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Aggregates of Yeast Mitochondrial Cytochrome *b* Observed After Electrophoresis*

Diana S. Beattie and Liviu Clejan†

Department of Biochemistry
Mount Sinai School of Medicine of
the City University of New York
New York, New York 10029

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Abstract

Mitochondrial translation products obtained from yeast cells labeled *in vivo* in the presence of cycloheximide were separated by dodecylsulfate polyacrylamide gel electrophoresis. The labeled band, with a molecular weight of 30,000 corresponding to cytochrome *b*, was excised and subsequently transferred to a second gel. After electrophoretic separation, two labeled polypeptides with apparent molecular weights of 67,000 and 27,000 became visible in addition to the cytochrome *b* band of 30,000 molecular weight. Heating of the cytochrome *b* band prior to transfer resulted in an increase in the amount of the labeled polypeptides migrating with a molecular weight of 67,000.

Longer exposure during autoradiography of the gels of mitochondrial translation products resulted in the appearance of a double band with an apparent molecular weight of 67,000. Limited proteolysis of this 67,000 dalton protein with *Staphylococcus aureus* V8 protease revealed a peptide map similar to that obtained after proteolysis of cytochrome *b*. These results suggest that the polypeptide with an apparent molecular weight of 67,000 represents an aggregate of cytochrome *b* that is either present as such in the membrane or is formed *in vitro* during the experimental manipulations to prepare mitochondria for gel electrophoresis.

Introduction

Mitochondria have the ability to synthesize 8–10 hydrophobic proteins located in the inner mitochondrial membrane [1, 2]. These mitochondrial translation products have been shown in yeast to include the three large

*Abbreviations used: SDS, sodium dodecylsulfate.

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subunits of cytochrome oxidase [3, 4], three to four subunits of the oligomycin-sensitive ATPase complex [4], cytochrome *b* [5–7], and VAR-1, a subunit of the 30S mitochondrial ribosome [8, 9]. To date, these polypeptides are the only ones which have been positively identified as products of protein synthesis on mitochondrial ribosomes; however, suggestions have been made that other mitochondrially synthesized proteins may exist, especially of higher molecular weight [10]. Indeed, examination of published gels indicates the cycloheximide-resistant synthesis of several proteins which apparently are not labeled as rapidly or as extensively as the majority of mitochondrial products [11, 12].

In a previous study in our laboratory [7] we reported the presence of polypeptides of higher molecular weight, other than cytochrome *b*, which are coprecipitated by the specific antiserum to cytochrome *b*. These proteins are products of mitochondrial protein synthesis but do not appear to be associated with cytochrome *b* in a partially purified preparation or to be precursors of cytochrome *b* [13]. In the current study, the presence of aggregates of cytochrome *b*, with an apparent molecular weight of 67,000, has been demonstrated. These results suggest that some mitochondrially synthesized proteins of higher molecular weight may be aggregates of known mitochondrial products.

Materials and Methods

Materials

Acrylamide, bisacrylamide, and TEMED (*N,N,N',N'*-tetramethylenediamine) were from Eastman. Cycloheximide, phenylmethanesulfonyl fluoride, and 2-mercaptoethanol were purchased from Sigma. *Staphylococcus aureus* V8 protease was a Miles Laboratories product. ^{35}S-Methionine (specific activity >1000 Ci mmole^{-1}) was obtained from Amersham. The molecular weight standard proteins (range 14,400–94,000 daltons) were from Pharmacia Fine Chemicals. The chemicals were of highest purity available.

