IDENTIFICATION OF A MAJOR MEMBRANE FRACTION AS A PRODUCT OF SYNTHESIS BY ISOLATED YEAST MITOCHONDRIA

Sophia Yang and R. S. Criddle
Department of Biochemistry and Biophysics
University of California, Davis, California 95616

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Summary: A fractionation procedure has been developed to separate mitochondrial membrane components into well-resolved fractions. One fraction of the membrane, which can be isolated by gel electrophoresis, accounts for a major portion of the total mass of the membrane protein. This protein was labeled to a much greater extent than other fractions during protein synthesis by isolated mitochondria. Inhibition of the synthesis of this component by chloramphenicol accounts for essentially all of the observed effect of chloramphenicol on protein biosynthesis in isolated mitochondria.

It is well documented that isolated mitochondria from a variety of sources have the ability to incorporate radioactive amino acids into protein (c.f. Roodyn *et al.*, 1961; Wheeldon and Lehninger, 1966; Beattie *et al.*, 1967; Beattie, 1968; Lamb *et al.*, 1968; Neupert *et al.*, 1967). Most of the radioactivity has been shown to be associated with the insoluble membrane proteins. Haldar and Work (1966) have shown that several protein species separatable on disc gel electrophoresis have incorporated radioactive label, however, no specific protein product of mitochondrial synthesis has been resolved. This communication describes a reproducible method for separation of membrane components by extraction and electrophoretic procedures and demonstrates that one of the membrane protein fractions contains the major product(s) of protein synthesis in isolated yeast mitochondria.

MATERIALS AND METHODS

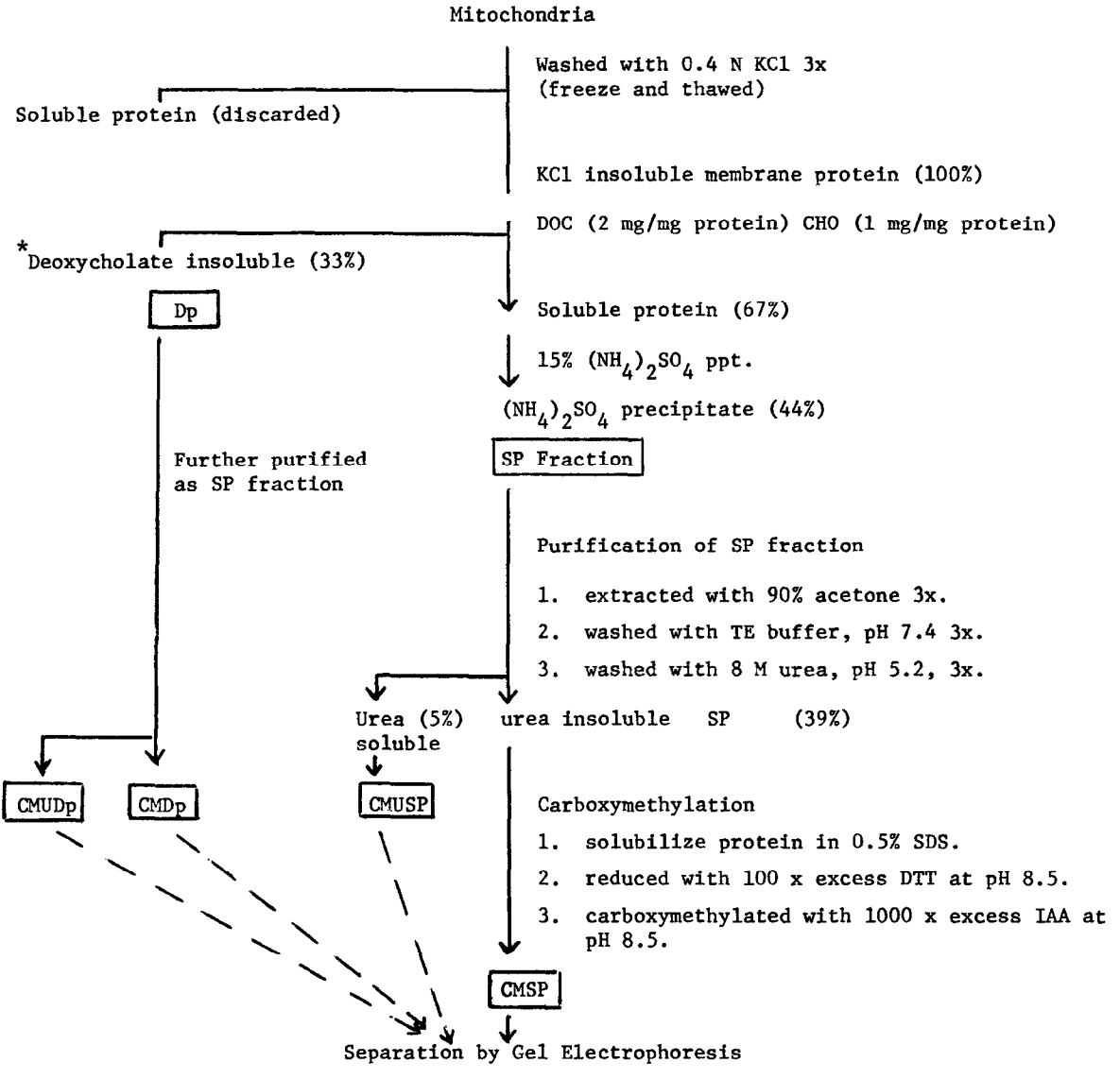
A double mutant (strain of lys^- met^-) *Saccharomyces carlsbergensis* (SCML-1) was employed in this study. The yeast cells were grown aerobically at room temperature in 1% peptone-yeast extract-glucose medium in two liter flasks on a rotary shaker and were harvested in late log phase. Spheroplasts

