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Proteolipid of Adenosinetriphosphatase from Yeast Mitochondria Forms Proton-Selective Channels in Planar Lipid Bilayers†

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ABSTRACT: Proteolipid isolated from yeast mitochondrial adenosinetriphosphatase by butanol extraction is reincorporated into lipid vesicles from which planar membranes are formed. The proteolipid permits electric conductance through the membrane. This conductance occurs through membrane channels which are highly selective for protons. Proton channels in the membrane are directly observed at high proton concentrations in the aqueous phases. Channels open and close independently from each other; their open-state conductances and lifetimes are monodisperse but influenced by the applied voltage (12 pS and 3 s, respectively, at pH 2.2 and 100 mV). Proton channels do not occur in single proteolipid molecules; the conducting structure consists of at least two polypeptide chains since channels form in a (reversible) bimolecular reaction of nonconducting forms of proteolipid. The number of proton channels at a constant proteolipid concentration changes in sharp transitions and by orders of magnitudes upon critical changes of membrane composition and pH. These transitions are caused by transitions of proteolipid organization in the membrane from a dispersed state (equilibrium between channel-forming "dimers" and a large pool of "monomers") to a state of almost complete aggregation of proteolipid which stabilizes large proton-conducting structures (probably associates of channel-forming dimers). This self-association of isolated proteolipid into structures containing proton-selective channels suggests that the six proteolipids in the adenosinetriphosphatase complex exist as a self-associating entity containing most likely three proton channels.

The *N,N'*-dicyclohexylcarbodiimide (DCCD)[1]-binding protein has been recognized as a main constituent of the proton channel in proton-translocating ATPases from bovine heart mitochondria (Racker, 1976, 1977), *Escherichia coli* membranes (Altendorf, 1977; Fillingame, 1980), and thermophilic bacteria (Kagawa, 1978). The first successful reconstitution was reported for DCCD-binding protein ("proteolipid") from

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[1] Abbreviations: ATPase, adenosinetriphosphatase; DCCD, *N,N'*-dicyclohexylcarbodiimide; $NaDodSO_4$, sodium dodecyl sulfate; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine.

0006-2960/82/0421-5787$01.25/0

butanol-extracted chloroplast membranes: the reconstituted lipid–proteolipid vesicles exhibited a high and DCCD-sensitive proton permeability (Nelson et al., 1977a,b). This work has been confirmed and extended in more recent studies that employed a variety of techniques (Sigrist-Nelson et al., 1978; Sigrist-Nelson & Azzi, 1979, 1980; Nelson, 1980). Criddle et al. (1977) used the method of phospholipid-impregnated Millipore filters to demonstrate oligomycin-sensitive proton conductance induced by the purified proteolipid from yeast mitochondria. However, it was subsequently reported that this preparation also induced permeability to potassium ions (Criddle et al., 1979). There is some debate as to whether the Millipore filter technique is suitable for measuring proton conductance across the filters (Moran et al., 1980). As yet, there has been no report on the reconstitution of the proteolipid into planar lipid bilayers which is the most suitable system for studying membrane channels [for a review, see Montal et al. (1981)]. Here, we describe the formation of active and specific proton channels upon reconstitution of the proteolipid from yeast mitochondria into planar bilayers.

Materials and Methods

The phospholipids 1-oleoyl-2-palmitoyl-3-alkyllecithin (hereinafter designated as [18(1),16(0)]-ether lecithin) and 1,2-dioleoylglycerolecithin were purchased from Berchtold (Biochemical Laboratories, Bern, Switzerland). 1,2-Dioleoyl-3-alkyllecithin (designated [18(1),18(1)]-ether lecithin) was a gift from Dr. F. Paltauf (Technical University of Graz, Austria). Cholesterol (purissium) was obtained from Fluka (Buchs, Switzerland). Asolectin (Sigma) was purified according to Kagawa & Racker (1971). The aqueous phases used for vesicle suspensions and planar bilayer formation contained, if not stated otherwise, 50 mM KCl and 20 mM citric acid–phosphate buffer from the potassium salts of citrate and phosphate adjusted by the addition of phosphate to pH values between 2.2 and 6.8 (buffer A).

Yeast mitochondria were prepared as previously described (Maccecchini et al., 1979) and stored at −70 °C. Published procedures were used for protein determination (Lowry et al., 1951) and $NaDodSO_4$–polyacrylamide gel electrophoresis (Douglas & Butow, 1976).

Isolation of Proteolipid. Yeast mitochondria containing about 50 mg of protein were thawed and diluted 20-fold with 10 mM Tricine–NaOH, pH 8. The suspension was centrifuged at 200000*g* for 20 min, and the pellet was homogenized in 2 mL of 10 mM Tricine–NaOH at pH 8. The suspension was injected into 100 mL of vigorously stirred 1-butanol at 0 °C. After incubation at 4 °C for 16–20 h under stirring, the suspension was centrifuged at 20000*g* for 10 min, and the pellet was discarded. The supernatant was recentrifuged as before. Special care was taken to avoid carry-over of chunks from the pellets to the supernatant. The volume of the supernatant was reduced to about 5 mL by a stream of nitrogen gas, and the preparation was stored at 4 °C until used.

