ASSOCIATION OF PANTOTHENIC ACID WITH A PROTEIN SUBUNIT OF YEAST MITOCHONDRIAL ATPase

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1. Introduction

Recent studies demonstrating a role for lipoic acid in ATP synthesis via mitochondrial ATP synthase enzymes suggest thioacyl intermediates are involved in the mechanism of oxidative phosphorylation [1–3]. Dihydrolipoate-dependent ATP synthesis by isolated oligomycin-sensitive ATPase preparations requires catalytic amounts of oleate and oleoyl CoA but the requirement for oleoyl CoA can be replaced by catalytic amounts of oleoyl-*S*-lipoate. These findings indicate the involvement of a protein-bound form of CoA or a pantothenyl protein which may serve as an acyl carrier protein. This paper describes studies indicating the presence of a pantothenate moiety in the oligomycin-sensitive ATPase complex from yeast mitochondria and its association with a specific protein subunit.

Yeast cells were depleted of pantotheine by growth in pantothenate-free media, then labeled with [^{3}H]-pantothenate. The mitochondrial oligomycin-sensitive ATPase complex was subsequently purified and shown to contain ^{3}H-label. Analysis of the protein subunits of ATPase by SDS–gel electrophoresis showed the label to be specifically associated with a 21 000 dalton protein subunit. It is concluded that the mitochondrial oligomycin sensitive ATPase complex (ATP synthase) contains a pantothenyl moiety tightly bound to a polypeptide subunit (subunit 6).

2. Materials and methods

Saccharomyces cerevisiae strains (D243-4A (*a, ade, lys,* ρ^+) and D22 (*a,* ad_2, ρ^+) were used. Cells were grown aerobically at 30°C in one liter batch cultures with rapid swirling in minimal medium containing 2% glucose [4]. Cells were harvested in late log phase by centrifugation at 1200 × *g* for 5 min and were then washed twice with minimal media minus pantothenate. Washed cells, 8 g wet wt, were transferred to 200 ml fresh pantothenate minus minimal media and incubated for about 30 min until growth stopped. In experiments with strain D22 this further incubation step was omitted. Then 0.5 mCi [^{3}H]pantothenate (sodium D-[^{3}H(*N*)] pantothenate, New England Nuclear, 30 Ci/mmol) was added and cells were incubated for approx. 6–8 h while monitoring cell growth and uptake of ^{3}H. The labeled yeast cells were harvested, washed and broken with glass beads in a Braun homogenizer. Mitochondria were prepared by differential sedimentation [5,6] and submitochondrial particles were prepared by sonication of the purified mitochondrial preparation [6]. Oligomycin-sensitive ATPase was then prepared by density gradient purification of Triton X-100 extracts of submitochondrial particles [7]. Alternatively, oligomycin sensitive ATPase was isolated using the method of Ryrie which employs a Triton-deoxycholate extraction and careful elimination of protease activity by addition of

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inhibitors [8]. Oligomycin sensitive ATPase was also purified by immunoprecipitation as described [9].

ATPase activities were measured using the coupled spectrophotometric method [10] or by release of inorganic phosphate [6]. Protein was analyzed by the Lowry method.

Identification of the pantothenate binding component of the ATPase complex was carried out by first heating the purified enzyme complex in 2% SDS and 0.01 M mercaptoethanol, then subunits were separated by electrophoresis on 10% or 12.5% acrylamide gels containing SDS and the buffer system of [11]. Gels were stained with Coomassie blue to locate protein or were sliced into 1 mm or 2 mm sections and separated into scintillation vials. A 0.2 ml aliquot of 30% H_2O_2 was added to each vial and samples were capped and heated 2 h at 90°C. PSC scintillation fluid (Amersham/Searle) was added and radioactivity determined in a Beckman scintillation counter.

To identify the nature of the radioactive component associated with the ATPase complex, material migrating with R_M in the range of 0.61–0.74 was pooled, the gel slices were macerated and extracted overnight with 4 ml 0.125 M Tris–HCl, pH 6.8, 2% SDS buffer. The gel particles were then removed by centrifugation. BSA, 1 mg, was added to the solution which was chilled to 0°C. Protein was then precipitated by addition of 80% perchloric acid to a final concentration of approx. 40%. The precipitate was extracted two times with chloroform. Precipitated protein was collected from the surface of the chloroform and was solubilized in 0.2 ml 0.05 M triethanolamine, pH 11.9. This solution was incubated for 1 h at 70°C, employing the method [12] for hydrolysis of phosphopantotheine moieties from protein. Hydrolysis products were separated by thin-layer chromatography using precoated cellulose plates and the solvent system *n*-butanol/acetic acid/water (65:25:13). Fractions from the thin-layer plate were collected by scraping and these were then counted in Bray's scintillation fluid.