Methods

The growth of yeast, the *in vivo* labeling of cells, the preparation of mitochondria, and the dodecylsulfate (SDS) polyacrylamide slab gel electrophoresis of the mitochondrial translation products were performed according to published procedures [13]. Linear concentrations of polyacrylamide were used for electrophoresis. The mitochondria were dissociated overnight at room temperature in the medium described by Douglas and Butow [10] but

contained 5% SDS and 2-mercaptoethanol. For the limited proteolytic digestion of the electrophoretically separated polypeptides, the method of Cleveland et al. [14] was followed with the modifications described by Cabral et al. [15]. The position of the polypeptides, either to be digested or transferred only to a second gel, was localized precisely in the following way: after electrophoresis, the whole gel was quickly stained with Coomassie Blue 0.025 g% in methanol:acetic acid (4.9:1) and destained in a CANALCO slab gel destaining apparatus. One part of the gel which contained the duplicates of the samples that had to undergo digestion (or to be transferred only) was cut apart, dried, and autoradiographed for 14–16 hr. This portion of the gel contained on one of its edges half of the lane on which the marker proteins were separated. The positions of these proteins were marked with ^{35}S-methionine along the dried gel and served to match the autoradiograph with the undried portion of the gel. The undried gel contained the second half of the lane with the markers and the polypeptides to be digested or transferred only. Digestion of the polypeptides in the excised slices was achieved at 37°C for 30 min, either in the stacking layer of the 15% polyacrylamide gel or in plastic tubes, using 100 μg of *S. aureus* V8 protease. In some experiments, stepwise dilutions of 1–100 μg protease were used.

Results

Prior to proteolytic digestion by the method of Cleveland et al. [14], it is necessary to transfer the radioactive band corresponding to a given protein from the original gel to the stacking gel of a new slab gel. The band corresponding to cytochrome *b*, indicated by the arrow in Fig. 1, is widely separated from other labeled proteins when total mitochondrial translation products are subjected to SDS-electrophoresis in 10% acrylamide gels. This separation permits the excision of cytochrome *b* from the original gel without contamination by other proteins, especially subunit II of cytochrome oxidase. Autoradiography of the gel after the excisions confirmed that no other radioactive bands were removed. After electrophoresis of the excised band in a new gel without proteolytic digestion, several bands other than cytochrome *b* were observed, depending on the concentration of acrylamide in the second gel. In a 12% acrylamide gel, a labeled band with a molecular weight of 67,000 was present in addition to the band of 31,000 daltons corresponding to cytochrome *b* (Fig. 2A). Similar results were observed when the original band was transferred to a 10% acrylamide gel (data now shown). Electrophoresis of the excised cytochrome *b* band on either a 15% (Fig. 2B) or 8% acrylamide gel revealed the presence of a third labeled band with a molecular weight approximately 3000 less than that of cytochrome *b*. It should be recalled that

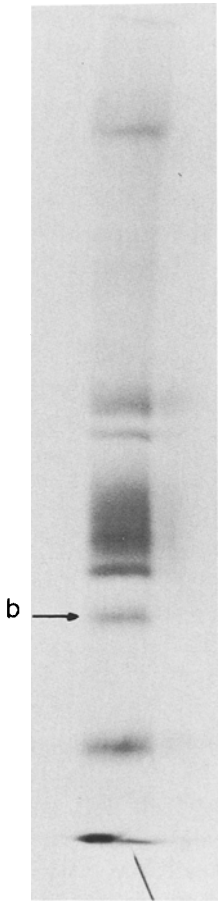

Fig. 1. Autoradiogram of yeast mitochondria labeled *in vivo* with ^{35}S-methionine in the presence of cycloheximide and separated on a 10% polyacrylamide slab gel, as described in ref. 13. The arrow indicates the position of cytochrome *b*.

cytochrome *b* migrates with an apparent molecular weight greater than 30,000 in a 15% acrylamide gel and less than 30,000 in a 10% gel [13, 16]. Again, a labeled band with a molecular weight of 67,000 is clearly visible in addition to the band corresponding to cytochrome *b*.

These observations suggested that protein(s) which had migrated with an apparent molecular weight of 30,000 in the presence of SDS contained a polypeptide with an apparent molecular weight of 67,000 after a second electrophoresis. A possible explanation for this observation is that cytochrome *b* aggregates during the experimental manipulations, especially during fixation of the gel with methanol and acetic acid, and migrates as if it were a larger protein. To test this possibility, mitochondria were heated for 20 min at 60° in dissociation buffer prior to the initial electrophoresis. Our usual