Radiolabeled Proteolipid. Yeast spheroplasts [2 g (wet weight)] were prepared and suspended to 100 mg wet weight/mL in labeling medium, as described (Nelson & Schatz, 1979). Labeling was for 30 min at 28 °C with 1 mCi of [^{35}S]methionine (100–300 Ci/mmol) in the presence of 100 μg of cycloheximide/mL. The spheroplasts were then washed once with 100 mL of 1.3 M sorbitol. Mitochondria and proteolipid were isolated from the labeled spheroplasts as described above. For labeling with [^{14}C]DCCD, isolated yeast mitochondria containing about 5 mg of protein in 1 mL of 10 mM Tricine, pH 8, were incubated for 30 min at room temperature with approximately 20 μCi of [^{14}C]DCCD (Centre d'Etudes Nucléaire de Saclay, Gif-sur-Yvette; 50 Ci/mol). Two 10-μL aliquots were removed, dissociated with 2% $NaDodSO_4$ and 2% mercaptoethanol, and electrophoresed in 10–15% exponential gradient polyacrylamide gels (Douglas & Butow, 1976). The remaining membranes were used for purification of proteolipid.

Preparation of Vesicles. Vesicles containing proteolipid were made by two methods. Both started from lipid and proteolipid in butanol (5 mg of lipid/mL of butanol and aliquots of proteolipid stock solution containing 20 μg of proteolipid/mL).

(A) Vesicles were formed (Schindler & Quast, 1980) by drying lipid–proteolipid solutions to surface films in a round-bottom flask by nitrogen and resuspending the film into buffer A to typically 0.5–1 mg of lipid/mL. Filtration of these vesicles through a 200-nm Unipore filter increased the size uniformity and stability of the vesicles as judged from negative-stain electron micrographs and from vesicle spreading into monolayers at the air–water interface (Schindler, 1979). Such samples were used for all results except for the proteolipid concentration dependence of the membrane conductance where an alternate technique B was used.

(B) Vesicles were prepared by the injection method (Kremer et al., 1977) adapted to the use of butanol instead of ethanol. One part of a solution containing lipid or lipid and proteolipid was injected into 20 parts of buffer A (4 times diluted with water). This suspension was sonicated in a bath sonicator for 3 min. The solvent was removed under reduced pressure by a rotary evaporator at 35 °C. Evaporation was continued until the buffer volume had diminished 4 times. The resulting vesicle suspension (1 mg/mL) was filtrated through a 200-nm filter and stored at room temperature. The proteolipid to lipid molar ratio in the vesicles (X_0) was estimated from the known molar ratio in butanol.

Planar bilayers were formed from these vesicle samples as described (Schindler, 1980), and electrical measurements were according to Schindler & Feher (1976). Membrane area was 2×10^{-4} cm^{-2}. All experiments were carried out at room temperature.

Results

Properties of the Purified Proteolipid. When a sample of the purified proteolipid was electrophoresed in a $NaDodSO_4$–polyacrylamide gel slab, only a single band was detected by protein staining. However, the proteolipid was poorly stained. So that the sensitivity of detection could be enhanced, the proteolipid was isolated from yeast cells that had been labeled with [^{35}S]methionine in the presence of cycloheximide in order to specifically label mitochondrially synthesized proteins, such as the proteolipid. Figure 1 shows that ^{35}S-labeled purified proteolipid (lane 3) gave a major labeled band which comigrated with proteolipid from [^{14}C]DCCD-labeled mitochondria (lane 4). In contrast, [^{14}C]DCCD-labeled mitochondrial membranes yielded a major radioactive band of much higher molecular weight (lane 5). This band almost certainly represents the proteolipid hexamer (Tzagoloff et al., 1976; Hoppe & Sebald, 1982) which is known to be depolymerized by organic solvents. A second radioactive band migrated with the tracking dye and is probably soluble DCCD. These observations, together with the fact that histidine could not be detected in the proteolipid preparation by amino acid analysis (not shown), indicate that the preparation predominantly contained the proteolipid, also termed "subunit 9", of the yeast mitochondrial H^+–ATPase complex.

Formation of Planar Membranes. The formation of planar membranes from lipid and proteolipid was carried out in two

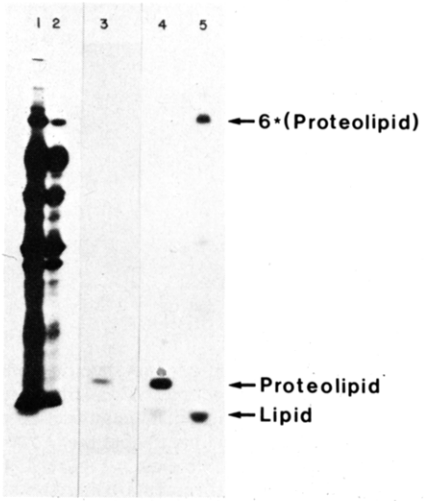

FIGURE 1: Isolation of proteolipid from yeast cells labeled with [^{35}S]methionine in the presence of cycloheximide and from mitochondria labeled with [^{14}C]DCCD. (Lanes 1–5) A radioautogram of a $NaDodSO_4$–polyacrylamide gel slab (see Materials and Methods) is shown. (Lane 1) [^{35}S]Methionine-labeled mitochondrial membranes (50 μg of protein); (lane 2) as in lane 1, but with 10 μg of protein; (lane 3) proteolipid (2 μg) from [^{35}S]methionine-labeled spheroplasts; (lane 4) purified proteolipid (2 μg) from mitochondria labeled with [^{14}C]DCCD; (lane 5) mitochondria (50 μg) labeled with [^{14}C]DCCD. The positions of lipid, of proteolipid, and of the proteolipid hexamer (6 × proteolipid) are indicated by arrows.

