycin and Ca^{2+} on the turbidity of mitochondrial suspensions.** Time courses of the turbidity change in non-treated mitochondrial suspension (trace *a*) and suspensions of mitochondria treated with 2.5 μM valinomycin (trace *b*) or with 100 μM Ca^{2+} (trace *c*) are shown. Mitochondria were suspended to make a final protein concentration of 0.7 mg/ml in $+P_i$ medium supplemented with 10 mM succinate (plus 0.5 μg of rotenone/mg of protein) as a respiratory substrate, and absorbance of this suspension at 540 nm was monitored. After incubation at 25 °C for 1 min, each agent was then added to the mitochondrial suspension at the time indicated by the *arrow*. A typical result of three independent runs is shown.

To prepare samples of the proteins released from mitochondria, we first centrifuged mitochondria treated with 100 μM Ca^{2+} or with 2.5 μM valinomycin under the standard conditions (15,000 $\times$ *g* for 10 min at 4 °C). However, the

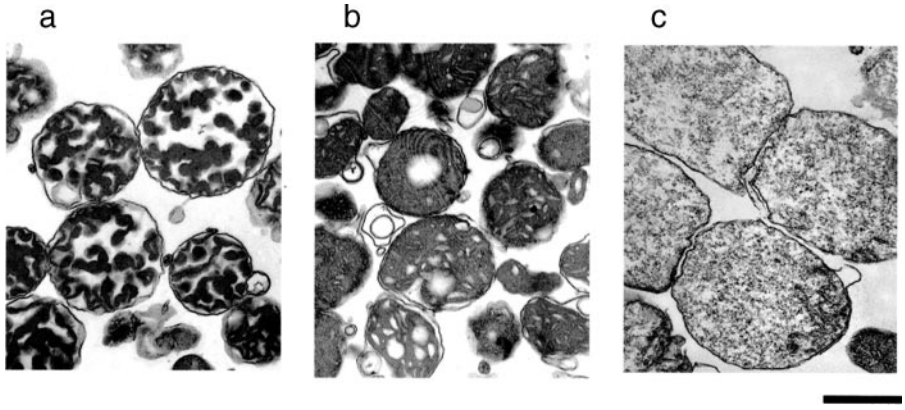

FIG. 2. **Effects of valinomycin and Ca^{2+} on mitochondrial configuration.** The transmission electron microscopic appearance of non-treated mitochondria (*a*), valinomycin-treated mitochondria (*b*), and Ca^{2+}-treated mitochondria (*c*) is shown. After treatment of mitochondria as described in the legend of Fig. 1, the mitochondria were promptly harvested and subjected to the fixation processes. The *bar under* photograph "*c*" indicates 1 μm. Of ~30 randomly selected sections observed for each condition, typical images are shown.

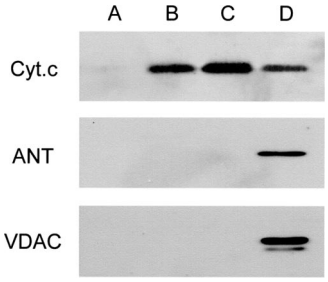

FIG. 3. **Quality check of the prepared protein samples by immunoblotting against various proteins.** For *lanes A–D*, supernatants (15 μl/lane) of non-treated, valinomycin-treated, and Ca^{2+}-treated mitochondria or whole proteins of rat liver mitochondria (5 μg/lane), respectively, were subjected to SDS-PAGE. After separation, the proteins were transferred to nitrocellulose membranes. Membranes thus prepared were first blocked with medium containing 5% skim milk and then incubated with specific antibodies against cytochrome *c* (*Cyt.c*), adenine nucleotide translocase (*ANT*), or VDAC. The immunoreactive proteins bands were visualized by using the ECL kit. A typical result of three independent runs is shown.

supernatant thus obtained was not applicable for subsequent analysis due to remarkable contamination by non-pelleted mitochondria (data not shown). To settle this issue of contamination, we applied high speed centrifugation (230,000 $\times$ *g* for 1 h at 4 °C) to the reaction mixtures. With this application, contamination of the supernatant with whole mitochondria was significantly reduced (data not shown). To examine whether the supernatant samples prepared by this procedure were suitable for subsequent analyses, we subjected these samples to Western blotting (Figs. 2 and 3) using antibodies against cytochrome *c*, adenine nucleotide translocase, and voltage-dependent anion channel (VDAC). None of these proteins were detected in the supernatant of non-treated mitochondria (*lane A*). In the supernatant of valinomycin-treated and Ca^{2+}-treated mitochondria (*lanes B* and *C*, respectively), cytochrome *c*, which is known to be released from mitochondria treated with these agents, was detected, whereas neither adenine nucleotide translocase nor VDAC, which are both located in the inner and outer membranes, was detected. The doublet bands of VDAC observed with whole mitochondrial protein reflect its isoforms (17). The results of this immunoassay showed that these supernatant samples were suitable as samples containing proteins released from mitochondria.

Identification by LC-MS/MS Analysis of the Proteins Released from Valinomycin- and Ca^{2+}-treated Mitochondria—Next to elucidate the protein species present in the supernatant samples, we subjected these samples to SDS-PAGE followed by CBB staining (Fig. 4). SDS-PAGE was performed by using 10 and 20% polyacrylamide gels containing SDS to enable high resolution analysis of proteins covering a wide range of molecular weights. Although their staining intensities with CBB were faint, several protein bands were still detected in the samples of the supernatant of non-treated mitochondria; these bands were considered to be proteins from mitochondria broken during the preparation and from non-mitochondrial compartments (*lane A*). The band pattern of the supernatant prepared from valinomycin-treated mitochondria was similar to that from the non-treated mitochondria, whereas that from the Ca^{2+}-treated mitochondria was apparently different (*lanes B* and *C*, respectively). For identification of the bands of proteins specifically released from mitochondria by valinomycin or Ca^{2+}, the band densities in individual lanes were measured by using an image analyzer, and differential analysis against the densitogram for the non-treated mitochondria was performed. The differential densitograms, obtained by the subtraction of the densitogram of *lane A* from that of *lane B* or *C*, are shown at the *right margins* of *lanes B* and *C* in Fig. 4. From these densitograms, protein bands 1–3 in *lane B* (B1–3) and 1–14 in *lane C* (C1–14) were identified as those showing higher signal intensity in valinomycin- and Ca^{2+}-treated mitochondria, respectively, than in non-treated mitochondria. These bands identified here were considered to contain the proteins released from mitochondria treated with each agent. The bands were cut out, the proteins present in each band were subjected to in-gel digestion with trypsin, and then the peptide mixtures obtained were analyzed by LC-MS/MS followed by a database search. In addition, the gel pieces in *lane A* corresponding to bands B1–3 and C1–14 were also cut out and analyzed as the background. Besides the above described bands, all of the visible bands were also analyzed as shown in supplemental Fig. S1 and Tables SI and SII.