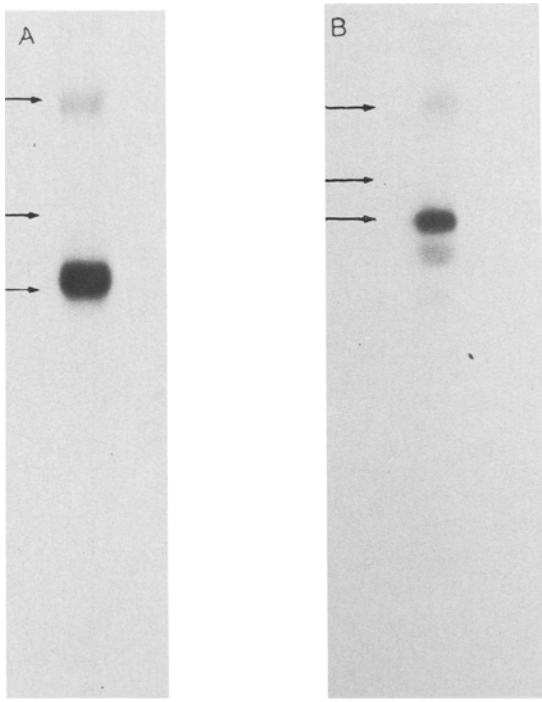

Fig. 2. Electrophoresis of cytochrome *b* excised from a 10% polyacrylamide gel and transferred to a second slab gel for a subsequent electrophoresis: (A) 12% polyacrylamide gel; (B) 15% polyacrylamide gel. Arrows indicate the position of standards with molecular weights of 67,000, 43,000, and 30,000.

procedure is to leave the mitochondria in dissociating buffer overnight at room temperature. As seen in Fig. 3, the band migrating at 67,000 was more pronounced after this treatment when compared to a similar band containing cytochrome *b* from mitochondria which had not been heated prior to the initial electrophoresis.

These results suggested that the proteins of higher molecular weight which were observed previously in the gels of mitochondrial translation products and in immunoprecipitates formed with the specific antiserum against cytochrome *b*, might indeed be aggregates of cytochrome *b* [7, 13]. The autoradiogram of Fig. 4 clearly indicates a labeled doublet with a molecular weight similar to that of bovine serum albumin. The gel of Fig. 4 was exposed to the X-ray film for a longer time than usual in order to visualize these bands. Hence, the major labeled proteins are overexposed. The area of the gel with 67,000 molecular weight was also excised and transferred

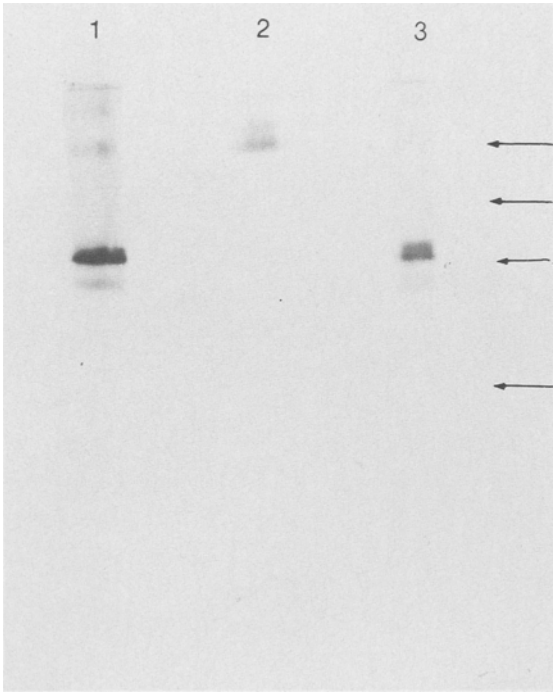

Fig. 3. Electrophoresis of cytochrome *b* and the polypeptide of 67,000 molecular weight excised from a 10% polyacrylamide gel and transferred to a 15% polyacrylamide gel for electrophoresis. Lane 1: mitochondria were dissociated for 20 min at 60°C prior to electrophoresis in 10% polyacrylamide. The band corresponding to cytochrome *b* was subsequently transferred to a 15% polyacrylamide gel for electrophoresis. Lane 2: polypeptide of 67,000 molecular weight transferred to a 15% polyacryl-amide gel. Lane 3: mitochondria were dissociated at room temperature overnight prior to electrophoresis in a 10% polyacrylamide gel. Cytochrome *b* was transferred to a 15% gel. Arrows indicate molecular weight standards of 67,000, 43,000, 30,000, and 20,100, respectively.

