Vol. 107, No. 1, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1982 Pages 258-264

EVIDENCE FOR PARTICIPATION OF THE INNER MEMBRANE IN THE IMPORT OF SULFITE OXIDASE INTO THE INTERMEMBRANE SPACE OF LIVER MITOCHONDRIA

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Received June 3, 1982

SUMMARY: Sulfite oxidase, a soluble enzyme in mitochondrial intermembrane space, was synthesized as a precursor protein larger than the authentic enzyme when rat liver RNA was translated in vitro using reticulocyte lysate. When the in vitro translation products were incubated with isolated rat liver mitochondria, the precursor of sulfite oxidase was converted to the size of the mature enzyme. The in vitro processed mature enzyme was no longer susceptible to externally added proteases and was extractable by a hypotonic treatment of the mitochondria, suggesting its location in the intermembrane space. When mitochondria were subfractionated, most of the processing activity was recovered in the mitoplast fraction. The import-processing activity of mitochondria was inhibited by CCCP, oligomycin, or atractyloside in the presence of KCN. These results suggest that the import of sulfite oxidase into mitochondrial intermembrane space requires the participation of inner membrane.

Most mitochondrial proteins are synthesized on cytoplasmic ribosomes as precursors, which are larger than the corresponding mature proteins found in mitochondria, and then imported post-translationally into mitochondria (1,2). Recently, we reported that hepatic sulfite oxidase, a soluble enzyme located in the space between the inner and outer membrane of mitochondria (3-5), is mainly synthesized by free ribosomes in the cytoplasm (6). In vivo label-chase experiments of the enzyme in the rat liver demonstrated the presence of a larger precursor form with an extra-peptide of about 3,000 dalton in the cytosol, which was then incorporated into mitochondria to be converted to the mature protein (7).

In this paper, we report in vitro import and processing of the precursor form of sulfite oxidase into the intermembrane space of rat liver mitochondria using a reticulocyte lysate translation system.

Abbreviations: SDS, sodium dodecyl sulfate; IgG, immunoglobulin G; SO, mature sulfite oxidase; pSO, putative precursor of sulfite oxidase; CCCP, carbonyl cyanide m-chlorophenylhydrazone.

0006-291X/82/130258-07$01.00/0

MATERIALS AND METHODS

Animals —— Male Sprague-Dawley rats weighing about 200 g were used.

Preparation of ^{3}H-Labeled Sulfite Oxidase and Antibody against Sulfite Oxidase —— Sulfite oxidase was purified according to the method of Johnson and Rajagopalan (8) and the purified enzyme was labeled with ^{3}H-$NaBH_4$ by the method of Tack et al. (9). Antibody against sulfite oxidase (anti-sulfite oxidase IgG) was prepared by immunizing a rabbit with purified enzyme as described (6).

Cell-free Protein Synthesis —— Total RNA was prepared from rat livers by SDS-phenol extraction (10) and translated in a nuclease-treated rabbit reticulocyte lysate system with [^{35}S]methionine (about 200 μCi/ml) for 90 min at 30°C as described (10).

In Vitro Import and Processing —— The translation mixture was adjusted to 0.48 M mannitol and incubated with 1 mg/ml (final concentration) of rat liver mitochondria for 60 min at 30°C. After centrifugation at 10,000 x g for 5 min, the pellet was dissolved in Tris-HCl buffer (pH 7.6) containing 0.9 % NaCl, 1 % Triton X-100, 10 mM EDTA and 0.15 % SDS, and the supernatant was also added with the same buffer to dissociate peptides.

Immunoprecipitation —— The samples were added with anti-sulfite oxidase IgG and incubated for 30 min at 30°C. Formaldehyde-fixed Staphylococcus aureus cells (100 μl of a 10 % w/v suspension) were then added, and incubation was continued for 60 min at 30°C. The cells were washed and immunoadsorbed sulfite oxidase was extracted in 2 % SDS containing 10 % 2-mercaptoethanol.