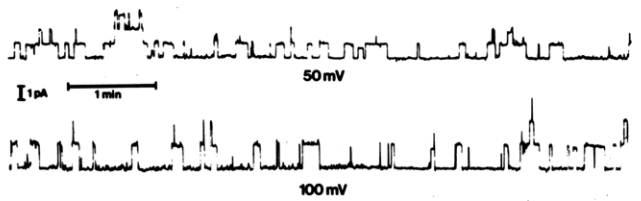

FIGURE 2: Discrete current transitions in a lipid–proteolipid planar membrane at pH 2.2 for two voltages. The molar ratio X_0 of proteolipid to lipid was 10^{-7}. Lipids contained [18(1),16(0)]-ether lecithin and 10 wt % cholesterol. For the aqueous phases, buffer A was used.

steps: First, vesicles were formed from lipid and proteolipid; second, the resulting proteoliposomes were converted into planar membranes via the spontaneous formation of two monolayers from the proteoliposomes followed by the apposition of the monolayers to a planar bilayer (Schindler, 1980). This paper describes the electrical properties of the resulting planar membranes with respect to pH dependence and membrane composition. Results are confined to the analysis of symmetrical membranes; that is, both vesicle suspensions and thus the two monolayers had the same composition.

Proteolipid Forms Proton-Selective Channels at Low pH. Detection of proton channels in planar membranes requires proton concentrations sufficiently high to resolve currents flowing through single channels. Electrical measurements on lipid–proteolipid membranes were, therefore, done at low pH values. Below pH 3, it became apparent that changes in membrane current indeed occurred between discrete levels. At pH 2.2 and for molar proteolipid to lipid ratios below 10^{-6}, the stepwise current changes were sufficiently resolved for quantitation. Figure 2 shows current traces observed at pH 2.2 for electrical potential differences (voltage) of 50 and 100 mV, respectively. It is generally accepted that such stepwise changes in membrane current reflect transitions, such as opening and closing, of single ion pathways in the membrane. The following results indicate that the observed activity of ion pathways represents the operation of a single type of pathway: (a) For a given applied voltage, the observed conductance steps

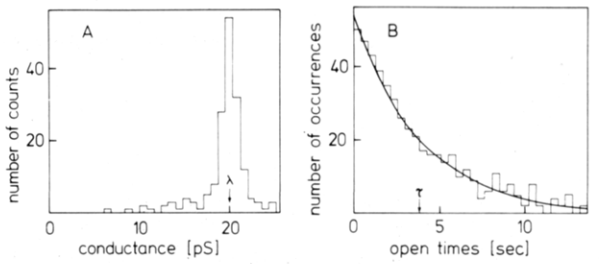

FIGURE 3: Statistical properties of discrete conductance transitions at pH 2.2. Conditions as in Figure 2 at 50 mV. (A) Probability of single discrete conductance changes. The mean conductance change λ is indicated and given in units of picosiemens (1 pS = $10^{-12}/\Omega$). The standard deviation from λ is ±0.75 pS. (B) Probability of time intervals the pathways stay open. The curve is a single exponential fitted to the data with the characteristic lifetime τ.

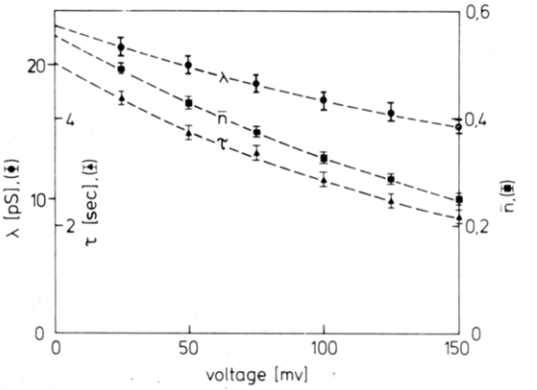

FIGURE 4: Voltage dependences of the single channel characteristics λ and τ and of the mean number of channels $\bar{n}$. Conditions as in Figure 2 at different voltages.

Table I: Probability of Occurrence of n Simultaneously Open Pathways[a]

n open channels	exptl probability, $W_e(n)$	Poisson probability, $W_p(n)$
0	0.420	0.412
1	0.347	0.365
2	0.165	0.162
3	0.054	0.041
4	0.008	0.011

[a] The $W_e(n)$ values were obtained from areas under the peaks in an amplitude histogram of a channel trace with multiple niveaus. 2^{12} samples were taken at intervals of 0.1 s. Poisson probabilities were calculated for the mean $\bar{n}$ of 0.886.

were sharply distributed about a mean step size, λ (Figure 3A). (b) The probability that pathways stayed open for time t (open times) fell on a single exponential with a characteristic time τ (lifetime) as shown in Figure 3B. (c) The probability for the simultaneous occurrence of more than one open pathway followed the Poisson statistics (Table I). This result indicates that the observed activity of the pathway system corresponds to the independent action of individual pathways. Also, compared to the average number of channels in the open state ($\bar{n}$), there must be many more pathways in the closed state. Homogeneity of these independent pathways is evident from their common open-state conductance λ and their common open-state lifetime τ. In the following, this pathway will be termed "channel" without any structural connotation yet.

Channel Characteristics Are Dependent on Voltage. The three characteristics λ, τ, and $\bar{n}$ of the single channel traces were evaluated at different voltages. From the two traces in Figure 2, recorded at different voltages, it may be directly seen that the current through the channels does not increase in strict

proportion to a voltage increase or, equivalently, the single channel conductance is dependent on voltage. The measured single channel conductances (λ) for voltages up to 150 mV are plotted in Figure 4 (upper trace, left outside scale). From the same single channel recordings, the channel lifetimes (τ) and the average number of open channels ($\bar{n}$) were estimated. These two characteristics exhibit about the same dependences on voltage as shown in Figure 4. Each of these three sets of data for λ, τ, and $\bar{n}$ could be well fitted to a single exponential (connecting lines in Figure 4) with half-maximal values at the voltages $V_{1/2}$ of 240 ± 20 mV for λ, 130 ± 10 mV for $\bar{n}$, and 125 ± 10 mV for τ. The error bars in the figure refer to the analysis of recordings from the same membrane. From membrane to membrane, larger variations of the $V_{1/2}$ values were found (up to 50%); the ratios of $V_{1/2}$ values for λ, $\bar{n}$, and τ remained, however, about 2:1:1. This indicates a high sensitivity of the channel to changes in membrane properties but also a mechanistic linkage between its characteristics.

