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Electrophoretic Behavior of the H^+-ATPase Proteolipid from Bovine Heart Mitochondria

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Abstract

The proteolipid subunit of H^+-ATPase was labeled by [^{14}C]*N,N'*-dicyclohexylcarbodiimide in bovine heart mitochondria. The radioactive labeling was followed using various systems of sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). When using discontinuous SDS-PAGE (Laemmli, U.K., 1970, *Nature (London)* **227**, 680–685) a monomeric (Mr 7600 ± 1500) and a dimeric form (Mr 17,800 ± 1200) of the proteolipid were detected, while only the monomeric form was found on urea (8 M) containing gels (SDS-PAGE according to Laemmli; or Swank, R. T., and Munkers, K. D., 1971, *Anal. Biochem.* **39**, 462–477). When using SDS-PAGE with Na–P_i buffer (Weber, K., and Osborn, M., 1969, *J. Biol. Chem.* **244**, 4406–4442), only a dimeric form of the proteolipid (Mr 15,000 ± 1000) was detected. Experimental data indicate that the different patterns of proteolipid separation are related to the presence of the two distinct proteolipid conformations in the SDS solution.

Key Words: Mitochondrial H^+-ATPase; bovine heart; proteolipid conformations; gel electrophoresis; dicyclohexylcarbodiimide.

Introduction

In H^+-ATPase of mitochondria, bacteria, and chloroplasts the vectorial movement of protons across the membrane is coupled to the catalytic function of the enzyme (for review see Nelson, 1981; Fillingame, 1981; Kagawa, 1982; Kopecký *et al.*, 1985). The major component of the H^+ channel is a hydrophobic subunit (proteolipid; see Cattell *et al.*, 1971) of the membrane sector $(F_0)^2$ of the enzyme, which is highly homologous in all energy-transducing membranes. Its Mr ranges from 6500 in bacteria to 8000 in chloroplasts and the proteolipid molecule consists of 76–81 amino acids.

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While the amino acid composition and the primary structure of the proteolipid are well characterized, the topological arrangement of the proteolipid in the membrane is not yet fully understood (Hoppe and Sebald, 1984; Modyanov *et al.*, 1984), although it is clear that each F_0 contains several proteolipid subunits (Sebald *et al.*, 1979; Foster and Fillingame, 1982), that are close to each other and form an oligomer (Sigrist-Nelson and Azzi, 1979). The other F_0 subunits participating in H^+ translocation (Sebald *et al.*, 1982; Downie *et al.*, 1979) are probably asymmetrically attached (Hoppe *et al.*, 1984) to this oligomer. The H^+ transport through F_0 (Hinkle and Horstman, 1971), and hence the catalytic activities of H^+-ATPase (Beechey *et al.*, 1967; Penefsky, 1985), are inhibited by *N,N'*-dicyclohexylcarbodiimide (DCCD)[2]. due to a covalent modification of a single acidic residue (GLU/ASP) of the proteolipid (Sebalt and Wachter, 1978; Altendorf *et al.*, 1979).

When H^+-ATPase is inhibited by [^{14}C]-DCCD in mammalian mitochondria, only three proteins of Mr around 33,000–35,000, 16,000–18,000, and 6500–9000 are significantly labeled (Glaser *et al.*, 1981; Houštěk *et al.*, 1981a) as resolved by polyacrylamide gel electrophoresis (PAGE)[2] in the presence of sodium dodecylsulfate (SDS)[2] according to Laemmli (Laemmli, 1970). Only two of these DCCD-reactive proteins, with lower Mr (referred to further as 18K and 8K proteins; see Table I), represent the proteolipid subunit of F_0 (Kužela *et al.*, 1980; Glaser *et al.*, 1981; Houštěk *et al.*, 1981b; Drahota *et al.*, 1981), while the largest protein of 33,000 Mr is most probably identical with porine from the outer mitochondrial membrane (De Pinto *et al.*, 1985). Concerning the F_0 subunit, the 8K protein is evidently a monomer and the 18K protein is probably a dimer of the proteolipid (Kužela *et al.*, 1980; Glaser *et al.*, 1981).