were prepared from glusulase-digested yeast cells according to the scheme of Duell and Utter (1964) with a slight modification. Mitochondria were obtained by differential centrifugation after the lysis of spheroplasts and were then purified by banding at 4°C in a 20-60% (w/w) linear sucrose density gradient in a Spinco SW-27 rotor at 27,000 rpm for 5 hours.

The incubation medium used for amino acid incorporation into isolated mitochondria was a modification of that described by Lamb *et al.* (see Table I for details) (1968). All incubations were carried out at 32°C in a metabolic shaker. Fifty ml aliquots of mitochondrial suspension were incubated with protein synthesis inhibitors (as noted) for 15 min prior to the addition of radioactive amino acids. The incorporation was stopped by addition of an equal volume of cold buffer (0°C) containing 200 x excess of unlabeled amino acids, 0.25 M sucrose, 0.01 M Tris, and 1 mM EDTA, pH 7.4 (STE). Mitochondria were then washed three times with STE buffer containing unlabeled amino acids. A modified method of Criddle *et al.* (1962) with additional washing steps (Richardson *et al.*, 1966; Lenaz and Green, 1968) was used to separate the total mitochondrial membrane proteins into major fractions (Fig. 1). The system of Ornstein and Davis (1964), modified to contain 0.03% SDS in the upper buffer, was then employed in disc gel electrophoresis to further fractionate the major fractions. Protein was stained with 0.05% Coomassie Blue in 10% TCA and was destained in 10% TCA. Segments of gel were cut and then eluted with 0.2 M NaOH. The eluted protein was dialyzed against 0.01 M Tris buffer, pH 8.5, and then concentrated. Radioactivity of the protein solutions was counted in a Nuclear Chicago scintillation counter using Bray's scintillation fluid and protein concentrations were determined by the method of Lowry *et al.* (1951).

RESULTS

Fractionation of Membrane Proteins. The isolation scheme of Fig. 1 employed general methods for the preparation of the mitochondrial "structural



*Deoxycholate, cholate insoluble material was fractionated further using the same condition as used for structural protein fraction.

Abbreviations used: SP = structural protein fraction; Dp = deoxycholate-cholate insoluble fraction; U = urea soluble; CM = carboxymethylated. Thus CMUSP = carboxymethylated urea soluble structural protein fraction. SDS = Sodium dodecyl sulfate; DTT = dithiothreotol; IAA = iodoacetic acid. DOC = deoxycholate. CHO = cholate. TE buffer = 0.01 M Tris, 0.001 M EDTA, pH 7.4.