Channel Is Proton Selective. Titration of pH symmetrically on both sides of the membrane revealed a linear decrease of the single channel conductance λ with decreasing proton concentration. The titration was done between pH 2.2 and 3, which was the limit of channel resolution. Increases of KCl or NaCl concentrations on both sides of the membrane from 10 to 500 mM did not change the single channel conductance λ within experimental error. We conclude that the channel is permeable to protons and that the permeabilities for potassium, sodium, or chloride ions are at least 1000-fold lower than the permeability for protons. About 10^7 protons pass each channel per second at 100-mV membrane potential and pH 2.2.

Number of Channels Increases with the Square of the Proteolipid Concentration. Single or multiple channel traces do not contain direct information about the number of proteolipid molecules required to form a channel. Since each H^+–ATPase complex may contain six proteolipid molecules (Nelson, 1980; Hoppe & Sebald, 1982), it would be of considerable value to know how many proteolipid molecules are needed to form a proton channel under our experimental conditions. As a first step, the dependences of proton conductance on proteolipid concentration were measured in order to determine the molecularity of the reactions which open and close the channels. This required adjustment of the proteolipid concentration in the membrane in a sufficiently quantitative manner. For this, the following strategy was used. Vesicles were formed from lipid and proteolipid in molar ratios of typically 10^6 to 1. Different aliquots of this vesicle sample were mixed with pure lipid vesicles. This dilution of proteolipid at constant lipid concentration allowed adjustment of the proteolipid content in the vesicle sample used for planar bilayer formation. The molar proteolipid to lipid ratio X ranged from that in the undiluted sample (referred to as X_0) to any lower value. Figure 5 shows conductance values observed at pH 2.2 and 50 mV for different dilutions of the same lipid–proteolipid sample ($X_0 = 2.5 \times 10^{-6}$). The data for the highest dilution ($X = X_0/25$) matched well with the average conductance levels in single channel traces (square symbol) obtained from undiluted samples with $X_0 = 10^{-7}$. The observed conductances depend on approximately the second power of the proteolipid concentration. The broken line in Figure 5 represents the best fit to the data and indicates a power of 2.1 with 0.2 standard deviation. The reaction, which leads to channel opening, has, therefore, a molecularity of 2.

Proteolipid-Induced Conductance at Different pH Values. Evidently it is of importance to relate these observations at

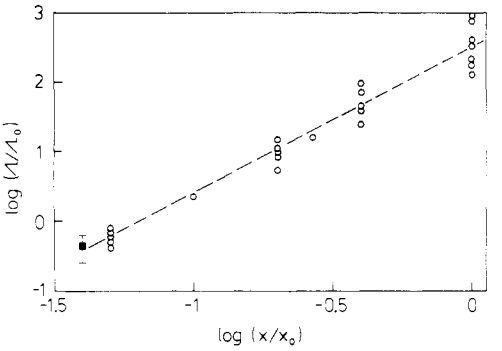

FIGURE 5: Dependence of proton conductance on proteolipid concentration at pH 2.2. The conductance Λ was measured at 50 mV and normalized to the single channel conductance of 20 pS at 50 mV (Λ_0). The molar ratio X_0 of proteolipid to lipid was 2.5×10^{-6}. Lipid- and aqueous-phase compositions were as in Figure 2. Open circles are results of independent measurements using the same proteolipid-containing vesicle sample with ratio X_0 at different dilutions with lipid vesicles. The broken line represents the best fit to the data and indicates an increase of conductance with the power of 2.1 of proteolipid concentration. The square symbol refers to the average conductance in single channel traces (like the ones in Figure 2) for an undiluted sample with a low proteolipid to lipid ratio of 10^{-7}. The latter data have been included to demonstrate that the dilution method allows a proper adjustment of proteolipid to lipid ratios by using only one proteolipid-containing vesicle sample.

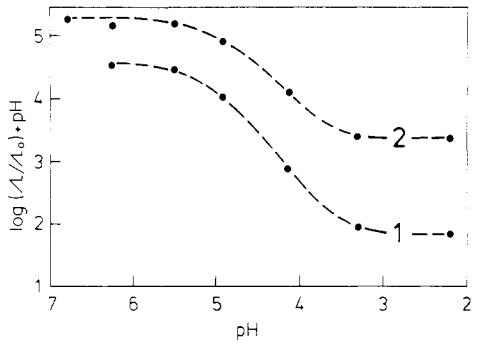

FIGURE 6: pH dependence of conductance for two proteolipid concentrations. The conductance Λ was measured at 50 mV and normalized to 20 pS (Ω_0) as well as to the proton concentration. Proteolipid to lipid ratio X_0 was 10^{-7} (curve 1) and 5×10^{-7} (curve 2). Broken lines are hand drawn. The data points along each curve were obtained on the same membrane by stepwise pH titration from pH 2.2 to higher values. For lipid composition, see the legend to Figure 2.