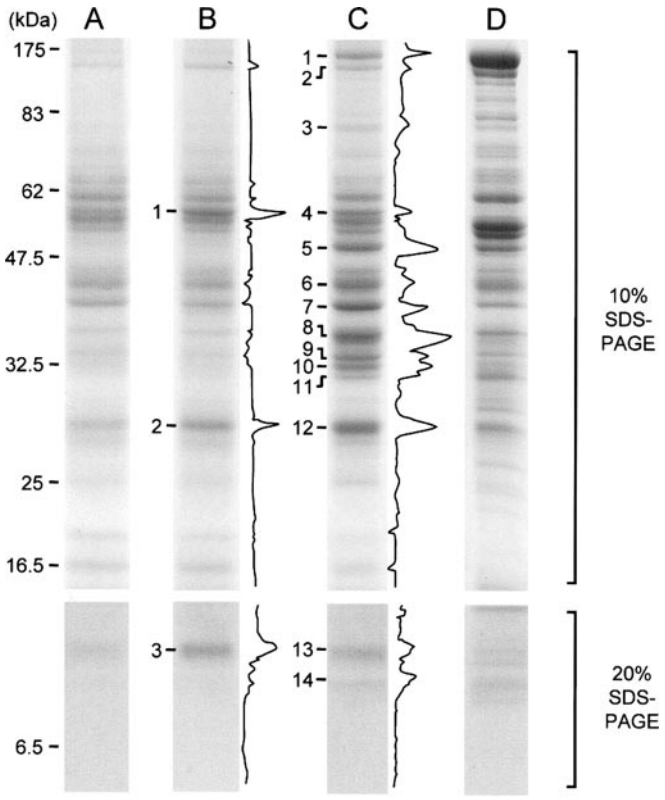

FIG. 4. **SDS-PAGE analysis of proteins present in the supernatants of treated and non-treated mitochondria.** For *lanes A–D*, supernatants (15 μl/lane) of non-treated, valinomycin-treated, and Ca^{2+}-treated mitochondria or whole proteins of rat liver mitochondria (5 μg/lane), respectively, were subjected to SDS-PAGE. *Upper* and *lower* photographs represent the results of SDS-PAGE using 10 or 20% acrylamide gel, respectively. *Numbers* shown at the *left margin* of *lane A* represent the size of proteins used as molecular mass markers and their migration position. Densitograms shown at the *right margins* of *lanes B* and *C* represent the differential densitograms obtained by the subtraction of the densitogram of *lane A* from that of *lane B* or *lane C*. The protein bands in *lanes B* and *C numbered* at their *left margins* were analyzed by LC-MS/MS. A typical result of three independent runs is shown.

As a result of LC-MS/MS analysis of protein bands B1–3, multiple proteins were identified for individual protein bands (Table II). As features of the detected protein species, the following three points are noteworthy. First, all of the identified proteins were water-soluble proteins, and no membrane proteins were included. Second, the molecular masses of the identified proteins were almost consistent with those expected from mobilities of the analyzed protein bands with the following few exceptions. For example, carbamoyl-phosphate synthase (166 kDa) was identified in band B1, showing the mobility of ~60 kDa; however, this band was assumed to be that of the degraded protein because this protein has been reported to be detectable as multiple fragments in immunoblotting analysis of rat liver mitochondria (18). Hydroxymethylglutaryl-CoA synthase (57 kDa) identified in band B2, showing the mobility of ~30 kDa, was also considered to have been detected at that position for the same reason. Third, non-mitochondrial proteins such as hemoglobin or fatty acid-binding protein were observed in band B3. The reason for these ectopic proteins in this fraction is uncertain.

We next determined the protein species specifically released from mitochondria by valinomycin treatment by comparing protein species identified from each protein band detected in the supernatant of valinomycin-treated mitochondria with those from the corresponding band in non-treated mitochondria. The rationale used for this determination is as follows. If the content of the peptides derived from the specific proteins in a certain gel piece of valinomycin-treated mitochondria was much higher than that in the non-treated mitochondria, those proteins could be considered as proteins selectively released from mitochondria. On the contrary, if that in valinomycin-treated mitochondria was similar to that in non-treated mitochondria, all proteins identified in the band could be considered to be unselectively released from mitochondria. As shown in the results of the LC-MS/MS analysis of each band (Table II), the proportions of the number of the peptides derived from sulfite oxidase, adenylate kinase 2, and cytochrome *c* to the total number of the peptides detected in bands B1–3 of valinomycin-treated mitochondria were 70, 56, and 46%, respectively (shown with underlines in Table II), which are about 2 times higher than the corresponding data for non-treated mitochondria (38, 29, and 24%, respectively). Therefore, we concluded that the protein species of sulfite oxidase, adenylate kinase 2, and cytochrome *c* were specifically released from mitochondria by valinomycin. This conclusion on the specific release of cytochrome *c* by valinomycin accords well with the result of the immunological analysis shown in Fig. 3. Sulfite oxidase, adenylate kinase 2, and cytochrome *c* are all known to be located in the mitochondrial intermembrane space, suggesting that valinomycin caused the release of proteins residing there.