to another gel for electrophoresis. Only one band with a molecular weight of 67,000 was observed (Fig. 3). No material of lower molecular weight was observed, suggesting that if these proteins do consist of aggregates of cytochrome *b*, they do not dissociate in SDS and glycerol under the usual conditions.

A direct comparison of the mitochondrial translation products and cytochrome *b* was next attempted after proteolytic digestion with *S. aureus* V-8 protease. The high-molecular-weight proteins were not readily digested, as is clear in Figs. 5 and 6. In some cases, larger amounts of protease were added and the time of digestion increased. A comparison of the peptide

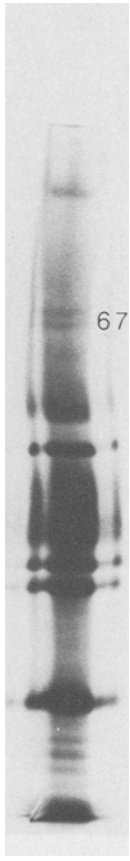

Fig. 4. Autoradiogram of mitochondrial translation products after longer exposure to reveal the double polypeptide in the 67,000 dalton region.

pattern after digestion of cytochrome *b* and the 67,000 molecular weight doublet proteins indicate several similar peptides (Fig. 5). One prominent band appearing in the digest of the 67,000 dalton protein(s) has an identical migration as undigested cytochrome *b*. At least five to six other bands with similar molecular weights were present. In a subsequent experiment, the two bands with a molecular weight close to 67,000 (Fig. 6, lanes 3 and 4) were separated prior to digestion in comparison with cytochrome *b* (Fig. 6, lanes 1 and 2). While considerable material of 67,000 molecular weight was not digested, at least four bands in the digest were of similar mobility.

Discussion

The present study was prompted by our interest in the identity of the polypeptides of higher molecular weight which are present in the immunoprecipitates formed with the specific antiserum against cytochrome *b* [7, 13].

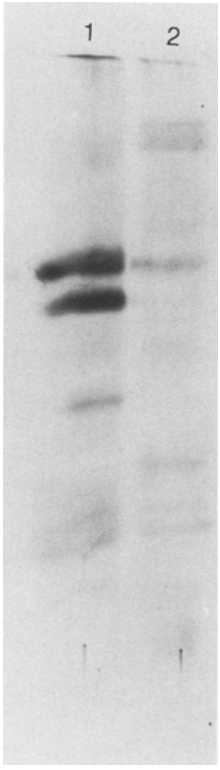

Fig. 5. Peptide maps of digests of cytochrome *b* and the 67,000 molecular weight polypeptide. Limited proteolytic digestion was achieved with 100 μg of *S. aureus* V-8 protease, as described in Materials and Methods. Lane 1: cytochrome *b*. Lane 2: 67,000 molecular weight polypeptide.

The results obtained suggest that certain of these polypeptides which migrate with an apparent molecular weight of 67,000 represent aggregates of cytochrome *b* which do not dissociate completely in SDS. This conclusion is based on the following observations: when the labeled band corresponding to cytochrome *b* is excised from a 10% polyacrylamide slab gel and transferred to a new gel for electrophoresis, a band migrating with an apparent molecular weight of 67,000 is clearly visible. Similar results were observed in different strains of yeast including mutants which lack the 30,000-dalton cytochrome *b* peptide but have new translation products of lower molecular weight [17]. Secondly, gels of total mitochondrial translation products contain a double band of approximately 67,000 molecular weight clearly visible with longer exposure during autoradiography. Limited proteolysis of this double band with *S. aureus* protease revealed similar peptides to those obtained after proteolysis of cytochrome *b*, suggesting that the protein in the double band of 67,000 molecular weight is indeed identical to cytochrome *b* and hence represents an aggregate of cytochrome *b*.