pH 2.2 to data at physiological pH values. This was approached by pH titration symmetrically at both sides of proteolipid-containing planar bilayers. Results are shown in Figure 6 for two different proteolipid to lipid ratios. The observed conductances, normalized to the proton concentration, exhibited a transition to higher values from pH 3 to pH 6 with midpoints at pH 4.5. The relative conductance increase during the transition depended on the proteolipid concentrations as evident from the two curves in Figure 6. At pH 5.5 (see Figure 7A, filled circles), an approximately linear relation between conductance and proteolipid concentration was found except for a slight systematic deviation at higher proteolipid concentrations. Thus, the second-order dependence found at low pH (Figure 5) changed to a first-order relation when the transition was passed to higher pH values. Concomitant with this, the current–voltage characteristic of the membrane also changed as shown in Figure 7B. The lower curve was recorded at pH 2.2 and the upper curve at pH 5.5. During these changes of conductance magnitude and dependencies on proteolipid and on voltage, the conductance remained, however, highly proton selective. Nernst potentials were measured in

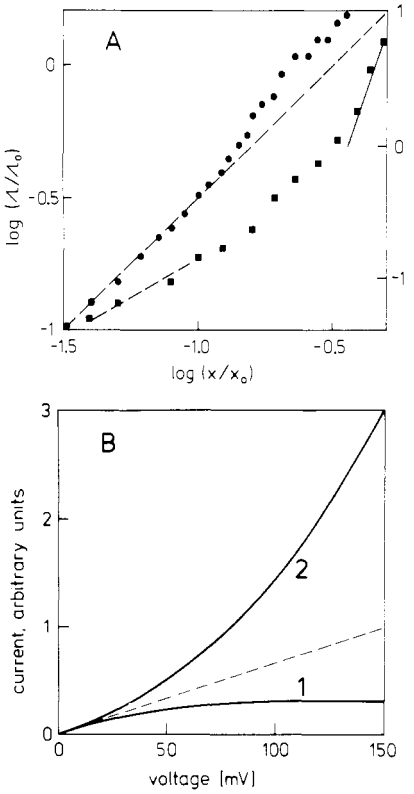

FIGURE 7: Comparison of membrane properties at different pH values. (A) Dependencies of proton conductance on proteolipid concentration X at pH 5.5 (circles and left scale) and pH 4.5 (squares and right scale). The data were obtained under conditions identical with the corresponding data in Figure 5 for pH 2.2 (at 50 mV, Λ_0 = 20 pS and $X_0 = 2.5 \times 10^{-6}$). Dashed lines indicate linear dependence, and the fully drawn line indicates a sixth power dependence of conductance on proteolipid concentration. (B) Current–voltage relation at pH 2.2 (curve 1) and pH 5.5 (curve 2). The relations are virtually independent of proteolipid concentration. The dashed line indicates ohmic behavior.

the presence of a 10-fold proton concentration gradient (pH 6 and pH 5); their values ranged between 58 and 58.5 mV. In the presence of only a KCl gradient (0.1 and 1 M KCl at pH 5.5), the Nernst potential assumed values between 56 and 58 mV which indicates that the permeability for potassium ions exceeds that for chloride ions by at least a factor of 100. Application of both a pH and a KCl gradient (pH 5 and 0.1 M KCl at side 1, pH 6 and 1 M KCl at side 2) resulted in potential differences (side 1 minus side 2) of 29, 30, and 32 mV in three independent experiments. When these three sets of data are introduced into the Goldman equation, the permeability ratio p_H/p_K is calculated to be at least 10^4.

These properties of proteolipid-containing membranes at both sides of the transition are distinguished from conductance features found within the transition region. The conductance–proteolipid concentration dependence showed, at pH 4.5, no unique power law but ranged from the first order up to the tenth order with increasing proteolipid concentration. This is shown in Figure 7A (square symbols). Spontaneous transient breakdown of the proton selectivity in favor of potassium conductance occurred frequently in the transition region. In this context, it should be mentioned that small amounts of chloroform or methanol (>0.1% in the aqueous phases) abolished proton selectivity in an irreversible manner. This effect was especially pronounced at pH 4.5.

Effect of DCCD. At pH 5.5, the effect of *N,N'*-dicyclohexylcarbodiimide (DCCD) on the proton conductance was investigated. This compound is known to bind covalently to the proteolipid and to block proton conductance of the H^+–

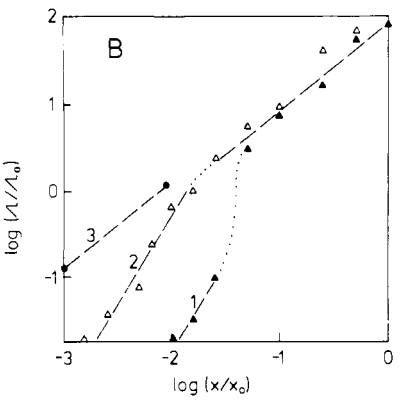

FIGURE 8: Relation between conductance (Ω) and proteolipid concentration (X) for different choices of lipids. Filled triangles (curve 1) refer to [18(1),16(0)]-ether lecithin without addition of cholesterol, open triangles (curve 2) refer to asolectin, and curve 3 indicates the data from Figure 7A (filled circles) obtained for ether lipids and 10 wt % cholesterol. The proteolipid to lipid ratio X_0 was 10^{-4}, and Ω_0 was equal to 20 pS. The dashed straight lines indicate a power index of 1 (two upper lines) and of 2 (two lower lines). The pH value was 5.5.

ATPase complex (Hoppe & Sebald, 1982). The response to the application of 10^{-6} M DCCD from a stock solution of 10^{-4} M DCCD in dimethylformamide was barely reproducible on a quantitative level but always was biphasic. Initially, the conductance rose 2–10-fold. During this phase, the membranes became transiently permeable to ions other than protons, such as to potassium ions as evaluated from Nernst potentials and titrations of potassium ions. This was followed by a slow (5 min) decrease of conductance to zero or, sometimes, to residual levels of up to 10% of the maximal value which partly or wholly represented K^+ conductance.

