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STUDIES ON THE ATPase COMPLEX FROM BEEF-HEART MITOCHONDRIA

I. ISOLATION AND CHARACTERIZATION OF AN OLIGOMYCIN-SENSITIVE AND AN OLIGOMYCIN-INSENSITIVE ATPase COMPLEX FROM BEEF-HEART MITOCHONDRIA

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Summary

1. A new method for the isolation of the oligomycin-sensitive ATPase from beef-heart mitochondria is described.

2. A Triton-soluble ATPase complex was isolated as a by-product of the standard procedure, or as the main product when the submitochondrial particles were pretreated with 1% Triton. The ATPase activity of this complex is sensitive neither to oligomycin nor to dicyclohexylcarbodiimide.

3. The ATPase activity of the oligomycin-sensitive ATPase complex is nearly completely dependent on added phospholipids. The highest activation was found with asolectin.

4. The oligomycin-sensitive complex can be integrated into phospholipid vesicles resulting in an ATP- and Mg^{2+}-dependent energization of the vesicles as monitored with the fluorescent dye 9-amino-6-chloro-2-methoxyacridine.

5. Aurovertin-binding studies based on fluorescence measurement reveal the presence of 1.5 μmol aurovertin-binding sites per g protein for the oligomycin-sensitive complex and about 2.2 μmol for the oligomycin-insensitive complex.

6. The preparation of the oligomycin-sensitive complex contains at least 6—7 polypeptides in addition to those derived from F_1. One of these polypeptides, with an apparent molecular weight of 31 000, is virtually absent from the oligomycin-insensitive complex.

7. Some of these polypeptides have been identified and isolated.

Abbreviations: SDS, sodium dodecyl sulphate; DCCD, dicyclohexylcarbodiimide; OSCP, oligomycin sensitivity-conferring protein.

Introduction

Investigations of the nature of the link between the redox reactions of the respiratory chain and the phosphorylating system have focussed attention on the role of the membrane part of the ATPase complex, called CF_0 or F_0. According to the chemiosmotic hypothesis [1,2] the ATPase acts as a reversible proton pump, and the membrane part of the ATPase contains a channel transporting protons from one side of the membrane to the other. A proton gradient built up by the operation of the respiratory chain drives the ATPase in the direction of ATP synthesis, whereas the ATPase reaction transports protons in the opposite direction. This process is inhibited by energy transfer inhibitors such as oligomycin and dicyclohexylcarbodiimide.

In order to be able to study the factors that make the ATPase oligomycin sensitive a purified preparation of the ATPase in its oligomycin-sensitive form is needed. The preparations described in the literature [3—6] until recently all have a relatively low specific activity and were obtained in a low yield. The two methods most commonly used [3,6] yield a preparation whose activity is not dependent on added phospholipid, whereas in phospholipid-depleted submitochondrial particles the requirement for phospholipids is well established [7,8]. We have developed a procedure for the preparation in good yield of a highly active ATPase complex from beef-heart mitochondria. As a by-product of this procedure a Triton-soluble, oligomycin-insensitive preparation was obtained, that lacks at least one of the polypeptides present in the oligomycin-sensitive preparation. The oligomycin-sensitive preparation is very similar, both in activity and composition, to the preparation recently described by the group of Racker [9]. Some of the polypeptides not belonging to the F_1 part of the complex have been identified and isolated.

Materials and Methods

Beef-heart mitochondria were prepared according to the procedure of Crane et al. [10]. Both the heavy and the light fraction may be used. The mitochondria were sonicated in 250 mM sucrose/50 mM Tris · HCl buffer (pH 7.5) for 5 min in an ice-bath and the pellet obtained after centrifugation at 20 000 × *g* was resonicated in the same buffer. The combined supernatants were centrifuged at 100 000 × *g* and the precipitated submitochondrial particles were washed and then suspended in 250 mM sucrose/50 mM Tris · HCl buffer (pH 7.5).

The ATPase activity of the preparations was measured in an ATP-generating system [11] by determining the phosphate liberated [12] from ATP.

SDS-polyacrylamide disc gel electrophoresis was carried out as described by Weber and Osborn [13]. The percentage acrylamide used, if not specified in the legends, was 13%. 10% mercaptoethanol was always present. Heating the samples in the SDS medium did not improve the separation of the various polypeptides on the gel and was usually omitted. The gels were stained with Coomassie Brilliant Blue, destained with 20% methanol/10% acetic acid for 2 days, and scanned at a wavelength between 500 and 550 nm, using a Zeiss spectrophotometer equipped with an automatic scanner and a scale expander.