In contrast to the two forms of the proteolipid (De Jong *et al.*, 1979; Kužela *et al.*, 1980; Houštěk *et al.*, 1981a; Glaser *et al.*, 1981) found in the mammalian H^+-ATPase preparations or in the whole mitochondria using the Laemmli system (Laemmli, 1970), only a single form of the proteolipid was detected when other SDS–PAGE methods (Weber and Osborn, 1969; Swank and Munkers, 1971) were applied (Cattell *et al.*, 1971; Stekhoven *et al.*, 1972; Serrano *et al.*, 1976; Berden and Voorn-Brouwer, 1978; Dianoux *et al.*, 1978; Galante *et al.*, 1979; Ludwig *et al.*, 1980; Kiehl and Hatefi, 1980). In spite of substantial differences of the apparent Mr values of the proteolipid found in these studies (Mr 6300–13,000) the proteolipid was generally assumed to exist in SDS solution as a monomer.

[2]Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; F_0, membrane integral sector of mitochondrial H^+-ATPase; SDS, sodium dodecylsulfate; PAGE, polyacrylamide gel electrophoresis.

The present study summarizes our attempts to clarify the differences in the separation pattern of the proteolipid found by the previous studies. As shown in this paper, the anomalous proteolipid migration probably results from two distinct conformational states of the proteolipid molecule differing in their tendency to form monomers and/or dimers in various SDS-PAGE systems.

A part of the results has already been published as a symposium abstract (Drahota *et al.*, 1982).

Materials and Methods

Materials

[^{14}C]-DCCD (50 Ci/mol) was purchased from Service des Molécules Marquées (France); acrylamide, *N,N'*-methylene-bisacrylamide, SDS, and urea were from Bio-Rad (USA). The following proteins were used as Mr standards: phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soyabean trypsin inhibitor (20,100), and lactalbumin (14,000) from Pharmacia (Sweden); insulin (5700) and mellitin (2850) from Sigma. All other chemicals were of analytical grade.

Preparation of Mitochondria

Bovine heart mitochondria were isolated according to procedure 3 of Smith (Smith, 1967). Prior to further use, mitochondria were frozen-thawed three times and sedimented by centrifugation (Houštěk *et al.*, 1981a).

Incubation of Mitochondria with [^{14}C]-DCCD

Mitochondria (5 mg protein/ml) were incubated (20 h, 0°C) with 1–5 nmol [^{14}C]-DCCD per mg protein (added as an ethanolic solution; 10 μl/ml) in a medium containing 0.25 M sucrose, 1 mM EDTA, and 10 mM Tris/Cl, pH 7.4. Glass test tubes were used (Kopecký *et al.*, 1983a). This resulted in binding of 0.5–1.0 nmol [^{14}C]-DCCD/mg mitochondrial protein as estimated by liquid scintillation counting of aliquots of labeled mitochondria washed with 90% acetone (v/v) and 2.5% (w/v) trichloroacetic acid (Houštěk *et al.*, 1981a; Kopecký *et al.*, 1982).

Electrophoretic Techniques

Before SDS-PAGE, 0.15 mg protein aliquots of [^{14}C]-DCCD-labeled mitochondria were washed with 90% (v/v) acetone in order to remove mitochondrial phospholipids and hence to abolish the effect of phospholipids on the electrophoretic migration of the proteolipid (Houštěk *et al.*, 1981a;

Dianoux *et al.*, 1978). Samples were solubilized with appropriate lysis buffers (Weber and Osborn, 1969; Laemmli, 1970; Swank and Munkers, 1971) by heating on a boiling water bath for 3 min. Even though acetone treatment increased a portion of radioactivity which did not enter the gels, more than 60% of DCCD radioactivity was usually recovered from gel slices (not shown).

Electrophoresis was performed on slabs of polyacrylamide gels or in tube gels (in two-dimensional electrophoresis). All the SDS-PAGE procedures (Laemmli, 1970; Weber and Osborn, 1969; Swank and Munkers, 1971) were performed using either a fixed concentration or linear gradient of polyacrylamide (see text to figures). In the two-dimensional analysis the first-dimension tube gels were equilibrated (under shaking) for 30 min at 20°C in 10 ml of the sample lysis buffer and fixed on the top of the second dimension slab gel using 0.7% agarose dissolved in the same buffer (O'Farrell, 1975).