Lipid Dependencies. The single channel characteristics evaluated at low pH were, qualitatively, independent of the choice of lipids. Exchange of [18(1),16(0)]-ether lecithin, used for all results presented so far, for [18(1),18(1)]-ether lecithin, and both phospholipids with or without 10 wt % cholesterol, yielded only small changes of the number and properties of the channels. A drastic lipid dependence was found, however, at the high pH side of the conductance transition. This is exemplified in Figure 8 for conductance–proteolipid concentration dependencies (Λ–X relations) at pH 5.5 for three choices of lipids. There are two main aspects: (1) At sufficiently high proteolipid concentration (the X_0 value is 10^{-4} in Figure 8), the Λ–X relations approach a common linear dependence as indicated by the dashed line in the upper right part of the figure. For these high proteolipid concentrations, the results for different lipids were similar within the entire pH range investigated, including the conductance transition at about pH 4.5. (2) This similarity was lost toward lower proteolipid concentrations. For [18(1),16(0)]-ether lipids with 10 wt % cholesterol at pH 5.5 (curve 3, data from Figure 7A), the linearity of the Λ–X relation extended to the lowest conductance levels which could be resolved. When the same ether lipid was used without any cholesterol (curve 1), the conductance dropped rather sharply around X values of 4×10^{-6} and decreased with a higher power than 1 (the dashed line indicates a power of 2). In this instance, no, or only a slight, conductance transition at pH 4.5 was found. The conductance continuously decreased with increasing pH, and the square power law, between Λ and X, found at low pH, was preserved within the large experimental error at the correspondingly low conductance levels at pH 5.5. This continuity of conductance properties with pH was found also for [18(1),18(1)]-ether lecithin and proteolipid to lipid ratios X below 2×10^{-6}. A

similar result was obtained by using asolectin as the host lipid for the proteolipid (curve 2). The power of the Λ–X relation changed around X values of about 2×10^{-6} from 1 to about 2 without a steep conductance drop as in curve 1. A systematic study of the Λ–X relation for asolectin at low proteolipid concentrations revealed a spread of power indexes between 2 and 3 with a mean of 2.3. It should be added that curve 1, in contrast to the other data, was difficult to obtain due to membrane instability and occasional breakdown of proton selectivity; only those data were chosen where the membranes were stable for at least 0.5 h without evidence for artifactual potassium conductances assayed by a 10-fold increase of the potassium concentration at the end of each experiment.

Discussion

The purified proteolipid from yeast mitochondria H^+–ATPase was shown to form proton-selective channels in planar phospholipid bilayers at pH values sufficiently low to resolve single proton channels. The observed activity of channels represents the operation of one type of channel with characteristic values for the open-channel conductance and lifetime. The average number of channels simultaneously open follows the Poisson statistics. This implies that (a) the activities of the individual channels are uncorrelated and (b) that only a small proportion of all available proteolipid molecules form open channels at any given time.[2]

The formation of channels from the large pool of proteolipid molecules was shown to involve a bimolecular reaction. The obvious choice for the reacting species in the bimolecular reaction is the proteolipid monomer. In principle, however, the reacting species might already be a dimer or trimer. Consistency with the data requires that such an oligomer should, on the one hand, be stable but not contain a proton channel. On the other hand, association of oligomers should form one proton-selective pathway of limited lifetime. In the absence of any direct evidence on this point, we prefer the simple picture of nonconducting proteolipid monomers reacting reversibly to a proton-conducting proteolipid dimer.

The stabilization of one proton pathway per "dimer" seems the most plausible interpretation of the results. The existence of more than one channel per dimer would require these channels to open and close in a strictly synchronous fashion in order to be consistent with the observed channel transition. For symmetry reasons, the assumption of one channel per dimer implies that the proton-conducting pathway is not confined to one proteolipid polypeptide chain but that both chains of the dimer contribute to the channel. The rate of proton transfer may then be expected to depend on the interaction energy or stability of the dimer. This corresponds well with the observed concerted changes in the lifetime and conductance of the open pathway.

If the pH was increased from 2 to 7, conductance by the proteolipid remained highly selective for protons. Also, for low proteolipid concentrations and in the absence of cholesterol, the conductance, normalized to the proton concentration, remained constant and characterized by a bimolecular reaction. This continuity of electrical properties with pH clearly indicates that the conductance occurs at any pH value between pH 2 and 6 through proton-selective channels formed from proteolipid molecules in a bimolecular reaction.

However, at high proteolipid concentrations and in the presence of cholesterol, a conductance transition occurred at pH 4.5 during titration from low to high pH. The observed increase in (normalized) conductance toward higher pH is so large (several orders of magnitude) that it probably does not simply result from a corresponding increase of proton permeability per channel. It is much more probable that the average number of open channels increases as the pH is increased. In other words, the equilibrium between nonconducting and conducting species most likely shifts toward saturation of the conducting species. Two findings argue for such a saturation. First, the estimated number of proteolipid molecules in the planar bilayer[2] approaches the number of channels formed within 1 order of magnitude (assuming constant normalized channel conductance). Second, saturation of the numbers of open channels should change the apparent concentration dependence of the conductance from the square to a linear relation; this is indeed found.