Subfractionation of Mitochondria —— Mitochondria were prepared from rat liver by the method of Schnaitman et al. (11) without bovine serum albumin. Mitochondria were subfractionated into outer membrane, intermembrane space, and mitoplast fractions by the hypotonic treatment (12). Mitoplasts were suspended in 220 mM mannitol containing 70 mM sucrose and 10 mM Hepes-KOH buffer (pH 7.6), disrupted by sonication, and centrifuged at 105,000 x g for 60 min. The pellet and the supernatant were used as inner membrane fraction and matrix fraction, respectively.

Other Methods —— 7.5 % Polyacrylamide slab gel electrophoresis in the presence of 0.2 % SDS (13) and fluorography (14) were performed as described. Protein was determined by the method of Lowry et al. (15) using bovine serum albumin as a standard.

Enzyme Assay —— Sulfite oxidase was assayed as described by Cohen and Fridovich (16), monoamine oxidase as described by Kraml et al. (17), succinate-cytochrome c reductase as described by Stotz (18), glutamate dehydrogenase as described by Arnold (19).

Materials —— [^{35}S]Methionine (>800 Ci/mmole) was obtained from the Commissariat a L'Energie Atomique, France. Sodium boro[^{3}H]hydride was from The Radiochemical Centre, Amersham, England.

RESULTS

In Vitro Synthesis of Putative Precursor of Sulfite Oxidase —— Total RNA of rat liver was translated in a messenger RNA-dependent reticulocyte lysate system in the presence of [^{35}S]methionine and sulfite oxidase synthesized was immunoprecipitated by its antibody (Fig. 1). As expected from the previous findings (7), sulfite oxidase was synthesized as a larger precursor whose apparent molecular weight was about 3,000 dalton larger than the mature enzyme. Control experiments, in which the antibody was replaced by non-immune IgG or a large excess of unlabeled mature enzyme was added to compete with

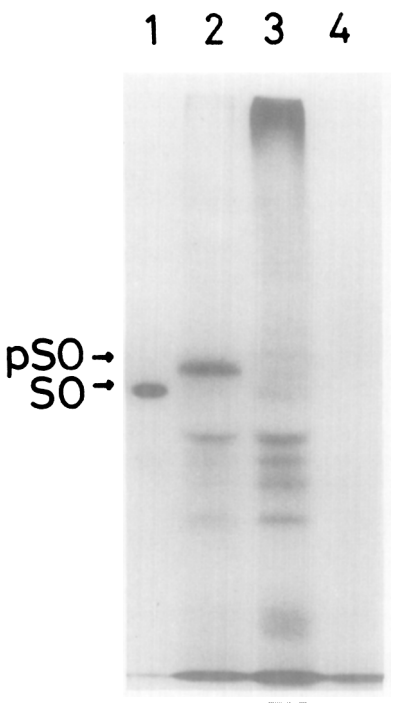

Fig. 1. RNA-dependent In Vitro Synthesis of Precursor of Sulfite Oxidase by Rabbit Reticulocyte Lysate. Cell-free translation was performed with total RNA isolated from rat liver. Immunoprecipitable products recovered with 40 μg of anti-sulfite oxidase IgG in the absence (lane 2) or presence (lane 3) of 40 μg of purified sulfite oxidase were analyzed on 7.5 % polyacrylamide gel electrophoresis in the presence of 0.2 % SDS followed by fluorography. Lane 4 shows the result of experiment in which IgG obtained from non-immune rabbit was used. Lane 1 shows the position of mature sulfite oxidase.

the radioactive precursor, gave no radioactive band at the expected position, confirming that the larger polypeptide was a precursor of sulfite oxidase subunit (pre-sulfite oxidase).