Radioactive proteins on the stained gels (Coomassie brilliant blue R 250) were detected by fluorography (Bonner and Laskey, 1974).

Results

Separation of Mitochondrial DCCD-Reactive Proteins in Various SDS-PAGE Systems

In accordance with the literature data (see Introduction) significant differences in the resolution of DCCD-reactive proteins were found in heart mitochondria incubated with [^{14}C]-DCCD at concentrations that are inhibitory for H^+-ATPase (Beechey *et al.*, 1967; Sebald *et al.*, 1979; Kopecký *et al.*, 1982; Glaser *et al.*, 1982) when comparing various SDS-PAGE systems (Fig. 1). When the discontinuous system of Laemmli was used (running buffer: Tris-glycine, pH 8.3; separating gel buffer: Tris-Cl, pH 8.8; stacking gel buffer: Tris-Cl, pH 6.8) three radioactive proteins were detected (Fig. 1A). In contrast, only two labeled proteins were resolved (Fig. 1C) when the Weber–Osborn (Na-P_i buffer, pH 7.0), the Swank–Munkers (Fig. 1E; Tris-glycine buffer, 8 M urea, pH 6.8), or the Laemmli system modified by addition of 8 M urea (Fig. 1F) were applied.

In all the systems compared the apparent Mr of the largest DCCD-reactive protein detected varied between 33,100–33,600 (Table I), indicating that this protein does not belong to H^+-ATPase (Houštěk *et al.*, 1981a, b; Drahota *et al.*, 1981), but it is rather identical with porine, a protein of the outer mitochondrial membrane (De Pinto *et al.*, 1985). As far as the proteolipid subunit of F_0 is concerned, on gels according to Laemmli the two forms (8K and 18K proteins) of the proteolipid were detected whereas only a single