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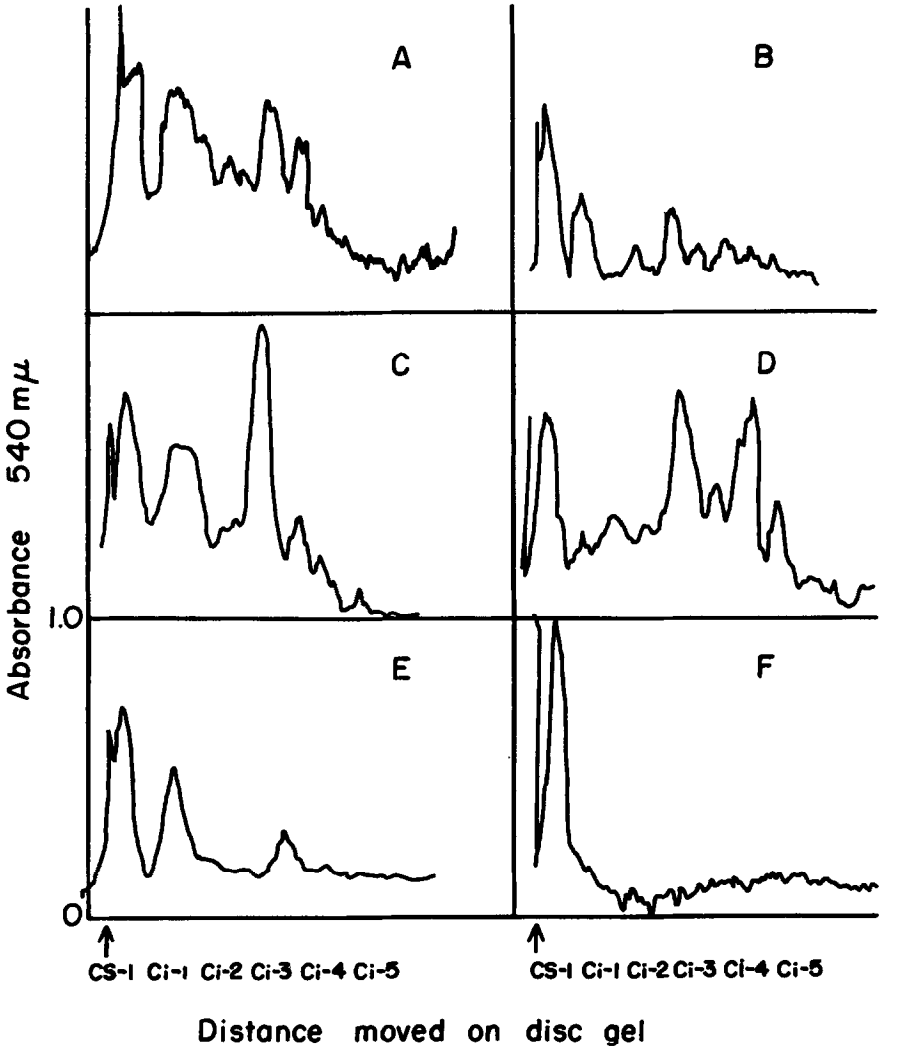
protein" fraction followed by further washing and fractionation to obtain more readily identifiable fractions. The yield of the SP fraction is 39% of the total membrane protein.

The disc gel electrophoresis pattern of the reduced, carboxymethylated SP fraction is shown in Fig. 2E. The most rapidly migrating band, termed CS-1, contains 45% of the protein of this fraction. The remainder of the material is distributed among the bands Ci-1 (30%), Ci-3 (12%) and Ci-2 plus Ci-4 (∿10%). In contrast to previously reported gel electrophoresis separations of mitochondrial proteins (Haldar *et al.*, 1966; Lenaz and Green, 1968), no protein material remained precipitated at the gel interface after electrophoresis, thus allowing quantitative determination of protein in each gel component.



To follow the fractionation process in detail and quantitate the yield at each step, the other major membrane fractions were each collected, reduced and carboxymethylated and separated by gel electrophoresis. As shown in Fig. 2A through 2F, all of the fractions had some of each of the components CS-1 and Ci-1 through Ci-5 plus additional components. The relative amounts of each of these varied in each fraction. The urea soluble extract of SP is particularly rich in Ci-3 while Ci-5 occurs in high concentration only in CMDp. CS-1 is a major component in all fractions and accounts for 25-30% of the total membrane protein.

Incorporation of Labeled Amino Acids Into Membrane Proteins. The results shown in Table I indicate that, as noted by previous workers, C^{14} amino acids are incorporated into all major membrane protein fractions by isolated mitochondria. This incorporation is sensitive to inhibition by chloramphenicol but not by cycloheximide. All incorporation experiments were run in the presence of cycloheximide in order to minimize the effect of any "microsomal type"

TABLE I

Distribution of radioactivity incorporated into major mitochondrial subfractions during synthesis in the presence of cycloheximide and cycloheximide + chloramphenicol.

Fractions	cycloheximide cpm/mg	chloramphenicol + cycloheximide cpm/mg	% inhibition by chloramphenicol
Whole mitochondria	640	327	49%
KCl insoluble	692	384	45%
SP fraction	650	208	68%
USP	290	143	51%
UDp	705	452	36%

The standard incubation mixture contained 40 mM Tris (pH 7.4), 250 mM sorbitol, 10 mM $MgCl_2$, 100 mM KCl, 5 mM PEP, 1.5 mM ATP, 5 mM PO_4 (pH 7.4), and 0.18 mM cycl. Each 1 ml system contained 0.2 µc ^{14}C-Val (195 mc/mmole), 0.2 µc ^{14}C-Tyr (34 mc/mmole), 0.2 µc ^{14}C-Arg (192 mc/mmole), 0.2 µc ^{14}C-Leu (23 mc/mmole), 10 µg oligomycin and 2.4 mg mitochondrial protein. In the incubation mixture containing chloramphenicol, the concentration of chloramphenicol was 5 mM. Incubation was at 32°C for 30 min.