Multiple protein species were also identified in the protein bands of Ca^{2+}-treated mitochondria (Table III). Similar to the protein samples of valinomycin-treated mitochondria, the molecular weights of most of the identified proteins of Ca^{2+}-treated mitochondria were consistent with the mobility of the analyzed bands. Non-mitochondrial hemoglobin and fatty acid-binding protein were also detected in band C13. To clarify the proteins specifically released from mitochondria by Ca^{2+}, the proportions of peptides derived from each protein to all peptides detected in each band were compared with those in the corresponding gel pieces for the non-treated mitochondria. As a result, unlike the bands for the valinomycin-treated mitochondria, in most of the protein bands except for bands C4, C6, and C13, the proportions of peptides derived from each protein were not markedly different from those in the corresponding gel pieces for the non-treated mitochondria. Therefore, Ca^{2+} was concluded to cause the nonspecific release of all identified mitochondrial proteins in these protein bands. These protein species included not only proteins lo-

TABLE II

LC-MS/MS analysis of proteins released from valinomycin-treated mitochondria

Protein species present in bands B1–3 (supernatant of valinomycin-treated mitochondria) were determined by LC-MS/MS analysis. Those present in the gel pieces in *lane A* (supernatant of non-treated mitochondria) corresponding to the positions of bands B1–3 were also determined as backgrounds and are shown in the column labeled "Non-treated." AC, UniProt accession number.

Band no. in *lane B*	Identified protein				Number of detected peptides[c] (%)[d]	
	Name	AC	Molecular mass[a]	Loc[b]	Non-treated	Valinomycin-treated
1	Sulfite oxidase	Q07116	55	IMS	18 (38)	14 (70)[e]
	Dihydrolipoyl dehydrogenase	Q6P6R2	55	M	12 (25)	0 (0)
	Glutamate dehydrogenase 1	P10860	62	M	10 (21)	3 (15)
	Carbamoyl-phosphate synthase	P07756	166	M	8 (17)	3 (15)
2	Enoyl-CoA hydratase	P14604	32	IMS	15 (39)	7 (26)
	Adenylate kinase isoenzyme 2	P29410	27	M	11 (29)	15 (56)[e]
	Electron transfer flavoprotein subunit β	Q68FU3	28	M	4 (11)	4 (15)
	3,2-*trans*-Enoyl-CoA isomerase	P23965	32	M	3 (6)	1 (3)
	Hydroxymethylglutaryl-CoA synthase	P22791	57	M	2 (5)	0 (0)
3	Ribonuclease UK114	P52759	14	Mt, Cy	4 (24)	4 (15)
	Cytochrome *c*	P62898	12	IMS	4 (24)	12 (46)[e]
	Hemoglobin subunit β -1	P02091	12	Bl	3 (18)	3 (12)
	Fatty acid-binding protein, liver	P02692	12	Cy	3 (18)	7 (27)
	Hemoglobin subunit α	P01946	12	Bl	3 (18)	0 (0)

[a] In kDa.

[b] Location of the individual proteins is shown by following abbreviations: Bl, red blood cell; Cy, cytosol; IMS, intermembrane space of mitochondria; M, mitochondrial matrix; Mt, whole mitochondria.

[c] Number of peptides derived from each protein identified by LC-MS/MS analysis.

[d] Relative proportions of the peptide number derived from the protein to the total number of the peptides detected in each protein band are shown.

[e] Peptides (their number and proportion to the total number of peptides) showing predominantly higher proportions in valinomycin-treated mitochondria than in the non-treated mitochondria are underlined.

cated in the intermembrane space such as adenylate kinase 2 (band C12) but also those situated in the matrix such as malate dehydrogenase (band C9), electron transfer flavoprotein subunit α (band C11), and enoyl-CoA hydratase (band C12).

In protein bands C4 and C13 of Ca^{2+}-treated mitochondria, the proportions of peptides derived from sulfite oxidase and cytochrome *c* were dominantly higher than those in non-treated mitochondria, respectively; and hence these two protein species were considered to be released from the Ca^{2+}-treated mitochondria. As for the reason why the content of sulfite oxidase in band C4 was much higher for Ca^{2+}-treated mitochondria than for non-treated mitochondria, we offer the following interpretation: Ca^{2+} treatment increases the permeability of the outer mitochondrial membrane even for proteins of the size of sulfite oxidase (50 kDa) but increases that of the inner mitochondrial membrane only for proteins smaller than sulfite oxidase. These differential effects of Ca^{2+} on the permeability of outer and inner mitochondrial membrane may result in the specific release of sulfite oxidase in band C4. We consider this interpretation to be reasonable because the staining intensities of protein bands smaller than this size in *lane C* in Fig. 4 were much stronger than those in *lane D*, but those of protein bands larger than this size in *lane C* were much weaker than those in *lane D*. As for the specific increase in cytochrome *c* in band C13 in Ca^{2+}-treated mitochondria, high contents of non-mitochondrial proteins such as hemoglobin or fatty acid-binding protein in this fraction may be one of the major reasons causing the observed results. Other visible protein bands in the case of Ca^{2+}-treated mitochondria showing signal intensities similar to those of the corresponding gel pieces in non-treated mitochondria mainly contained non-mitochondrial proteins such as catalase and Cu,Zn-superoxide dismutase, which were suspected to be contaminants arising during the preparation of the mitochondria (supplemental Tables SI and SII). Consequently our data suggest that, unlike valinomycin, Ca^{2+} causes the release of not only proteins located in the intermembrane space (*e.g.* adenylate kinase 2) but also those residing in the matrix (*e.g.* malate dehydrogenase).