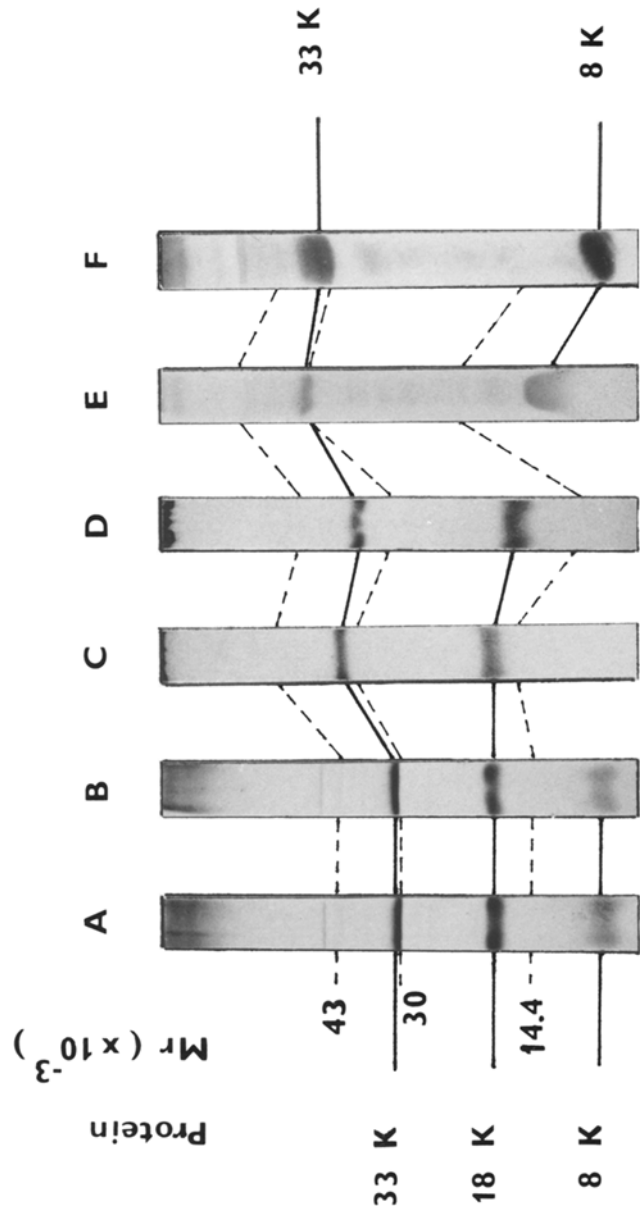

Fig. 1. SDS-PAGE of mitochondria labelled with [^{14}C]-DCCD. Analysis was performed according to Laemmli on 12–20% gel (A), on 15% Weber–Osborn gel (C), and on 15% Swank–Munkers gel (E). In (B) the (A) system was used with 8 M urea included only during solubilization of the sample before electrophoresis. In (D) the (C) system was modified so that the pH of the Na-P_i buffer was changed from 7.0 to 8.8. In order to compensate for the lowered buffering capacity of the electrophoretic medium at pH 8.8, the volume of the upper and lower buffer reservoirs was enlarged from 0.3 to 2.0 liters. In (F) the (A) system was used with 8 M urea present in stacking and separating gels and also during the solubilization of the samples. The figure shows the distribution of radioactivity in the gel as detected by fluorography.

Table I. Apparent Mr's of Mitochondrial DCCD-Reactive Proteins[a]

Gel system	Mr of DCCD-reactive protein detected			Number of estimations
Laemmli	32,100 ± 1700	17,800 ± 1200	7600 ± 1500	8
Weber–Osborn	33,600 ± 1700	15,500 ± 1000		5
Swank–Munkers	33,500		7200	2
Laemmli/urea	32,200		6600	2

[a]SDS-PAGE was performed as in Fig. 1A, C, E, and F and apparent Mr were estimated using various protein standards (see Materials and Methods).

proteolipid form was found using the other SDS-PAGE techniques (Fig. 1; Table I).

The apparent Mr (Table I) of the single form of the proteolipid on urea-containing gels (Fig. 1E, Swank–Munkers; Fig. 1F, Laemmli gels with urea) clearly indicates the presence of the monomeric form of the proteolipid. The same holds for the 8K protein on gels according to Laemmli (Table I). In contrast, the apparent Mr (close to 16,000; Table I) of the single form of the proteolipid detected on Weber–Osborn gels is very similar to that of the 18K protein (Laemmli; Table I). This suggests a dimeric nature of these two components (see below).

The detection of only the monomeric form of the proteolipid on urea-containing SDS-gels (Fig. 1E, F) is not surprising as urea increases the dissociation of noncovalent bonds between protein subunits, especially of those which are of hydrophobic nature. However, the presence of 8M urea only during solubilization of the sample did not affect the separation pattern, and, thus, on Laemmli gels both 8K and 18K proteins were detected under these conditions (Fig. 1B). This indicates that the effect of urea is reversible (see Discussion).

The existence of the two forms of the proteolipid on Laemmli gels and their absence on Weber–Osborn gels is less understandable. It was observed that the following modifications of these two techniques had no effect on the typical separation patterns (not shown): changes of the polyacrylamide concentration (10–20%) in the separating gels, the absence of mercaptoethanol during solubilization, mutual replacement of the sample lysis buffer of the two systems, or an omission of the stacking gel. As the different ionic composition of the electrophoretic buffers used in these two SDS-PAGE techniques might influence the efficiency of SDS to monomerize the proteolipid molecules, the effect of SDS concentration was also tested. However, in both methods changes of SDS concentration during solubilization (0.05–4.0%; not shown) or during separation (0.05–0.4%) in the Laemmli system (Fig. 2) had again no effect. When the SDS concentration was below 0.05%

