SHORT COMMUNICATIONS

BBA 43231

Identification of denatured mitochondrial ATPase in "structural protein" from beef heart mitochondria

Several years ago, CRIDDLE *et al.*[1] isolated from beef heart mitochondria an insoluble, colorless protein without detectable enzymic function. Since this protein accounted for roughly one third of the mitochondrial dry mass[1] and formed tight complexes with mitochondrial cytochromes[1,2] it was viewed as a mitochondrial "structural protein" which ensured the correct alignment of the respiration carriers[1]. Reports from different laboratories indicated, moreover, that "structural protein" is homogeneous[3–5] and that it is a product of the mitochondrial protein synthesizing system[5,6].

Subsequent studies revealed, however, that acrylamide gel electrophoresis separated mitochondrial "structural protein" into numerous bands[7,8]. Ultracentrifugal studies[9] also indicated considerable heterogeneity. These observations raise the possibility that the preparations of mitochondrial "structural protein" described thus far[1,5,10] represent a mixture of denatured mitochondrial proteins. Alternately, most of the subfractions detected by electrophoresis or ultracentrifugation could be simply different aggregates of "structural protein" rather than chemically distinct contaminants (*cf.* ref. 11).

The present study offers direct evidence that "structural protein" from beef heart mitochondria contains substantial amounts of denatured mitochondrial ATPase. It is shown that submitochondrial particles reconstituted with ^{3}H-labeled ATPase yield a "structural protein" which is radioactive and contains most of the ATPase protein initially present in the particles.

If highly purified mitochondrial ATPase (F_1)* is reacted with [^{3}H]acetic anhydride under the conditions specified in Table I, the enzyme accepts approximately one [^{3}H]acetyl group per mole of protein and does not suffer any loss of its ATPase activity (Table I). The labeled enzyme also binds to ATPase-deficient submitochondrial particles, thereby regaining the oligomycin sensitivity typical of membrane-bound F_1 (*cf.* also ref. 12). The specific ATPase activity of the reconstituted particles indicates that 12.3% of the total particle protein is accounted for by bound F_1. Essentially the same value (12.6%) is obtained by radioactivity measurements (Table I). The recombination of the labeled ATPase with the particles is thus not accompanied by significant denaturation or masking of the enzyme.

"Structural protein" isolated from the labeled particles by the method of CRIDDLE *et al.*[1] lacked ATPase activity, yet was strongly radioactive (Table II); thus, it contained inactivated or denatured F_1. In the experiment documented in Table II,

* F_1, coupling factor 1. In this paper, the terms F_1 and mitochondrial ATPase are used interchangeably.

TABLE I

PREPARATION OF SUBMITOCHONDRIAL PARTICLES CONTAINING RADIOACTIVE ATPase

ATPase was purified from beef heart mitochondria by a modification[13] of the method of PULLMAN *et al.*[14]. The enzyme was reacted with acetic anhydride as described by KAGAWA AND RACKER[12] with the following modifications: (1) only 1.6 moles of [^{3}H]acetic anhydride (50 mC/mmole) were added for each mole of enzyme and (2) the labeled enzyme was freed from radioactive impurities by passage (at room temperature) through a Sephadex G-200 column equilibrated with 20 mM Tris sulfate (pH 7.4)–2 mM EDTA–4 mM ATP. ATPase-deficient submitochondrial particles from beef heart (SU-particles) were prepared according to RACKER AND HORSTMAN[15]. Reconstitution of these particles with labeled ATPase was achieved by mixing the following components in a final volume of 8.0 ml: 80 μmoles Tris sulfate (pH 7.4); 80 μmoles potassium succinate; 4 μmoles EDTA; 54 mg SU-particles; and 53.2 mg [^{3}H]ATPase. After 45 min at room temperature, the mixture was centrifuged for 20 min at 100000 $\times$ *g* at room temperature and the particle pellet purified further as described by SCHATZ[16]. The assays for protein and ATPase have been specified earlier[17]. For the radioactivity measurements, the protein samples were precipitated with an equal volume of 10% trichloroacetic acid, dried *in vacuo*, dissolved in 0.3 ml of concentrated formic acid and counted in a Packard liquid scintillation spectrometer at a counting efficiency of 13%.

Fraction	*ATPase activity (μmoles ATP cleaved per min per mg protein)*		*Radioactivity (counts/min per mg)*
	–oligomycin	*+oligomycin (5 μg/ml)*	
Purified ATPase	67	67	—
[^{3}H]Acetyl-ATPase	73	73	$1.9 \cdot 10^4$
ATPase-deficient particles	0.095	0.001	—
ATPase-deficient particles reconstituted with [^{3}H]ATPase	9.1	0.98	$2.4 \cdot 10^3$

TABLE II

ISOLATION OF "STRUCTURAL PROTEIN" FROM SUBMITOCHONDRIAL PARTICLES CONTAINING RADIOACTIVE ATPase

"Structural protein" was isolated from labeled particles (*cf.* Table I) as described by CRIDDLE *et al.*[1] except that the final removal of bile salts with methanol was omitted.