3. Results

Figure 1 illustrates the final step in purification of [^{3}H]pantothenate labeled, Triton-solubilized oligomycin sensitive ATPase. It can be seen that a portion

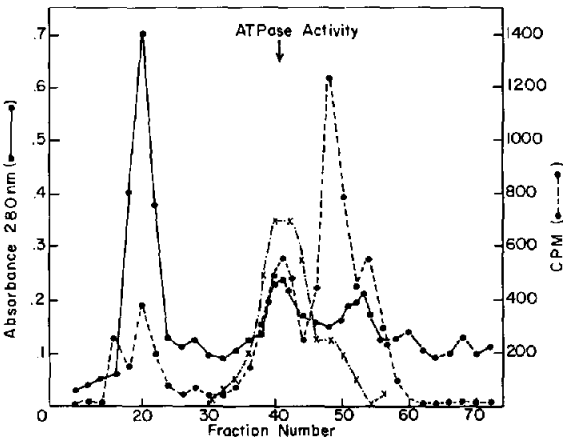

Fig.1. Chromatographic separation of proteins in a Triton X-100 extract of submitochondrial particles using Sepharose 6B [13]. The column elutant was monitored to determine protein $A_{280\,nm}$ (–•–•–) radioactivity (- -•– –•– –) and ATPase activity (–X–X–). Fractions, 1 ml, were collected and 50 μl aliquots from each sample were used for enzyme activity and radioactivity measurements.

of the ^{3}H-label chromatographs with the ATPase complex on Sepharose 6B. Additional label was found to elute later with the lower molecular weight protein fractions. Similar results were obtained when sucrose and glycerol gradient purification [7] was employed for these studies. Electrophoresis of the ATPase fraction on SDS gels yielded the distribution of label shown in fig.2. A significant fraction of the total label migrated with a mobility corresponding to a protein in the range 18 000–22 000 daltons with a mobility equivalent to a Coomassie blue staining band at 21 000 daltons. Some label was routinely noted with higher molecular weight components, the most prominent peak often nearly two times that of the 21 000 dalton band. If the ATPase solution was allowed to stand at 0–5°C for a few weeks, the label in the 21 000 dalton fraction gradually disappeared with a concurrent increase in labeled low molecular weight components migrating near the dye front. If the complex is treated with alkali (0.1 M NaOH for 30 min) there is a marked decrease in label associated with the 21 000 dalton component and an increase in label at the dye front.

When oligomycin sensitive ATPase was prepared by the method [8], a virtually identical distribution of

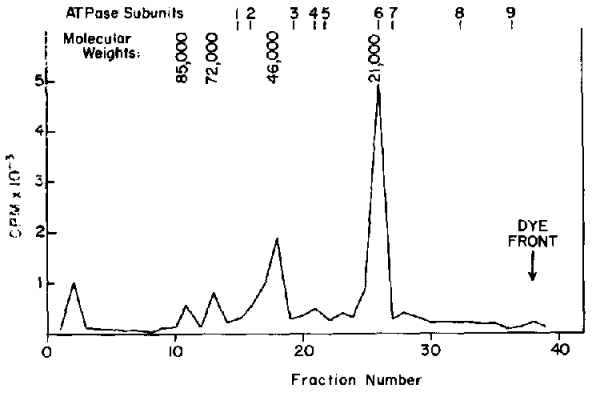

Fig.2. SDS–gel electrophoretic separation of subunits of [^{3}H]pantothenate-labeled ATPase. The position of protein bands were determined by staining with Coomassie blue. Molecular weights for the radioactive-labeled peaks were determined by comparison with the known molecular weights of the ATPase subunits and by comparison with relative mobilities of protein standards run simultaneously on identical gels.

radioactivity was found by gel electrophoretic analysis. Staining such samples to visualize the location of ATPase subunit proteins allowed individual subunit bands to be cut and counted. The results of this are shown in table 1. Subunit 6 appears to be clearly the major site of labelling by [^{3}H]pantothenate. Similar results are obtained on examination of a yeast ATPase complex purified by immunoprecipitation [9].

Table 1
Radioactivity migrating with subunits of ATPase

Subunit	Mol. wt (× 10^{-3})	cpm
1	58	236
2	55	236
3	39	442
4	31	207
5	28	131
6	21	1385
7	18.5	167
8	12	212
9	7.5	604
Top of gel	–	105
Dye front	–	590

SDS–gels of [^{3}H]pantothenate-labeled ATPase were stained with Coomassie blue then gel slices containing the individual protein bands removed, hydrolyzed and counted. In this experiment, considerable radioactivity migrated near the dye front and overlapped the subunit 9 band. This label was not covalently linked to protein

The amounts of subunit 6 protein on the SDS gels can be calculated from the total amounts of ATPase added and a presumed stoichiometry for subunit 6 in the complex. This number, together with the known specific activity of labeled pantothenate added, allows an estimate of the number of pantothenate molecules bound per subunit. Assuming two subunit 6 peptides per ATPase complex mol. wt 400 000 [13], two generations of yeast cells growth on labeled pantothenate, and 50 μg ATPase added per gel, yields a calculated value of 0.9–1.1 pantothenate molecules per subunit 6 on the gel.