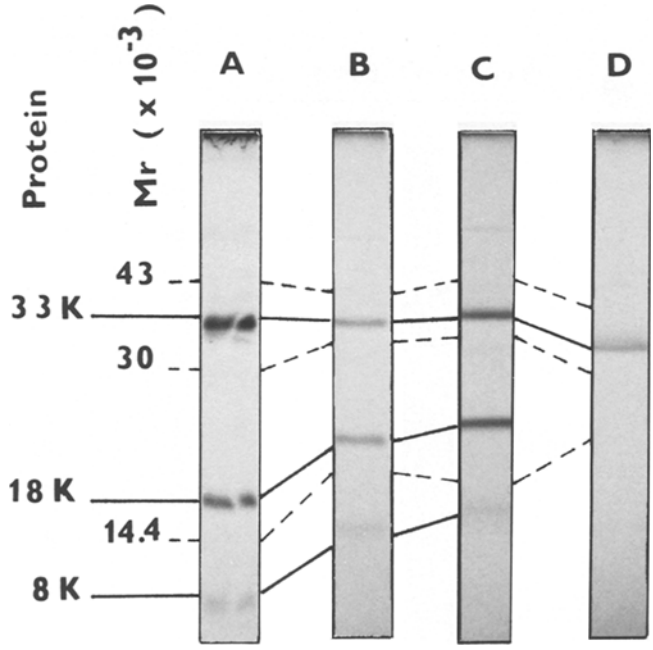

Fig. 2. Effect of SDS concentration in polyacrylamide gels on the separation of mitochondrial DCCD-reactive proteins. [^{14}C]-DCCD labeled mitochondria wee analyzed according to Laemmli (12–20% gradient gel) and radioactivity was detected by fluorography. The concentration of SDS in both separating and stacking gels was varied and was 0.40% (A), 0.10% (B), 0.05% (C), and 0.025% (D). The concentration of SDS in the running buffer (0.1%) and during sample solubilization (2.3%) remained unchanged.

neither 8K nor 18K proteins entered the gel whereas the largest (Mr 33,000) DCCD-reactive protein was still very well resolved (Fig. 2D). Also the change of pH of the Na-P_i buffer in the Weber–Osborn system from 7.0 to 8.8 had no influence on the separation pattern (Fig. 1D). Therefore, the reason for the different separation pattern of the proteolipid on the Laemmli and Weber–Osborn gels, respectively, is most probably not related to any of the above parameters (see Discussion).

Two-Dimensional SDS-PAGE of Mitochondrial DCCD-Reactive Proteins

With the aim of analyzing further the nature of the various electrophoretic forms of the proteolipid found in the Laemmli and Weber–Osborn systems, a two-dimensional SDS-PAGE was performed where the two methods were combined. When the Weber–Osborn gel was used in the first dimension, it was found that the single form of proteolipid separated in this way dissociates in the second dimension (Laemmli) yielding the 8K and 18K