Immunological Confirmation of the Protein Release from Valinomycin- and Ca^{2+}-treated Mitochondria—To reconfirm the results obtained from the above proteomics analysis, we also carried out immunological analysis. For this, we first raised antibodies against sulfite oxidase, adenylate kinase 2, hydroxymethylglutaryl-CoA synthase, and ornithine carbamoyltransferase: the former two are located in the intermembrane space, and the latter two are located in the matrix. These four proteins were the main proteins identified by LC-MS/MS analysis of bands B1, B2, C5, and C8, respectively. We used these antibodies for Western blot analysis of the supernatant samples. As shown in Fig. 5, adenylate kinase 2 and sulfite oxidase were detected in the supernatant prepared from mitochondria treated with valinomycin or with Ca^{2+}, whereas ornithine carbamoyltransferase and hydroxymethylglutaryl-CoA synthase were detected in that from the or-

TABLE III

LC-MS/MS analysis of the proteins released from Ca^{2+}-treated mitochondria

Protein species present in bands C1–14 (supernatant of Ca^{2+}-treated mitochondria) were determined by LC-MS/MS analysis. Those present in the gel pieces in *lane A* (supernatant of non-treated mitochondria) corresponding to the positions of bands C1–14 were also determined as backgrounds and are shown in the column designated as "Non-treated." AC, UniProt accession number; PH, pleckstrin homology.

Band no. in *lane C*	Identified protein				Number of detected peptides[c] (%)[d]	
	Name	AC	Molecular mass[a]	Loc[b]	Non-treated	Ca^{2+}-treated
1	Carbamoyl-phosphate synthase	P07756	166	M	31 (65)	61 (94)
	Aspartate aminotransferase	P00507	48	M	7 (15)	0 (0)
	Catalase	P04762	60	Pr	4 (8)	1 (2)
	Aldehyde dehydrogenase	P11884	57	M	2 (4)	0 (0)
	Hydroxymethylglutaryl-CoA synthase	P22791	57	Mt	2 (4)	1 (2)
	Malate dehydrogenase	P04636	36	M	0 (0)	1 (2)
	Thiosulfate sulfurtransferase	P24329	33	M	2 (4)	0 (0)
2	Carbamoyl-phosphate synthase	P07756	166	M	50 (84)	58 (93)
	Pyruvate carboxylase	P52873	130	M	5 (8)	0 (0)
	Bile acid CoA:amino acid *N*-acyltransferase	Q63276	47	Pr	3 (5)	1 (2)
	Catalase	P04762	60	Pr	2 (3)	3 (5)
3	Carbamoyl-phosphate synthase	P07756	166	M	18 (60)	22 (42)
	Dimethylglycine dehydrogenase	Q63342	96	Mt	6 (20)	14 (26)
	Aspartate aminotransferase	P00507	48	M	4 (13)	1 (2)
	Aconitate hydratase	Q9ER34	86	Mt	2 (7)	16 (30)
4	Sulfite oxidase	Q07116	55	IMS	18 (38)	11 (69)
	Dihydrolipoyl dehydrogenase	Q63342	55	M	12 (25)	2 (13)
	Glutamate dehydrogenase 1	P10860	62	M	10 (21)	0 (0)
	Carbamoyl-phosphate synthase	P07756	166	M	8 (17)	1 (6)
	Hydroxymethylglutaryl-CoA synthase	P22791	57	M	0 (0)	1 (6)
	Methylmalonate-semialdehyde dehydrogenase	Q02253	58	M	0 (0)	1 (6)
5	Hydroxymethylglutaryl-CoA synthase	P22791	57	M	24 (58)	18 (100)
	Carbamoyl-phosphate synthase	P07756	166	M	6 (15)	0 (0)
	Fumarate hydratase	P14408	55	Mt	5 (12)	0 (0)
	Acyl-coenzyme A oxidase 1	P07872	75	Pr	4 (10)	0 (0)
	Retinoid-inducible serine carboxypeptidase	Q920A6	51	S	2 (5)	0 (0)
6	Long chain-specific acyl-CoA dehydrogenase	P15650	48	M	23 (37)	11 (35)
	3-Ketoacyl-CoA thiolase	P13437	42	Mt	16 (25)	12 (39)
	Acetyl-CoA acetyltransferase	P17764	45	Mt	7 (11)	1 (3)
	Aspartate aminotransferase	P00507	48	M	5 (8)	5 (17)
	Medium chain-specific acyl-CoA dehydrogenase	P08503	47	M	5 (8)	1 (3)
	Cathepsin D	P24268	45	Ly	4 (6)	1 (3)
	Fumarylacetoacetase	P25093	46	?	3 (5)	0 (0)
7	Aspartate aminotransferase	P00507	48	M	32 (51)	37 (86)
	3-Ketoacyl-CoA thiolase A	P13437	44	Pr	11 (17)	0 (0)
	Short chain-specific acyl-CoA dehydrogenase	P15651	45	M	10 (16)	3 (7)
	α-Methylacyl-CoA racemase	P70473	42	Pr, Mt	6 (10)	3 (7)
	Cathepsin D	P24268	45	Ly	2 (3)	0 (0)
	PH domain leucine-rich repeat protein phosphatase	Q9WTR8	186	Cy	1 (2)	0 (0)
	Thiosulfate sulfurtransferase	P24329	33	M	1 (2)	0 (0)
8	Ornithine carbamoyltransferase	P00481	40	M	21 (50)	23 (64)
	Thiosulfate sulfurtransferase	P24329	33	M	13 (31)	13 (36)
	Arginase-1	P07824	35	Cy	4 (10)	0 (0)
	Carbamoyl-phosphate synthase	P07756	166	M	3 (7)	0 (0)
	Malate dehydrogenase	P04636	36	M	3 (7)	0 (0)
	Glyceraldehyde-3-phosphate dehydrogenase	P04797	36	Cy	1 (2)	0 (0)
9	Malate dehydrogenase	P04636	36	M	22 (58)	34 (76)
	Hydroxyacyl-CoA dehydrogenase	P22791	35	M	8 (21)	5 (11)
	L-Lactate dehydrogenase A chain	P04642	37	Cy	4 (11)	0 (0)
	Thiosulfate sulfurtransferase	P24329	33	M	3 (8)	0 (0)
	Cathepsin Z	NP_899159[e]	35	Ly	1 (3)	1 (2)
	Peroxisomal *trans*-2-enoyl-CoA reductase	Q9WVK3	33	Pr	0 (0)	3 (7)
10	Hydroxyacyl-Co A dehydrogenase	P22791	35	M	16 (52)	9 (40)
	Electron transfer flavoprotein subunit α	P13803	35	M	9 (29)	4 (17)
	Malate dehydrogenase	P04636	36	M	2 (6)	0 (0)
	3-Mercaptopyruvate sulfurtransferase	P97532	33	Cy, Mt	2 (6)	1 (4)
	Cathepsin Z	NP_899159[e]	35	Ly	1 (3)	1 (4)
	Thiosulfate sulfurtransferase	P24329	33	M	1 (3)	0 (0)
	Peroxisomal *trans*-2-enoyl-CoA reductase	Q9WVK3	33	Pr	0 (0)	4 (17)