The greater prominence of these higher-molecular-weight proteins in the immunoprecipitates, as compared to total mitochondrial translation products,

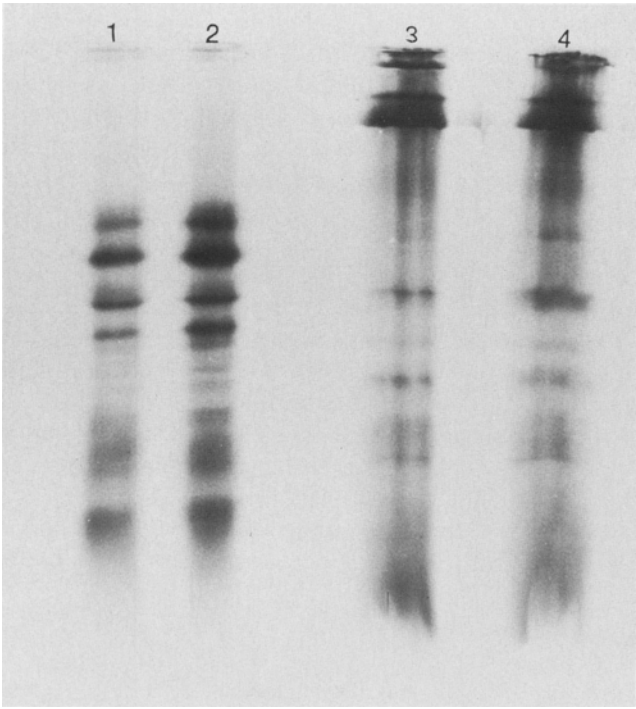

Fig. 6. Peptide maps of digests of different concentrations of cytochrome *b* and the doublet band with an apparent molecular weight of 67,000. Limited digestion was achieved with 20 μg of *S. aureus* V-8 protease, as described in Materials and Methods. Lane 1: cytochrome *b*, 48,000 counts per lane. Lane 2: cytochrome *b*, 60,000 counts per lane. Lanes 3 and 4: double band of 67,000 molecular weight, 48,000 and 60,000 counts per lane, respectively.

may have resulted because of the procedures involved in immunoprecipitation. Previous studies [16, 18] had indicated that boiling in dissociation medium caused the formation of aggregates of cytochrome *b* which would not enter the gel. We have reported similar observations [13], but did not observe an increase in material migrating at a molecular weight of 67,000 after boiling. In this context, it should also be noted that when cytochrome *b* is separated from complex III, labeled polypeptides with molecular weights of 31,000 and 67,000 were observed [13]. The higher-molecular-weight labeled proteins may be aggregates of cytochrome *b* formed during the preparation which involves cholate extraction of mitochondria followed by ammonium sulfate precipitation.

It should be stressed that these aggregates do not appear nonspecific as only one labeled protein of 67,000 molecular weight is observed after the transfer to the second gel and not many labeled high-molecular-weight

proteins. In addition, a protein migrating as a doublet at this molecular weight is observed in gels of total mitochondrial translation products *in vivo*.

The results of the present study do not preclude the possibility that cytochrome *b* may exist as a polymer in the mitochondrial membrane. The existence of a dimer of cytochrome *b* has been proposed in *Neurospora* mitochondria [19]. Indeed, in an isolated complex III there is a 2:1 molar ratio of cytochrome *b* to cytochrome c_1 [19–21]. Although 67,000 is more than double the 30,000 molecular weight of cytochrome *b*, the anomalous migration behavior of this protein in polyacrylamide prevents a precise calculation of these molecular weights. An additional explanation for these high-molecular-weight proteins is that they represent complexes of cytochrome *b* and other proteins of complex III. One possible candidate is the protein associated with cytochrome *b* during purification [22]. Incomplete dissociation of either this protein or some other protein from cytochrome *b* in the presence of SDS might result in material migrating with a higher apparent molecular weight. In any event, caution is necessary in examining labeling patterns after SDS gel electrophoresis. These data indicate clearly that treating samples with SDS does not always cause them to dissociate to monomers.

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