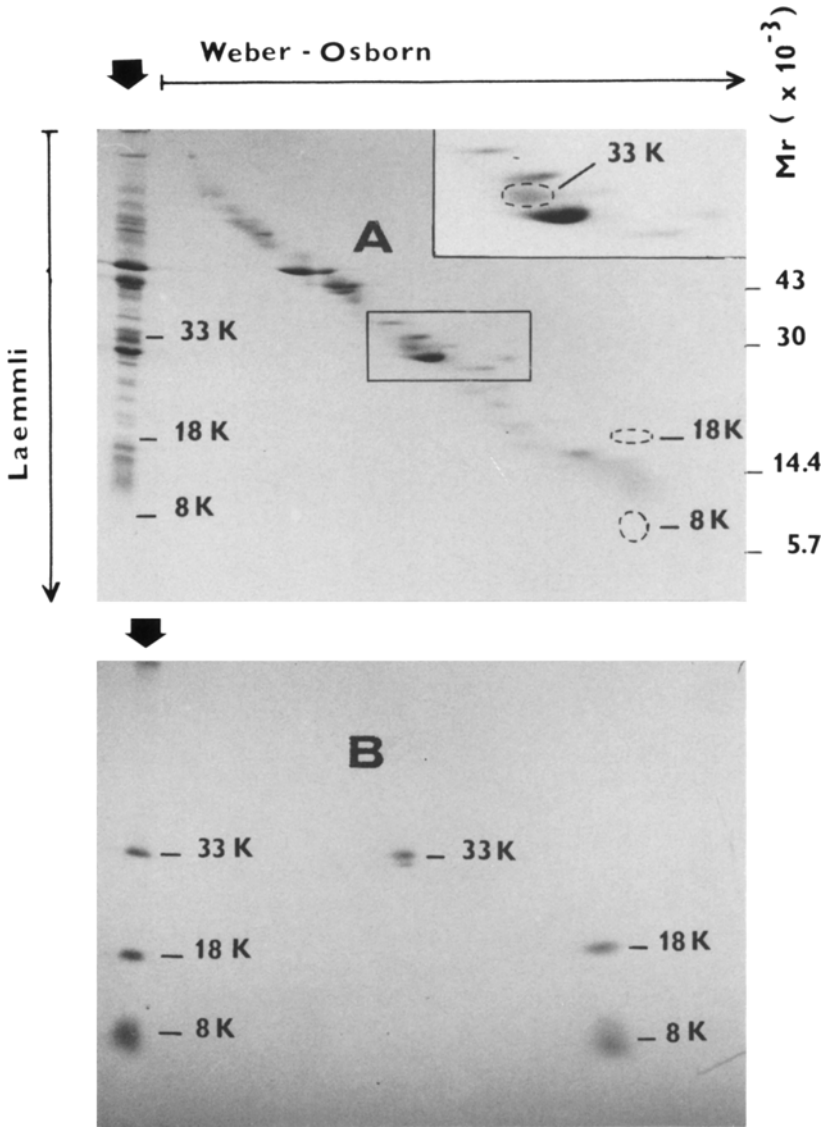

Fig. 3. Two-dimensional SDS-PAGE of [^{14}C]-DCCD-labeled mitochondria. First dimension, Weber–Osborn 10% gel; second dimension, Laemmli 12–20% gel. After staining for proteins (A), radioactivity was detected by fluorography (B) and radioactive spots were marked in (A). On the left side of the gel slab a sample of [^{14}C]-DCCD labeled mitochondria was also applied (↓).

proteins (Fig. 3B). The ratio between these two proteins was similar to that found after a direct analysis of [^{14}C]-DCCD-labeled mitochondria in the second-dimension gel.

In contrast to the 33,000–Mr DCCD-reactive protein, which could also be identified in the second dimension as a Coomassie blue stained band (Fig. 3A; inset), the 8K and 18K proteins were not stained by Coomassie blue (Fig. 3A; see Sebald *et al.*, 1979) and could be detected only by radiography (Fig. 3B). Also only in the case of the 33,000-Mr protein does a minor proteolytic digestion occur during reelectrophoresis as indicated by the appearance of a new radioactive protein with Mr around 30,000 (Fig. 3B).

Figure 4A shows the separation of proteolipid in a "reverse" two-dimensional system where the Laemmli technique was used in the first and the Weber–Osborn technique in the second dimension, respectively. Under these conditions the two species separated in the first dimension were recovered in the second dimension as two components with an equal mobility, corresponding to an Mr of 16,000. Their positions, with respect to the first