TABLE III—*continued*

Band no. in *lane C*	Identified protein				Number of detected peptides[c] (%)[d]	
	Name	AC	Molecular mass[a]	Loc[b]	Non-treated	Ca^{2+}-treated
	Hydroxymethylglutaryl-CoA synthase	P22791	57	M	0 (0)	2 (9)
	Hydroxymethylglutaryl-CoA lyase	P97519	35	M	0 (0)	1 (4)
11	Electron transfer flavoprotein subunit aα	P13803	35	M	15 (26)	20 (40)
	3-Mercaptopyruvate sulfurtransferase	P97532	33	Cy, Mt	11 (19)	10 (20)
	Hydroxyacyl-Co A dehydrogenase	P22791	35	M	9 (16)	3 (6)
	2,4-Dienoyl-CoA reductase	Q64591	36	Mt	7 (12)	7 (14)
	$\Delta^{3,5}$-$\Delta^{2,4}$-Dienoyl-CoA isomerase	Q62651	36	Mt, Cy	5 (9)	1 (2)
	Thiosulfate sulfurtransferase	P24329	33	M	5 (9)	2 (4)
	Betaine-homocysteine *S*-methyltransferase	O09171	45	Cy	3 (5)	0 (0)
	Retinoid-inducible serine carboxypeptidase	Q920A6	51	S	2 (3)	0 (0)
	Cathepsin Z	NP_899159[e]	35	Ly	1 (2)	0 (0)
	3-Hydroxyisobutyrate dehydrogenase	P29266	36	Mt	0 (0)	5 (10)
	Hydroxymethylglutaryl-CoA synthase	P22791	57	M	0 (0)	2 (4)
12	Enoyl-CoA hydratase	P14604	32	M	15 (43)	14 (26)
	Adenylate kinase isoenzyme 2	P29410	27	IMS	11 (31)	17 (32)
	Electron transfer flavoprotein subunit β	Q68FU3	28	M	4 (11)	10 (19)
	Hydroxymethylglutaryl-CoA synthase	P22791	57	M	2 (6)	0 (0)
	3,2-*trans*-Enoyl-CoA isomerase	P23965	32	M	3 (9)	11 (21)
	Adenylate kinase isoenzyme 4	Q9WUS0	25	M	0 (0)	1 (2)
13	Ribonuclease UK114	P52759	14	Mt, Cy	4 (24)	6 (20)
	Cytochrome *c*	P62898	12	IMS	4 (24)	13 (45)
	Hemoglobin subunit β -1	P02091	12	Bl	3 (18)	2 (7)
	Fatty acid-binding protein, liver	P02692	12	Cy	3 (18)	8 (28)
	Hemoglobin subunit α	P01946	12	Bl	3 (18)	0 (0)
14	10-kDa heat shock protein	P26772	11	M	6 (75)	14 (83)
	Betaine-homocysteine *S*-methyltransferase	O09171	45	Cy	2 (25)	2 (11)

[a] In kDa.

[b] Location of the individual proteins is shown by the following abbreviations: Bl, Red blood cell; Cy, cytosol; IMS, intermembrane space; Ly, lysosome; M, matrix; Mt, mitochondria; Pr, peroxisome; ?, unknown.

[c] Number of peptides derived from each protein identified by LC-MS/MS analysis.

[d] Relative proportions of the peptide number derived from the protein to the total number of the peptides detected in each protein band are shown.

[e] NCBI accession number.

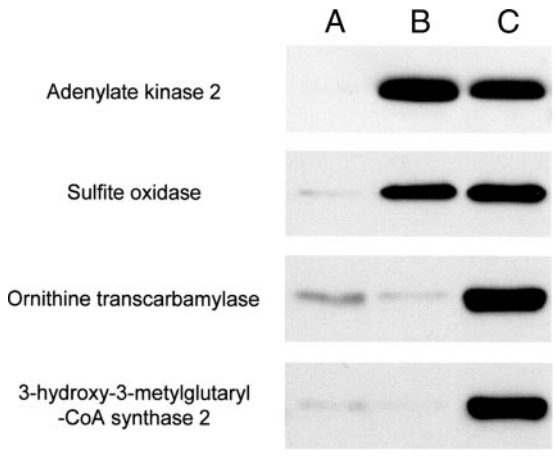

FIG. 5. **Immunoblot confirmation of release of proteins from mitochondria.** *Lanes A–C* represent the supernatants of mitochondria used in the experiments whose results are given in Fig. 3 or 4 (15 μl/lane). Proteins in these samples were subjected to immunoblotting under the same experimental conditions as indicated in the legend of Fig. 3. Adenylate kinase 2, sulfite oxidase, ornithine carbamoyltransferase, and 3-hydroxymethylglutaryl-CoA synthase 2 proteins present in individual samples were detected with their specific antibodies. A typical result of three independent runs is shown.

ganelles treated with Ca^{2+} but not in that from those treated with valinomycin. These results obtained by immunological analysis accord well with those found by our proteomics analysis.