Urea-SDS-polyacrylamide disc gel electrophoresis was carried out as described by Swank and Munkres [14]. The concentration acrylamide was 10% and that of bisacrylamide 1% (w/v).

As reference for the determination of the molecular weight a mixture of bovine serum albumin, egg albumin, catalase, aldolase, myoglobin, chymotrypsinogen and cytochrome *c* was used in the case of SDS gels, and insulin was additionally present in the case of urea-SDS gels. As reference for the F_1 subunits, F_1 prepared by J.L.M. Muller in this laboratory by the method described by Knowles and Penefsky [15] was used.

Aurovertin isolated in this laboratory by Bertina [16] was used, its concentration being determined via the absorbance at 367.5 nm, using 28.5 as the millimolar extinction coefficient at 367.5 nm [16,17]. Egg-yolk lecithin and egg-yolk phosphatidylethanolamine were prepared according to the procedure described by Litman [18]. The other phospholipids used were commercial products. The aurovertin fluorescence measurements were carried out with an Eppendorf fluorimeter, with primary filter 313 + 366 nm, secondary filter 470—3000 nm. The 9-amino-6-chloro-2-methoxyacridine fluorescence measurements were carried out with a Hitachi-Perkin-Elmer spectrofluorimeter.

Protein was determined using the biuret procedure after precipitation with trichloroacetic acid [19] or (in the case of soluble proteins or small amounts of material), by the method of Lowry et al. [20].

Triton X-100 was obtained from Packard, DEAE-cellulose from Serva (Heidelberg).

Results

Isolation of the ATPase complex

A suspension of submitochondrial particles (see Materials and Methods) was diluted to 6—10 mg protein/ml in 200 mM sucrose, 10 mM Tris · HCl buffer (pH 7.5), 0.2 mM EDTA, 1 mM dithiothreitol, 1 mM ATP, 2% methanol and 1% Triton X-100, and solid Na_2SO_4 was added to 0.5 M. After 5 min stirring the homogenate was centrifuged at 150 000 × *g* for 30 min and the pellet (N_1), which had only a slight ATPase activity and contained most of the $b \cdot c_1$ complex and all the cytochrome *c* oxidase, was discarded. The supernatant (S_1) was dialysed overnight against 50 volumes of the same medium, but without the Na_2SO_4. Variation of the Triton concentration during dialysis between 0.25 and 1% had no effect; 0.5% was mostly used. After addition of Triton and Na_2SO_4 to the submitochondrial particles, the ATPase activity usually declined by more than 50%, but when after dialysis the dialysate was centrifuged at 50 000 × *g* for 30 min nearly all the ATPase activity present in the particles was recovered in the precipitate. This could be explained either by a reversibility of the effect of Triton on the activity or to partial loss or inactivation of the natural F_1 inhibitor. The relatively low activity recovered in the supernatant (S_2) is, in contrast to that in the precipitate, largely oligomycin insensitive.

The precipitate (N_2) was suspended in 250 mM sucrose, 50 mM Tris · HCl buffer (pH 7.5), 1 mM dithiothreitol, 0.2 mM EDTA and 2% methanol to a final concentration of 3 mg/ml, and 0.4% cholate was added followed by saturated $(NH_4)_2SO_4$ to 30% saturation. After centrifugation the pellet (N_3) was

TABLE I

DISTRIBUTION OF ATPase ACTIVITY AND PROTEIN DURING THE ISOLATION OF THE ATPase COMPLEX

SMP, submitochondrial particle suspension.

Fraction	Volume (ml)	Protein (mg/ml)	Total protein (mg)	ATPase (μmol/min per mg)	Total ATPase	
					μmol/min	%
SMP	68	24.5	1666	2.74	4565	100
N_1	40	20.4	816	0.25	204	4.5
N_2	144	3.0	432	8.0	3456	76
S_2	405	1.13	458	0.76	348	7.6
N_3	6.5	14.5	94	1.6	150	3.3
N_4	9.7	29.1	282	14.2	4004	88

discarded and the supernatant brought to 50% saturation with saturated $(NH_4)_2SO_4$ and centrifuged. The precipitate (N_4), which contained the ATPase complex, was suspended in 250 mM sucrose/50 mM Tris · HCl buffer (pH 7.5)/ 1 mM dithiothreitol/0.2 mM EDTA. Storage at the temperature of liquid nitrogen does not inactivate the enzyme, but repeated freezing and thawing does. The activity and protein contents of the various fractions are given in Table I.