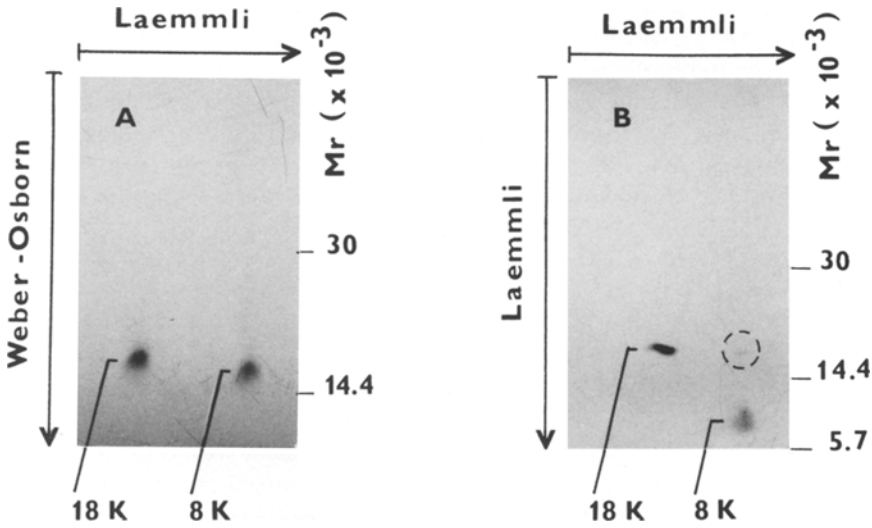

Fig. 4. Two-dimensional SDS-PAGE of [^{14}C]-DCCD-labeled mitochondria. For the separation in the second dimension only the anodic portion of the first-dimension gels were used containing proteins of Mr below 25,000. (A) First-dimension Laemmli 15% gel; second dimension Weber–Osborn 10–16% gel. (B) First-dimension 15% gel and second-dimension 12–20% gel; both according to Laemmli. In (B), the higher Mr aggregate of 8K form is marked by dotted lines; 8K and 18K refer to migration of the two forms of the proteolipid in the first dimension. The apparent Mr's estimated in the second dimension (not shown) were 16,000 (A; for both 8K and 18K form) and 7600 and 17,800 (B; for 8K and 18K form, respectively). Radioactivity was denoted by fluorography.

dimension (Laemmli), clearly indicated that they originated from 8K and 18K proteins.

These data show that the separation pattern of the proteolipid is always preserved in a given SDS-PAGE system, no matter whether the sample has previously been electrophoresed in another system. Concerning the nature of the single form of the proteolipid in the Weber–Osborn system, its formation from 8K protein of the Laemmli system (Fig. 4A) shows that it does not result from an aggregation of the proteolipid with some other protein (F_0 subunit) but rather represents a dimer of the proteolipid. In fact, the apparent Mr 15,500 $\pm$ 1000 of the single proteolipid form on Weber–Osborn gels found in this study is somewhat higher than that determined previously (9000–14,000; Cattell *et al.*, 1971; Stekhoven *et al.*, 1972; Serrano *et al.*, 1976; Dianoux *et al.*, 1978; Galante *et al.*, 1979; Kiehl and Hatefi, 1980; Ludwig *et al.*, 1980), when the proteolipid was assumed to migrate in the monomeric form. It should be stressed, however, that in this study the apparent Mr was carefully estimated not only on linear polyacrylamide gels (Fig. 1C; Table I), but also on gradient gels (Fig. 4A), yielding identical results.

When using the Laemmli system in both dimensions (Fig. 4B), both the 8K and 18K protein were recovered in the second dimension. Importantly, during this type of reelectrophoresis no dissociation of the 18K protein occurred. A marginal aggregation of the 8K and 18K protein detected indicates that the 18K protein is also a dimeric form of the proteolipid. This is also in accordance with the conversion of the 18K into 8K protein after performic acid treatment (Glaser *et al.*, 1981) and with identical isoelectric points (7.5) of the two proteins (Kužela *et al.*, 1980).

Discussion

In the present study the separation of the proteolipid subunit of mitochondrial H^+-ATPase was systematically reevaluated using various SDS-PAGE techniques. The results obtained are consistent with different separation patterns found previously under various electrophoretic conditions.

As demonstrated by two-dimensional SDS-PAGE (see below), the observed migration of the proteolipid in its monomeric and/or dimeric form in various SDS-PAGE systems cannot result from a different, but random aggregation in the SDS solution under different electrophoretic conditions. More likely, the data reflect the two distinct conformational states of the proteolipid in SDS solution. According to this concept, in the Laemmli system the two proteolipid conformations migrate as the monomeric (8K) and dimeric (18K) form, respectively. In the presence of urea all the

proteolipid molecules are present in the monomeric form (Fig. 1E, F), but the two conformational states of the proteolipid have to be retained, because when analyzing samples solubilized by SDS and urea by a regular Laemmli system, the 8K and 18K proteins are again retained (Fig. 1B). Similarly, the two proteolipid conformations have to be retained on Weber–Osborn gels (Fig. 3B), where all the proteolipid molecules migrate as dimers (Fig. 4A). The experimental data supporting the concept of the two conformational states include: (a) the virtual absence of any dissociation of the 18K protein and only a marginal aggregation of the 8K into 18K protein during reelectrophoresis in the Laemmli system (Fig. 4B); and (b) the quantitative recovery of the 8K and 18K proteins in the Laemmli system after prior urea treatment (Fig. 1B) or electrophoresis on Weber–Osborn gels (Fig. 3B).