Analysis of the Selectivity of the Protein Release from Mitochondria—To understand how proteins are released from mitochondria by valinomycin or Ca^{2+} treatment, it is important to reveal whether proteins are released from mitochondria in a selective manner or in a non-selective manner. We next tried to elucidate this problem by using proteome analysis. If the protein release occurs in an unselective manner, the released proteins would be those abundantly present in mitochondria because they would be expected to leak from mitochondria according to their expression or concentration levels. Conversely if the protein release occurs in a selective manner, the released proteins would not always necessarily be highly expressed proteins. To examine these possibilities, we first examined the abundantly expressed proteins in mitochondria. For this, all proteins of rat liver mitochondria were subjected to SDS-PAGE (Fig. 4, *lane D*), and then the gel was cut into pieces of ~5-mm vertical thickness. The proteins included in these gel pieces were analyzed by using LC-MS/

MS. The proteins identified here by LC-MS/MS can be roughly considered to be those abundantly expressed in mitochondria because the efficiency of identification of a protein by LC-MS/MS is generally considered to correlate coarsely with the amount of the protein. Table IV shows the 111 species of proteins identified by LC-MS/MS analysis of whole mitochondrial proteins. Mann and co-workers (19) identified ~700 species of proteins in the proteome analysis of mitochondria isolated from rat various tissues. Thus, the proteins described in Table IV can be regarded as a part of all of the mitochondrial proteins and may be considered to be abundantly expressed in rat liver mitochondria. Actually the proteins known to be abundantly expressed in the liver mitochondria, such as carbamoyl-phosphate synthase 1 (18), ATP synthase subunit (20), phosphate carrier (21), and voltage-dependent anion channel 1 (17), appear in Table IV. We next examined whether the proteins revealed to be released from valinomycin- and Ca^{2+}-treated mitochondria were also present in Table IV. As a result, 92% of the proteins released from mitochondria by valinomycin or by Ca^{2+} were present in Table IV. Therefore, only the protein species abundantly present in mitochondria were found to be released by valinomycin or Ca^{2+} treatment, and so we may conclude that the release of these proteins from valinomycin- and Ca^{2+}-treated mitochondria occurred in an unselective manner.

DISCUSSION

In this study, we performed proteome analysis of the proteins released from valinomycin- and Ca^{2+}-treated mitochondria. Until now, there was no report on proteomics studies on the proteins released from valinomycin-treated mitochondria. On the contrary, several studies reported the results of proteome analysis of the proteins released from PT-induced mitochondria. Patterson *et al.* (22) performed such an analysis of the proteins released from mitochondria with PT induced by atractyloside. Different from the results of our present study on the proteins released from mitochondria with PT induced by Ca^{2+} in which no membrane protein was detected as stated above, they detected three inner membrane proteins such as electron transfer flavoprotein α-subunit precursor in the released proteins. As was shown in our Fig. 4, the supernatant of non-treated mitochondria also showed numerous proteins, and complete elimination of proteins reflecting this background was difficult. Nevertheless Patterson *et al.* (22) simply applied the supernatant of mitochondria treated with atractyloside to the LC-MS/MS analysis without taking the background into account. For this reason, the samples used as the proteins released from mitochondria in their study seemed to be contaminated by a number of non-released proteins. To identify the proteins specifically released from PT-induced mitochondria, differential analysis between samples prepared from PT-induced mitochondria and from non-treated mitochondria, as performed in this study, is essential. Recently to eliminate the background proteins, Teilum *et al.* (23) prepared the released protein samples by using mitochondria immobilized on a sponge-like material, cryogel monoliths. As a result of the proteome analysis of thus prepared samples, they showed 68 proteins released from Ca^{2+}-treated mitochondria. Of these, 30 proteins were also identified in the present study; however, two important proteins, *i.e.* adenylate kinase 2 and sulfite oxidase, which were released from either valinomycin-treated or Ca^{2+}-treated mitochondria, were not identified in their study. The exact reason why these two proteins were not identified in their study is uncertain. One possible explanation for this discrepancy is that immobilization of mitochondria on the cryogel monoliths affects the property of the samples. Further careful examinations are necessary to understand the reason causing such differences. The two above cited studies were performed to enable effective screening of novel apoptosis-inducible proteins and to develop a novel method to prepare released protein samples, respectively, and selectivity of proteins released from mitochondria was not discussed.

In this study, we demonstrated that valinomycin caused the specific release of proteins located in the intermembrane space in an unselective manner. This result agrees with that of a previous study showing immunologically that not only cytochrome *c* but also adenylate kinase 2 is released from valinomycin-treated mitochondria (24). The exact mechanisms governing how these proteins located in the intermembrane space were released are unclear. However, valinomycin is thought to cause the specific rupture of the outer membrane at least under the experimental conditions used because the intermembrane space proteins having high molecular mass, such as sulfite oxidase (55 kDa), were also released.

Moreover we also demonstrated that treatment of mitochondria with Ca^{2+} caused the release of proteins located not only in the intermembrane space but also in the matrix. This finding indicates that both outer membrane and inner membranes are ruptured in Ca^{2+}-treated mitochondria. Possible rupture of the outer mitochondrial membrane accompanied by induction of PT has been proposed by multiple researchers (8, 25, 26). These conclusions were mainly made based on morphological analysis of PT-induced mitochondria by electron microscopy or tomography (27–29). In the present study, during the configuration analysis of Ca^{2+}-treated mitochondria by electron microscopy (Fig. 2), we also observed mitochondria with a ruptured outer membrane (data not shown). Thus, rupture of the outer mitochondrial membrane in PT-induced mitochondria was confirmed by both proteomics and structural analyses.

On the contrary, rupture of the inner mitochondrial membrane in PT-induced mitochondria has hardly ever been reported. Pfeiffer and co-workers (30) reported that the electrophoresis pattern of the proteins released from Ca^{2+}-treated mitochondria was similar to that of the soluble protein fraction of mitochondria. This result may indicate the release of matrix proteins from PT-induced mitochondria, but analysis of these

TABLE IV

LC-MS/MS profile of proteins expressed in mitochondria

The whole mitochondrial proteins obtained from gel pieces cut out from Fig. 4, *lane D* were subjected to LC-MS/MS analysis.