The 21 000 dalton component labeled with [^{3}H]-pantothenate was readily extracted from the gels and was precipitated with 40% perchloric acid. This material was hydrolyzed at pH 11.9 and chromatographed on thin-layer cellulose plates. With this chromatography system, protein and any residual protein-bound panthothenate remain at the origin. The hydrolysate yielded two labeled migrating components, one having a R_F of 0.79 and corresponding to standard pantothenate (1925 cpm) and one with an R_F of 0.38, identical to that of β-alanine (964 cpm). A total of 786 cpm of the tritium label remained at the sample origin.

4. Discussion

The mitochondrial ATPase enzyme complex was isolated by three different methods following the labeling of yeast cells with [^{3}H]pantothenate. In all cases, oligomycin-sensitive ATPase was labeled with ^{3}H, indicating that this labeling is probably not due to some contaminating pantothenyl protein which copurifies with ATPase. The major possible source of contamination by some other pantothenyl protein in yeast would be from the fatty acid synthetase enzyme complex. In yeast fatty acid synthetase, the acyl carrier protein is contained on a 180 000 dalton polypeptide [14]. Evidence has been presented that limited protease activity can cause a much lower molecular weight fragment containing 4′-phosphopantotheine to be produced by hydrolysis of this high molecular weight protein. However, neither the intact poly-

peptide nor the protease fragment containing pantotheine would be expected to copurify with ATPase and then migrate on SDS–gels with mol. wt 21 000. It should be noted that ATPase isolation by the method [8] employed protease inhibitors at every step of the isolation procedure. No differences were found in the size or yield of 21 000 dalton pantothenate labeled protein using this method.

The 21 000 dalton subunit of yeast mitochondrial ATPase is located in the membrane sector of the enzyme complex [7]. This peptide, referred to as subunit 6 in yeast, has been shown to be synthesized on mitochondrial ribosomes and is probably a mitochondrial gene product [7,15,16]. A temperature sensitive mutant of yeast apparently is unable to synthesize subunit 6 at restrictive temperatures and cells grown under these conditions cannot carry out oxidative phosphorylation [16]. This provides presumptive evidence that subunit 6, containing a bound pantothenate moiety, is required in the chemical steps of ATP synthesis by oxidative phosphorylation.

Figure 1 shows that a major portion of label in Triton X-100-solubilized extracts of mitochondrial particles migrates with a lower molecular weight than ATPase on gel chromatography. Electrophoresis of this material on SDS gels showed again a predominant peak of radioactivity with the 21 000 dalton protein. By analogy with other studies with labeled mitochondrial gene products [7,15] and fractionation of inhibitor labeled membranes [17] it is proposed that a major portion of the label in this column fraction is associated with membrane sector fractions of ATPase (F_0) which has been separated from the F_1-ATPase during extraction.

The chemical identity of the bound pantothenate-containing moiety is not yet clearly established. It appears covalently bound to the protein, as it remains associated through such treatments as isolation of the complex in Triton X-100, immunoprecipitation, SDS–gel electrophoresis and perchloric acid precipitation. The label is largely released from protein by hydrolysis in pH 11.9 triethanolamine at 70°C for 1 h. This hydrolysis procedure was selected since it has been shown to hydrolyze 4′-phosphopantotheine from various acyl carrier proteins [12]. The results suggest that a phosphopantotheine may be part of the ATPase complex. In contrast to studies with acyl carrier protein, however, the labeled hydrolysis products of subunit 6 could not be accounted for simply as 4′-phosphopantotheine. Considerable label was found in products migrating with β-alanine and pantothenate on thin-layer chromatography. This suggests that the ATPase protein product is somewhat less stable, or other solution components aid in hydrolysis. It was noted that storage of isolated ATPase at 5°C in Triton X-100-containing isolation buffer, at pH 7.5, resulted in a gradual loss of ^{3}H-label migrating with subunit 6 on SDS–gel electrophoresis.

The chemical role of subunit 6 pantotheinyl protein in ATP synthesis is not yet clearly defined, but it is presumed that it serves as an acyl group carrier, possibly forming an oleoyl-*S*-pantotheinyl protein intermediate in the chemical synthesis of ATP [1,3]. Such an enzyme might be expected to have a thioesterase (deacylase) activity measurable in vitro, though such activity would be coupled to other reactions in vivo, and our studies indicate that such an activity, sensitive to oxidative phosphorylation inhibitors, can be characterized. The specific stimulation of P_i–ATP exchange activity in mitochondrial membranes and oligomycin-sensitive ATPase by oleoyl derivatives, including oleoyl CoA and the differential sensitivity to inhibitors and uncouplers [18], provides further evidence for oleoylation and transoleoylation reactions catalyzed by mitochondrial ATPases [3]. Further studies of acylation, transacylation and deacylation reactions and attempts to prepare the postulated oleoyl-*S*-pantothenyl enzyme complex intermediate are in progress for characterization of possible roles of an acyl carrier function within the intact enzyme complex.

Acknowledgements

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