This change in the apparent concentration dependence upon altering the pH suggests that at pH values larger than 4.5 dimers are stabilized at the expense of monomers. However, a more appropriate picture emerges from the anomalous dependence of conductance on proteolipid concentration at pH 4.5, the midpoint of the pH-induced transition. The apparent molecularities of much larger than 2 indicate that the stabilization of proton pathways is not sufficiently explained by stabilized dimers but that the stabilization of pathways involves the formation of structures larger than dimers. Within the transition region, these structures are not yet stable, probably because of uncomplete deprotonation which results in association–dissociation reactions with high molecularities. At pH 5.5, deprotonation is completed, and the molecularity of the reaction approaches unity since dissociation of these associates becomes negligible. These proteolipid associates may be considered as oligomerized dimers since it is reasonable to assume that the pathway for proton translocation remains localized within the dimer, irrespective of the pH value, although its structure and properties may change with pH and dimer oligomerization.

This stabilization of proton-conducting oligomers may result from the deprotonation of aspartic acid or glutamic acid residues since their pK values are close to the midpoint of the observed transition. This assumption is especially intriguing since these amino acids are conservative structural elements of the proteolipid and the site for DCCD binding (Hoppe & Sebald, 1982). The effect of DCCD to abolish proton conductance in the H^+–ATPase complex and the destabilization of channels at pH values below 4.5 may thus both involve neutralization of the Glu–Asp residues in the proteolipid molecule. This is not in conflict with the rather complicated effect of DCCD reported here. The initial transient conductance increase elicited by DCCD suggests rearrangement of proteolipid molecules, generating defect structures which allow potassium ions to permeate. This is not altogether surprising since the spatial arrangement of proteolipid in oligomers of dimers is likely to be more susceptible to perturbations than the proteolipid oligomers in the H^+–ATPase complex. A perturbation by DCCD appears especially likely since its binding site is known to be located in a hydrophobic environment (Hoppe & Sebald, 1982). Loss of the proton selectivity in the presence of organic solvents also points to the ease with which the arrangement of proteolipids in oli-

[2] The planar bilayers of typically 2×10^{-4} cm^2 area contained about 5×10^{10} lipid molecules. At a proteolipid to lipid ratio X of 2×10^{-7} (single channel conditions), about 10^4 proteolipid molecules are expected to be present in the planar bilayer. The assumption that the proteolipid content in the monolayer and in the bilayer is about the same as that in the vesicles has not been explicitly tested for the proteolipid but has been shown to be valid in several other systems (Pattus et al., 1981; Schindler & Rosenbusch, 1981; Schindler & Quast, 1980).

gomers can be perturbed. Since potassium conductances occurred concomitantly with proteolipid oligomerization and in a transient, nonreproducible, and erratic fashion, it appears most likely that they occurred through organizational defects in proteolipid oligomers rather than through the proton channels. The true permeability ratio, p_H/p_K, of the channel may, therefore, exceed by far the minimal estimate of 10^4. This, indeed, would be required for a proton channel to conduct more protons than potassium ions at physiological conditions with 10^{-7} M protons and about 10^{-1} M potassium ions present.

The conductance transition has been interpreted above to arise from a stabilization of proton channels within dimers via oligomerization of these dimers and to reflect deprotonation of particular amino acids, probably aspartic and/or glutamic acid. This interpretation of the transition may also explain why the transition was found only with membranes of particular compositions. While deprotonation of proteolipids above pH 4.5 apparently favors increased interactions between proteolipids, these interactions may not be sufficiently strong to stabilize proteolipid oligomers in any membrane environment. In the experiments reported here, pH-induced conductance transitions were only found in the presence of cholesterol or sufficiently high proteolipid concentrations. Cholesterol may enhance the oligomerization of dimers to large structures by increasing the cohesiveness of the bilayer.[3] Similarly, at sufficiently high proteolipid concentrations, proteolipid oligomers larger than dimers may well represent organizations of minimal energy. Indeed, the particular shape of the Λ–X relation for ether lipids alone (curve 1 in Figure 8) is characteristic for a phase separation or a micellization (in two dimensions) at a critical concentration.

What is the significance of these findings for understanding the H^+–ATPase complex which appears to contain six copies of the proteolipid studied here? It is currently accepted that these proteolipids contribute to the membrane-embedded part of the ATPase complex and that they are intimately involved in H^+ conductance (Nelson, 1980; Hoppe & Sebald, 1982). Little is known about the nature of the proton pathway(s), the arrangement of proteolipid in the membrane, and the number of H^+ channels existing in a single ATPase complex. Our work shows directly that isolated proteolipid can form membrane channels which are highly proton selective, each of which should transport about 100 protons/s at pH 7 and 100 mV. These results match expectations for ungated proton translocation through the membrane-embedded part of the ATPase complex and, therefore, suggest that the proteolipids in the ATPase complex may also form channellike proton pathways. Our experiments also indicate that channels may occur in proteolipid dimers and that, at neutral pH, these dimers tend to form larger oligomers with open channels. This self-association to oligomers exhibiting H^+ conductance strongly suggests that the proteolipid hexamer in the ATPase may resemble such a proteolipid self-associate. The six proteolipids in the ATPase complex could, therefore, represent an associate of three proteolipid dimers, with each dimer constituting one channel. It is interesting that this stoichiometry of three channels, or three dimers, per hexamer matches the occurrence of three α and three β subunits in the ATPase complex (Todd et al., 1979). Moreover, it is likely that three protons are required to cross the membrane for the formation of each ATP molecule (McCarty, 1978). Also, assembly and stabilization of the proteolipid hexamer within the ATPase complex should be facilitated if its structure approaches that formed spontaneously in vitro. Of course, this does not imply that the proteolipid monomers have to associate before their integration. In fact, such a premature association would be undesirable since it would lead to ungated H^+ channels and thus collapse transmembrane H^+ gradients. For this reason, it has been proposed (Nelson et al., 1980) that the proteolipid does not form stable channels unless additional subunits of the ATPase complex are also present (some of them imported from outside the organelle). This hypothesis is supported by a recent study of an *Escherichia coli* mutant which contains the proteolipid but lacks one (or more) additional subunit(s) of the membrane-embedded part of the ATPase complex (Loo & Bragg, 1981). In the present study, the isolated proteolipid was indeed found to induce very little H^+ conductance, but only under certain conditions. Under these conditions, the proteolipid molecules existed predominantly as monomers in equilibrium with very few proton-conducting dimers. Sharp transitions from this situation to almost complete association of proteolipids to oligomers composed of conducting dimers occurred upon small alterations of pH or membrane composition. It is tempting to speculate that such a transition from a monomeric and nonconducting state to an oligomeric and conducting state also occurs during the biogenesis of the ATPase complex.