Protein name	Ac[a]	Molecular mass[b]	Loc[c]	Release[d]
Carbamoyl-phosphate synthetase 1	P07756	166	M	Ca^{2+}
Pyruvate carboxylase	P52873	130	M	
2-Oxoglutarate dehydrogenase E1 component	Q5XI78	117	M	
Ladybird homeobox corepressor 1	P84551	101	N	
Dimethylglycine dehydrogenase	Q63342	96	Mt	
Glycerol-3-phosphate acyltransferase	P97564	95	OM	
Aconitate hydratase	Q9ER34	86	Mt	
Trifunctional enzyme subunit α	Q64428	83	M	
NADH-ubiquinone oxidoreductase 75-kDa subunit	Q66HF1	80	IM	
Peroxisomal multifunctional enzyme type 2	P97852	80	Pr	
Long-chain fatty acid CoA ligase 1	P18163	79	OM	
Peroxisomal bifunctional	P07896	79	Pr	
Carnitine O-palmitoyltransferase 2	P18886	75	IM	
Stress-70 protein	P48721	74	Mt	
Chaperone-activity of bc1 complex-like	Q5BJQ0	73	Mt	
Succinate dehydrogenase [ubiquinone] flavoprotein subunit	Q920L2	73	IM	
Very long chain-specific acyl-CoA dehydrogenase	P45953	71	IM	
Electron transfer flavoprotein-ubiquinone oxidoreductase	Q6UPE1	69	IM	
Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex	P08461	68	M	
Apoptosis-inducing factor 1	Q9JM53	67	IMS	
Carboxylesterase 3	NP_579829[e]	62	ER	
Glutamate dehydrogenase 1	P10860	62	M	Ca^{2+}
60-kDa heat shock protein	P63039	61	M	
ATP synthase subunit α	P15999	60	IM	
Catalase	P04762	60	Pr	
UDP-glucuronosyltransferase 1-1	Q64550	60	Mic	
Amine oxidase (flavin-containing) B	P19643	59	OM	
Propionyl-CoA carboxylase β chain	P07633	59	M	
Seryl-tRNA synthetase, cytoplasmic	Q6P799	59	Cy	
Alanine-glyoxylate aminotransferase 2	Q64565	58	Mt	
Methylmalonate-semialdehyde dehydrogenase	Q02253	58	M	Ca^{2+}
Aldehyde dehydrogenase	P11884	57	M	
Hydroxymethylglutaryl-CoA synthase	P22791	57	M	Ca^{2+}
Protein-disulfide isomerase	P04785	57	ER	
4-Aminobutyrate aminotransferase	P50554	57	M	
Amyloid β A4 precursor protein binding family B member 3	O35827	56	?	
ATP synthase subunit β	P10719	56	IM	
Dihydrolipoyl dehydrogenase	Q6P6R2	55	M	Ca^{2+}
Fumarate hydratase	P14408	55	Mt, Cy	
Neural Wiskott-Aldrich syndrome protein	O08816	55	Cy, N	
Sulfite oxidase	Q07116	55	IMS	Ca^{2+}, val
Ubiquinol-cytochrome-*c* reductase complex core protein 1	Q68FY0	54	IM	
Trifunctional enzyme subunit β	Q60587	52	M	
Acyl-CoA thioesterase 2	O55171	50	M	
2-Oxoisovalerate dehydrogenase subunit α	P11960	50	M	
Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex	Q01205	49	Mt	
Ornithine aminotransferase	P04182	49	M	
Aspartate aminotransferase	P00507	48	M	Ca^{2+}
Kynurenine/α-aminoadipate aminotransferase	Q64602	48	Mt	
Long chain-specific acyl-CoA dehydrogenase	P15650	48	M	
Short/branched chain-specific acyl-CoA dehydrogenase	P70584	48	M	
Ubiquinol-cytochrome-*c* reductase complex core protein 2	P32551	48	IM	
Bile acid CoA:amino acid *N*-acyltransferase	Q63276	47	Pr	
Isovaleryl-CoA dehydrogenase	P12007	47	M	
Medium chain-specific acyl-CoA dehydrogenase	P08503	47	M	Ca^{2+}
Acetyl-CoA acetyltransferase	P17764	45	Mt	Ca^{2+}
Betaine-homocysteine *S*-methyltransferase	O09171	45	Cy	
Short-chain specific acyl-CoA dehydrogenase	P15651	45	M	Ca^{2+}