Fraction	*mg*	*Counts/min*	*Counts/min per mg*	*% of particle-bound radioactivity*	*% of specific radioactivity of [^{3}H]ATPase*
Labeled particles	39	$9.4 \cdot 10^4$	$2.4 \cdot 10^3$	(100)	12.6
Detergent-insoluble residue	5.2	$8.6 \cdot 10^3$	$1.7 \cdot 10^3$	9.2	8.4
Supernatant after 12% $(NH_4)_2SO_4$ fractionation	10.5	$5.8 \cdot 10^3$	$5.5 \cdot 10^2$	6.1	2.8
"Structural protein"	17.5	$6.6 \cdot 10^4$	$3.8 \cdot 10^3$	70.0	19.6
Sucrose washing of "structural protein"	0.71	$6.0 \cdot 10^2$	$8.4 \cdot 10^2$	0.63	4.2

about 20% of "structural protein" consisted of denatured ATPase. Other experiments yielded values as high as 35%. Similar results were obtained if "structural protein" was prepared by the method of RICHARDSON *et al.*[10]. With either method, between 60 and 80% of the ATPase protein originally bound to the particles was recovered in the "structural protein" fraction.

It has been claimed[18] that treatment of "structural protein" with acid methanol

and urea results in an electrophoretically homogeneous preparation. In our hands, however, this procedure removed only about two thirds of the radioactivity and thus still did not yield a pure product.

When the labeled, soluble ATPase was subjected to acrylamide gel electrophoresis according to TAKAYAMA *et al.*[19] about 90% of the radioactivity migrated as a single, sharp band (Fraction 8 of Fig. 1A). Essentially the same radioactivity peak was observed if "structural protein" from the labeled, reconstituted particles was analyzed by this procedure (Fig. 1B). However, a substantial fraction of the "structural protein" and about one fourth of the radioactivity failed to penetrate into the gel. As previously observed by others[7,8], the "structural protein" preparation was separated into at least 18 protein bands of varying intensity. The most prominently stained band coincided with the main peak of radioactivity, proving that our findings are not merely caused by acetyl transfer from F_1 to other membrane proteins. The present data also confirm the earlier observation of MACLENNAN AND TZAGOLOFF[20] that acrylamide gel electrophoresis of "structural protein" reveals a component that is indistinguishable from the main band of a partially purified F_1 preparation.

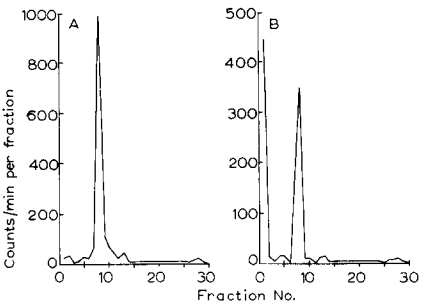

Fig. 1. Distribution of radioactivity after acrylamide gel electrophoresis of [^{3}H]acetyl-ATPase and labeled "structural protein". [^{3}H]Acetyl-ATPase (40 μg; $1.9 \cdot 10^4$ counts/min per mg; Trace A) and labeled "structural protein" (200 μg; $3.75 \cdot 10^3$ counts/min per mg; Trace B) were subjected to acrylamide gel electrophoresis according to TAKAYAMA *et al.*[19] except that acetone treatment of the [^{3}H]acetyl-ATPase was omitted. Electrophoresis was performed for 1.5 h at room temperature at 5 mA/tube. The gels were fixed with 10% trichloroacetic acid for 20 min, stained with a 0.05% solution of Coomassie Brilliant Blue R 250 in 10% trichloroacetic acid and cut into thirty 2-mm sections. These were dissolved in 0.1 ml 30% H_2O_2 and counted in a Nuclear-Chicago liquid scintillation spectrometer at a counting efficiency of 35–40%.

It might be argued that the labeled ATPase rebound to the particles is not equivalent to the endogenous, nonlabeled ATPase present *in vivo*. However, the mild labeling conditions adopted here neither affected the ATPase activity of the soluble enzyme nor its ability to assume the oligomycin sensitivity of membrane-bound F_1. Moreover, if added together with F_2 and F_3 (ref. 21) the labeled ATPase restored oxidative phosphorylation in the acceptor particles (*cf.* ref. 12). Finally, if "structural protein" from the F_1-deficient SU-particles was analyzed by acrylamide gel electrophoresis, the relative intensity of the F_1 band (as revealed by staining with Coomassie Brilliant Blue) was greatly diminished.

We have also considered the possibility that our results merely reflect the presence of radioactive impurities in our preparation of [^{3}H]acetyl-ATPase. Acrylamide gel electrophoresis of the labeled enzyme at three different gel concentrations

and at different pH values did indeed reveal some labeled contaminants. However, these accounted for less than 10% of the total radioactivity and, therefore, do not significantly affect the main conclusion of this study.

Mitochondrial "structural protein" from beef heart, as prepared by the two most commonly used procedures[1,10] is thus chemically heterogeneous. A significant fraction of it must also be considered an artifact since it represents the denatured form of a distinct and well characterized mitochondrial enzyme. While these results do not exclude the existence of a specific mitochondrial "structural protein", they leave little doubt that such a protein has not yet been isolated in a pure state.

Several laboratories have reported that mitochondrial "structural protein" from "petite" mutant yeast lacks at least one component which is present in the corresponding preparation from the wild-type strain[8,22,23]. Since the "petite" mutation abolishes the tight linkage between ATPase and the mitochondrial inner membrane[16] the present results support the possibility (already considered by TUPPY *et al.*[8]) that this difference between the "structural proteins" merely reflects a loss of ATPase from the mutant mitochondria during their isolation.

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