TABLE II

Distribution of Radioactive Label in Proteins of Carboxymethylated Structural Protein in Fraction

		Incubation with cycloheximide		Incubation with cycloheximide plus chloramphenicol	
Gel Fraction	% of protein	% of total radioactivity	cpm/mg	cpm/mg	% inhibition
CS-1	45	94.7	775	73	90.5
Ci-1	30	3.9	48	21	∿56
Ci-2	∿5	.4	31	22	∿29
Ci-3	12	.7	22	17	∿23
Ci-4	∿5	∿.2	16	16	∿0

TABLE III

Distribution of Radioactive Label in Proteins of Carboxymethylated Deoxycholate Insoluble Protein

		Incubation with cycloheximide		Incubation with chloramphenicol + cycloheximide	
Gel Fraction	% of protein	% of total radioactivity	cpm/mg	cpm/mg	% inhibition
CS-1	32	72	618	115	81.5
Ci-1	20	12	167	-	-
Ci-2	8.5	-	trace	-	-
Ci-3	14	6.3	125	79	∿37
Ci-4	5.5	1.8	89	51	∿43
Ci-5	7	3.1	123	163	∿0

incorporation by the mitochondrial preparation. Chloramphenicol inhibition noted in these experiments is somewhat less than reported with other mitochondrial systems. It is apparent that inhibition of all fractions occurs and that inhibition is greatest in the SP fraction.

The distribution of radioactivity in the gel electrophoretic components of CMSP is shown in Table II. It is apparent that most of the C^{14} label is found in CS-1 and that chloramphenicol strongly inhibits this incorporation. The incorporation into the Ci-components is relatively insensitive to this inhibitor.

Incorporation into other membrane subfractions shows a similar pattern. Table III shows that CS-1 of CMDp (from the same experiment as Table II) is again the most highly labeled gel component, having about the same specific activity and sensitivity to chloramphenicol inhibition as CS-1 from CMSP.

DISCUSSION

Haldar *et al.* have shown that C^{14} amino acids may be incorporated into several major protein fractions by isolated mitochondria. Our results confirm this observation, but in addition show that a specific protein component, CS-1, isolated from gel electrophoresis, is labeled to a much greater extent than any other observable component.

Numerous authors have demonstrated that mitochondrial protein synthesis was inhibited up to 90% by low levels of chloramphenicol *in vitro* (Linnane, 1968; Beattie, 1968; Lehninger, 1966). However, most of the major mitochondrial fractions in our experiments, with the exception of the SP fraction, were inhibited only 50% or less. The relative low level of inhibition may arise from differences in organisms or yeast strains used. Since the synthesis of CS-1 is inhibited 80-90% by chloramphenicol and it contributes 30-35% of the total mass of the mitochondrial membrane protein, the inhibition of this one fraction can account for most of the observed inhibition in our isolated mitochondrial system. On the basis of its specific activity, its total activity,

and its inhibition by chloramphenicol, one may conclude that CS-1 contains the major protein component(s) synthesized by isolated mitochondria.

Incorporation of C^{14} amino acids into other protein components of the mitochondrial membrane, while much smaller than into CS-1, is still significant.* The nature of the system involved in the synthesis of these species in the isolated mitochondria is not clear, but must in general be different from the chloramphenicol sensitive synthesis of CS-1.

CS-1 from all membrane fractions moves with identical relative mobility on disc gel electrophoresis. However the use of mobility as the only criterion to establish the identity of this material from different fractions is not sufficient because of its rapid migration rate. Stronger support comes from the high and nearly identical specific activity of CS-1 in all fractions following C^{14} amino acid incorporation and from the similarities in its sensitivity to chloramphenicol inhibition.

The homogeneity of the CS-1 component(s) may also be questioned on the basis of limited separation of rapidly moving electrophoretic components. Some degree of heterogeneity of this material has been suggested by studies in other buffer systems and this is now under further study.

In this study we demonstrate that ^{14}C amino acids are incorporated primarily into a clearly definable component(s) of mitochondrial membrane protein by isolated mitochondria. The sensitivity of this incorporation to protein synthesis inhibitors is consistent with the conclusion that this represents a major product of synthesis by the mitochondrial protein synthesizing system under the particular experimental conditions reported.

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*In addition to labeled insoluble membrane components, some highly labeled species have been observed in the ammonium sulfate supernatant fraction (Fig. 1).