It is possible that two distinct conformational states of the proteolipid in SDS solution are related to the native structure of the proteolipid. In fact, it has been suggested that in mammalian mitochondria two states of the native proteolipid exist, which remain preserved after solubilization by SDS (Glaser *et al.*, 1981; Houštek *et al.*, 1981a, 1982; Kopecký *et al.*, 1985). Support for this hypothesis comes from the different reactivity with DCCD of the two 8K and 18K proteins found in the Laemmli system (Glaser *et al.*, 1981; Houštěk *et al.*, 1981a; Kopecký *et al.*, 1983b), and from corresponding different sensitivities to DCCD of the catalytic functions of H^+-ATPase (Kopecký *et al.*, 1981; Glaser *et al.*, 1981; Houštěk *et al.*, 1981a; Kopecký *et al.*, 1982; Kopecký *et al.*, 1984) and passive H^+-translocation through F_0 (Kopecký *et al.*, 1981; Glaser *et al.*, 1982; Kopecký *et al.*, 1983b), which are related to the interaction of DCCD with the 8K and 18K proteins, respectively. Importantly, the two proteolipid forms are not induced by DCCD-ATPase interaction (Glaser *et al.*, 1981). It is also noteworthy that circular dichroism spectroscopy indicated the preservation of the native secondary structure of the proteolipid after solubilization by SDS (Mao *et al.*, 1982).

With respect to the specific capability of the Laemmli system to discriminate between the two proteolipid conformations, it is to be stressed, however, that the present approach cannot explain whether the dimeric form of the proteolipid was retained, or formed in the SDS solution. The higher resolving power of the Laemmli system in comparison with that of the Weber–Osborn technique might result from the discontinuous system of the electrophoretic buffers used only in the former technique (Tris-glycine-Cl versus Na-P_i), or, alternatively, the specific effect of some of the components of the electrophoretic might be involved. Interestingly, the two 8K and 18K proteins were also found using another discontinuous SDS-PAGE method (Sebald *et al.*, 1979) with the running (Tris-Cl) and gel (Tris-acetate) buffer of constant pH (8.0).

Even though the two 8K and 18K proteins were repeatedly observed in mammalian mitochondria or in H^+-ATPase preparations (De Jong *et al.*, 1979; Sebald *et al.*, 1979; Kužela *et al.*, 1980; Houštěk *et al.*, 1981a; Glaser *et al.*, 1981) in yeast and *Neurospora* mitochondria only the monomeric form of the proteolipid was found under the above SDS-PAGE conditions (Sebald *et al.*, 1979). On the other hand, in yeast a higher Mr (45,000) form of the proteolipid was described on Weber–Osborn gels (Tzagoloff and Meagher, 1972; Tzagoloff and Akai, 1972), representing probably the native hexameric arrangement of the proteolipid (Sebald *et al.*, 1979). In contrast to the 18K protein found in mammalian mitochondria (Glaser *et al.*, 1981; Houštěk *et al.*, 1981a), in the case of the yeast proteolipid hexamer a dissociation into the monomeric units was observed after the chloroform/methanol treatment (Tzagoloff and Akai, 1972). These data might indicate that the structural organization of the oligomeric proteolipid assembly in mitochondria from lower eukaryotes differs from that in higher eukaryotes.

In conclusion, the data of this study are consistent with the existence of the two different conformational states of the proteolipid in mammalian F_0, which remain preserved in SDS solution and cause a different separation pattern in various SDS-PAGE systems. Further studies are required to characterize the physicochemical properties of these two states of the proteolipid.

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