[3] A recent development allows us to assay and adjust the cohesive energy of a planar bilayer. It is based on the measurement of the surface pressure in monolayers at the exchange equilibrium with vesicles. Quantitative relations are obtained from, for example, the increase of cohesive energy of the bilayer with increasing cholesterol content in the lipids (H. Schindler, unpublished experiments).

Acknowledgments

We are grateful to Dr. G. Schatz, Biozentrum, Basel, Switzerland, for stimulating discussions and for critical reading of the manuscript. We thank Dr. F. Paltauf, Technical University, Graz, Austria, for providing us with ether lipids.

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Adenosine Cyclic 3′,5′-Monophosphate Dependent Protein Kinase: Kinetic Mechanism for the Bovine Skeletal Muscle Catalytic Subunit†

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ABSTRACT: The kinetic mechanism for adenosine cyclic 3′,5′-monophosphate dependent protein kinase was determined from initial velocity studies in the absence and presence of the product MgADP and dead-end inhibitors. Data are consistent with random addition of MgATP and Ser-peptide and ordered release of phospho-Ser-peptide and MgADP with a dead-end E–MgADP–Ser-peptide complex. In addition to the metal required for the nucleotide, we also characterized the binding of Mg^{2+} to a second site. Increasing the Mg^{2+} results in a 5–6-fold decrease in V_{max} in the presence or absence of 0.1 M KCl. There is a 5-fold increase in V/K_{MgATP} in the absence of KCl and a 13-fold increase in V/K_{MgATP} at 0.1 M KCl. The effect of increasing free Mg^{2+} on V_{max} and V/K was also obtained with MgITP (20% the V_{max} with MgATP) and MgGTP (10% the V_{max} with MgATP) as substrates. The dissociation constant for Mg^{2+} from E–Ser-peptide–Mg^{2+} and central complexes is 2–3 mM. At low concentrations of free Mg^{2+} and no added KCl, competitive inhibitors of MgATP (K_i = 160 μM) decrease in the order adenosine = MgADP ($K_i \simeq 40$ μM) > AMP ($K_i \simeq 8$ mM). At saturating free Mg^{2+}, K_i values are 10 μM (MgATP), 10 μM (MgADP), 40 μM (adenosine), and 850 μM (AMP). In addition, guanosine (1 mM) and $MgPPP_i$ (10 mM) failed to inhibit at low or high free Mg^{2+}. A mechanism for nucleotide interaction is proposed on the basis of a hydrophobic site or negatively charged group in the region of the α-phosphate in the nucleotide binding site and the presence or absence of a negative charge on the α-phosphate. We also made use of a spectrophotometric assay for protein kinase that couples the production of MgADP to the pyruvate kinase and lactate dehydrogenase reactions. On the basis of determinations of the amount of phosphorylatable Ser-peptide by spectrophotometric or radioisotopic determinations, we find that commercial samples contain 25–50% nonpeptide material by weight.

Adenosine cyclic 3′,5′-monophosphate dependent protein kinase catalyzes the phorphorylation of a variety of proteins according to

$$MgATP + protein \rightarrow phosphoprotein + MgADP$$

Activation by cAMP[1] occurs via dissociation of the inactive holoenzyme (R_2C_2) to a regulatory dimer (R_2·$cAMP_4$) and two catalytic monomers (C). A common feature of the amino acid sequence of many cAMP-dependent protein kinase substrates is the presence of one or two arginyl residues on the amino-terminal side of the phosphorylated serine or threonine (Zetterquist et al., 1976; Kemp et al., 1977; Feramisco et al., 1979, 1980; Kemp, 1980). Guanethidine sulfate, a guanidinium containing analogue, is a competitive inhibitor of protein kinase with respect to the protein substrate (Witt & Roskoski, 1980). The nucleotide specificity of the enzyme has been reported by Walsh & Krebs (1973).

A combination of magnetic resonance (Granot et al., 1979, 1980) and kinetic studies (Armstrong et al., 1979; Bolen et

† From the Department of Biochemistry, Louisiana State University Medical Center, New Orleans, Louisiana 70112-1393. *Received December 11, 1981; revised manuscript received April 19, 1982.* This work was supported in part by grants from the Research Corporation (P.F.C.), the Muscular Dystrophy Association (R.R.), and the U.S. Public Health Service (T32HL07098; K.E.V.).

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[1] Abbreviations: cAMP, adenosine cyclic 3′,5′-monophosphate; AMPPCP, 5′-adenylyl methylenediphosphate; Ser-peptide, Leu-Arg-Arg-Ala-Ser-Leu-Gly; R, regulatory subunit; C, catalytic subunit; E_t, total enzyme concentration; Mops, 4-morpholinepropanesulfonic acid; DPNH, reduced nicotinamide adenine dinucleotide.

0006-2960/82/0421-5794$01.25/0