Import and Processing of Pre-Sulfite Oxidase into Mitochondria —— When the translation mixture containing pre-sulfite oxidase was incubated with rat liver mitochondria for 60 min at 30°C and then the mitochondria were pelleted by centrifugation, most of the radioactivity of pre-sulfite oxidase was recovered in the mitochondria, and that the radioactivity was found at the position of the mature subunit of the enzyme in SDS-gel electrophoresis (Fig. 2). The processed product was resistant to trypsin added externally, whereas pre-sulfite oxidase was completely degraded by the treatment. To check further if the processed product was imported into the intermembrane space, mitochondria were suspended in a hypotonic medium (10 mM Tris-HCl

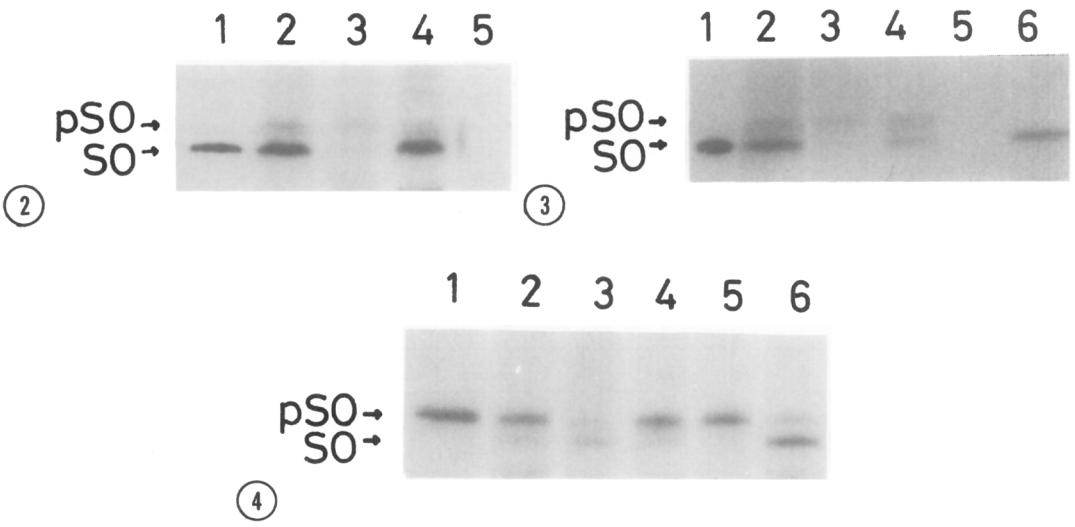

Fig. 2. Import of Pre-Sulfite Oxidase into Mitochondria. Mitochondria were incubated for 60 min at 30°C with in vitro translation products of total hepatic RNA. The suspension was then incubated in the absence or presence of trypsin (final concentration; 50 μg/ml) at 0°C for 15 min. Lane 1, mature sulfite oxidase; Lane2, sulfite oxidase recovered in mitochondria; Lane 3, sulfite oxidase recovered in supernatant; Lane 4, sulfite oxidase recovered in mitochondria after protease-treatment; Lane 5, supernatant after protease treatment.

Fig. 3. Location of Sulfite Oxidase Incorporated into Mitochondria. Mitochondria were incubated with in vitro translation products for 60 min at 30°C and then isolated by centrifugation at 10,000 x g for 5 min. The isolated mitochondria were subfractionated into outer membrane, intermembrane space, and mitoplast fractions by hypotonic treatment and ultracentifugation. Lane 1, mature sulfite oxidase; Lane 2, sulfite oxidase recovered in mitochondria; Lane 3, sulfite oxidase recovered in supernatant; Lane 4, sulfite oxidase recovered in mitoplast; Lane 5, outer membrane; Lane 6, sulfite oxidase recovered in the intermembrane space.

Fig. 4. Submitochondrial Distribution of Processing Activity of Pre-Sulfite Oxidase. Translation products were incubated for 60 min at 30°C with 100 μg each of submitochondrial fractions as follows. Lane 1, none; Lane2, outer membrane; Lane 3, intermembrane space; Lane 4, inner membrane; Lane 5, matrix; Lane 6, mitoplast. After incubation, immunoprecipitation was performed and the immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography.

buffer, pH 7.6) and the suspension was centrifuged at 105,000 x g to separate solubilized intermembrane space proteins. As shown in Fig. 3, the processed product was exclusively recovered in the supernatant.