TABLE IV—*continued*

Protein name	Ac[a]	Molecular mass[b]	Loc[c]	Release[d]
α-Methylacyl-CoA racemase	P70473	42	Mt, Pr	Ca^{2+}
3β-Hydroxysteroid dehydrogenase type 5	P27364	42	ER	
3-Ketoacyl-CoA thiolase	P13437	42	Mt	Ca^{2+}
NADH dehydrogenase (ubiquinone) 1 α subcomplex subunit 10	Q561S0	41	M	
Ornithine carbamoyltransferase	P00481	40	M	Ca^{2+}
Phosphate carrier	P16036	40	IM	
D-β-Hydroxybutyrate dehydrogenase	P29147	39	M	
Proto-oncogene tyrosine-protein kinase FER	P09760	37	?	
$\Delta^{3,5}$-$\Delta^{2,4}$-Dienoyl-CoA isomerase	Q62651	36	Mt, Pr	Ca^{2+}
Malate dehydrogenase	P04636	36	M	Ca^{2+}
Sideroflexin-1	Q63965	36	Mt membr	
2,4-Dienoyl-CoA reductase	Q64591	36	Mt	Ca^{2+}
3-Hydroxyisobutyrate dehydrogenase	P29266	36	Mt	Ca^{2+}
Electron transfer flavoprotein subunit α	P13803	35	M	Ca^{2+}
Hydroxyacyl-CoA dehydrogenase	Q9WVK7	35	M	Ca^{2+}
Hydroxymethylglutaryl-CoA lyase	P97519	35	M	Ca^{2+}
Uricase	P09118	35	Pr	
Tricarboxylate transport protein	P32089	34	IM	
Carnitine/acylcarnitine carrier	P97521	33	IM	
Peroxisomal *trans*-2-enoyl-CoA reductase	Q9WVK3	33	Pr	Ca^{2+}
Prohibitin-2	Q5XIH7	33	IM, Cy, N	
Thiosulfate sulfurtransferase	P24329	33	M	Ca^{2+}
3-Mercaptopyruvate sulfurtransferase	P97532	33	Mt, Cy	Ca^{2+}
Enoyl-CoA hydratase	P14604	32	M	Ca^{2+}
3,2-*trans*-Enoyl-CoA isomerase	P23965	32	M	Ca^{2+}
Voltage-dependent anion channel 1	Q9Z2L0	31	OM	
Voltage-dependent anion channel 3	Q9R1Z0	31	OM	
ATP synthase γ chain	P35435	30	IM	
Prohibitin	P67779	30	IM	
Ubiquinol-cytochrome-*c* reductase iron-sulfur subunit	NP_001008888[e]	30	IM	
ATP synthase B chain	P19511	29	IM	
Electron transfer flavoprotein subunit β	Q68FU3	28	M	Ca^{2+}
ES1 protein homolog	P56571	28	Mt	
NADH dehydrogenase (ubiquinone) flavoprotein 2	P19234	28	IM	
Adenylate kinase isoenzyme 2	P29410	27	IMS	Ca^{2+}, val
3-Hydroxyacyl-CoA dehydrogenase type-2	O70351	27	Mt	
Cytochrome *c* oxidase subunit 2	P00406	26	IM	
Serum amyloid P component	P23680	26	S	Ca^{2+}
Aldehyde dehydrogenase family 7 member A1	Q64057	25	?	
Glutathione *S*-transferase Kappa 1	P24473	25	M	
Superoxide dismutase (manganese)	P07895	25	M	
ATP synthase O subunit	Q06647	23	IM	
Peptidyl-prolyl *cis-trans* isomerase	P29117	22	M	Ca^{2+}
Cytochrome *c* oxidase subunit 4 isoform 1	P10888	20	IM	
ATP synthase D chain	P31399	19	IM	
ATP synthase δ chain	P35434	18	IM	
Succinate dehydrogenase (ubiquinone) iron-sulfur protein	P21913	17	IM	
Cytochrome *c* oxidase subunit 5A	P11240	16	IM	
Cytochrome *c* oxidase subunit 5B	P12075	14	IM	
Ribonuclease UK114	P52759	14	Mt, Cy, N	
Cytochrome *c*	P62898	12	IMS	Ca^{2+}, val
ATP synthase e chain	P29419	8	IM	
ATP synthase protein 8	P11608	8	IM	

[a] Accession number from UniProt.

[b] In kDa.

[c] Location of the individual proteins is shown by the following abbreviations: Cy, cytosol; ER, endoplasmic reticulum; IM, inner membrane; IMS, intermembrane space; M, matrix; Mic, microsome; Mt, mitochondria; Mt membr, mitochondrial membrane; N, nuclei; OM, outer membrane; Pr, peroxisome; S, secretase; ?, unknown.

[d] The proteins revealed to be released from valinomycin-treated or Ca^{2+}-treated mitochondria are shown as val and Ca^{2+}, respectively.

[e] NCBI accession number.

protein bands at the molecular level was not performed. In another report, possible rupture of the inner membrane of PT-induced mitochondria was proposed (31), but no experimental data supporting this statement were presented. In the present study, by using proteomics and immunological techniques, we showed that the proteins located in the matrix space could also be released by induction of PT. By the increased permeability of the mitochondrial inner membrane caused by Ca^{2+}, proteins smaller than 50 kDa seemed to be easily released as evident from the staining intensities of the released proteins (Fig. 4*C*) and from the results showing the specific release of sulfite oxidase (band C4 in Table III).

It has been well established that the PT pore allows the mitochondrial inner membrane to be permeable to solutes or ions smaller than 1.5 kDa. This contradicts with our finding that matrix proteins bigger than 1.5 kDa such as ornithine carbamoyltransferase (40 kDa) were released from Ca^{2+}-treated mitochondria. This contradiction may be interpreted as follows. The inner membrane of the Ca^{2+}-treated mitochondria becomes permeable to solutes or ions smaller than 1.5 kDa by opening of the PT pores, and the inner membrane of some of these mitochondria becomes permeable to macromolecules probably by rupture following PT. Up to now, the mitochondrial apoptosis inducers have been considered to be present in the intermembrane space. However, the finding that matrix proteins are also released from PT-induced mitochondria suggests that the novel apoptosis inducers may be present not only in the intermembrane space but also in the mitochondrial matrix.

In this study, apoptosis-inducible proteins besides cytochrome *c*, such as caspase 9, AIF, Smac/DIABLO, endonuclease G, and Omi/HtrA2 (32–37), were not identified in the supernatants by proteomics analysis, but Smac/DIABLO was detected in the supernatant of valinomycin- or Ca^{2+}-treated mitochondria by immunological analysis (supplemental Fig. S2). The main reason for the difficulty in observation of these proteins is possibly their low abundance. Actually proteins other than AIF were not observed by the proteome analysis of the whole mitochondrial proteins (Table IV). AIF is considered to be expressed more abundantly than the other apoptotic proteins (Table IV); however, it was not observed in the supernatants of Ca^{2+}- or valinomycin-treated mitochondria (Tables II and III), indicating that AIF is not released from valinomycin- or Ca^{2+}-treated mitochondria. Results on the localization of AIF in valinomycin-treated mitochondria are in accord with the results reported by Gogvadze *et al.* (24). On the contrary, two studies reported the release of AIF from PT-induced mitochondria by Ca^{2+} (38, 39). The exact reason for the discrepant behaviors of AIF in PT-induced mitochondria is uncertain. Possibly its distribution in PT-induced mitochondria may significantly dependent upon the experimental conditions used because AIF is also reported to be anchored to the inner mitochondrial membrane (40, 41).

In summary, we performed proteome analysis of the proteins released from valinomycin- and Ca^{2+}-treated mitochondria. As a result, we succeeded in demonstrating differences in protein release from mitochondria treated with valinomycin or with Ca^{2+}, suggesting differential permeabilization effects of Ca^{2+} and valinomycin on the inner and outer mitochondrial membranes. These findings are important to understand how the release of mitochondrial proteins is achieved.

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