Submitochondrial Location of the Processing Activity —— Rat Liver mitochondria were fractionated into five subfractions by hypotonic treatment and sonication. Monoamine oxidase, sulfite oxidase, succinate-cytochrome c reductase, and glutamate dehydrogenase were assayed in each submitochondrial fraction as marker enzyme, and each fraction showed the highest specific activity of the marker enzyme (data not shown). Figure 4 shows the sub-

Table I. Energy-dependent Incorporation of Pre-sulfite Oxidase into Isolated Mitochondria. Rat liver mitochondria (350 μg protein) were incubated with in vitro translation products (2×10^6 dpm) in the presence of uncouplers or respiratory inhibitors at 30°C for 60 min. After centrifugation at 10,000 x g for 5 min, sulfite oxidase peptides in mitochondria and supernatant were separated by immunoprecipitation as described in Materials and Methods, and analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. The fluorograph was quantitated with a densitometer. The bands of sulfite oxidase and pre-sulfite oxidase in mitochondria and supernatant were summed and the sum was taken as 100 %. (Mt, Mitochondria; Sup, Supernatant.)

	None		Oligomycin (50 μM)		Atractyloside (50 μM)		CCCP (50 μM)	
	Mt	Sup	Mt	Sup	Mt	Sup	Mt	Sup
Precursor (%)	15.5	8.6	38.5	13.2	37.8	12.6	65.4	7.3
Mature protein (%)	75.9	0	48.2	0	49.6	0	27.3	0

mitochondrial localization of the processing activity. Most of the activity was found in the mitoplast fraction, whereas little or no activity was recovered in the matrix fraction and in the inner membrane vesicles which were possibly inverted by sonication. Some activity was observed in the intermembrane space fraction. These results suggested that the processing activity was located mainly on the outside surface of inner mitochondrial membranes.

Energy Dependency of Import-Processing Activity —— Effect of various uncouplers and respiratory inhibitors on the import and processing of pre-sulfite oxidase is shown in Table I. When the translation mixture containing ATP and its generating system was incubated with mitochondria, KCN had little effect on the import-processing activity (data not shown). As shown in the table, the activity was clearly inhibited by atractyloside, oligomycin, and CCCP in the presence of 5 mM KCN. The result suggested the requirement for energy in the import of sulfite oxidase into mitochondrial intermembrane space.

DISCUSSION

Our previous in vivo study with rat liver (7) demonstrated the presence of a larger precursor form of sulfite oxidase with an extra-peptide of about 3,000 dalton in the cytosol, which was then transported into mitochondria to

be converted to the mature protein. Our present study confirmed those findings by *in vitro* experiments, and presented strong evidence for the participation of inner mitochondrial membrane in the import of this enzyme into the intermembrane space of the organelle.

When *in vitro* translation products were incubated with mitochondria isolated from rat liver, pre-sulfite oxidase was processed to the mature size and resulting mature protein was found in the intermembrane space, indicating that the precursor of the enzyme was transported *in vitro* into its correct mitochondrial location. Subfractionation of mitochondria showed that the processing enzyme was mainly localized in the inner mitochondrial membrane, probably on its outer surface, since the activity was found in the mitoplast but neither in the matrix fraction nor in the inner membrane vesicles inverted by sonication. It was also found that the import of sulfite oxidase was energy-dependent process and the process seems to require an electrochemical gradient across the inner mitochondrial membrane.

These findings suggest that the import of sulfite oxidase into mitochondria requires the participation of the inner membrane. Most recently, Schatz and collaborators reported that the precursor peptide of cytochrome b_2 (20), an enzyme in the intermembrane space of yeast mitochondria, first penetrates intc the mitochondrial matrix by a mechanism requiring an electrochemical gradient across the inner membrane, and then the precursor is cleaved to an intermediate form by a soluble protease in the matrix followed by the conversion of the intermediate form to the mature form. Although an intermediate form was not observed in the case of sulfite oxidase of rat hepatocyte, the precursor of the enzyme was converted to the mature form by a proteolytic activity on the outside surface of the mitoplast. It is most likely that the participation of the inner membrane is universal in the import of proteins into the intermembrane space of mitochondria.

ACKNOWLEDGMENT

The authors wish to thank Professor T. Omura for his valuable discussion and critical reading of the manuscript.

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