For the preparation of an oligomycin- and dicyclohexylcarbodiimide-insensitive complex the supernatant S_2 was brought on a DEAE-cellulose column, washed with the same medium as used for dialysis and then with the same medium containing in addition 50 mM KCl. The ATPase activity together with

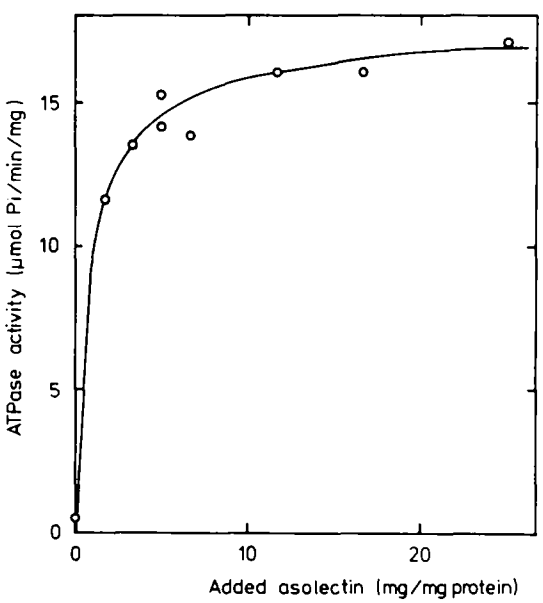

Fig. 1. Activation of the ATPase activity of the oligomycin-sensitive ATPase complex by asolectin. The ATPase activity of 8 μg ATPase complex was measured as described under Materials and Methods. The reaction was started 3 min after addition of the phospholipid vesicles. The vesicles were prepared by sonicating a suspension of asolectin (15 mg/ml) for 20 min.

some residual $b \cdot c_1$ complex was eluted with 100 mM KCl. Addition of solid $(NH_4)_2SO_4$ to 35% saturation gives a floating layer at the top containing both Triton and ATPase activity. When 1% cholate is added before the $(NH_4)_2SO_4$, the ATPase is precipitated (cf. ref. 21), but the activity is quite low (1—3 μmol P_i/min/per mg), and can be stimulated by phospholipids only about 2-fold. The preparation obtained in the absence of cholate contains a large amount of Triton, and a higher but very variable activity (between 3 and 40 μmol P_i/min per mg) which is not stimulated by phospholipids. The variability is not due to differences in purity, but to differences in the degree of inactivation by Triton. In all cases the measured ATPase activity is cold-stabile.

When submitochondrial particles are washed with 1% Triton before they are extracted with 1% Triton plus 0.5 M Na_2SO_4, very little ATPase activity is recovered in N_2 and the activity in S_2 (oligomycin insensitive) is increased. Both the yield and specific activity, however, vary considerably, due to a variation in the Triton-induced inactivation. Passing the final preparation over a column of Bio-Beads (cf. ref. 22) to remove residual Triton leads to binding of the ATPase complex (not the impurities) to the beads. The activity is now partially oligomycin sensitive, but cannot be removed from the beads by washing. This result indicates that it is the presence of Triton that causes the oligomycin insensitivity of the ATPase activity.

Requirement of phospholipids for ATPase activity

The oligomycin-sensitive ATPase activity of the fraction N_4 is very low (about 0.5 μmol P_i/min/per mg) when measured in the absence of added phospholipids, and can be enhanced about 30-fold by the addition of soy-bean phospholipids (asolectin), as shown in Fig. 1. Half-maximal stimulation occurs at about 4 mg phospholipids/mg protein. None of the other phospholipids tested was as effective. Egg phosphatidylcholine did not stimulate at all, egg phosphatidylethanolamine stimulated only about 3-fold (a mixture of these phospholipids also gave very poor stimulation), cardiolipin and lysolecithin gave about 60—70% of the activity with asolectin. From these results the specific requirements for activation are not clear. Since neither lecithin nor phosphatidylethanolamine is active, it is possible that negative charges are important and in fact are an absolute requirement, unless micellar structures (lysolecithin) are present (cf. refs. 5 and 23). It is possible that the low activation of the oligomycin-insensitive ATPase complex by phospholipids is due to a tighter association of the protein components in this form of the complex.

Reconstitution in vesicles

For the reconstitution of ATPase vesicles capable of energization by ATP we used the dialysis method as described by Kagawa [24]. A suspension of asolectin was briefly sonicated in the presence of cholate until clarification and then the ATPase complex together with bovine serum albumin was added. The final concentration of all constituents was: 15 mg/ml asolectin, 1.2% cholate, 1.5 mg/ml ATPase, 1.5 mg/ml albumin, 66 mM $(NH_4)_2SO_4$ and 10 mM Tris/EDTA buffer (pH 8). The mixture was dialysed for 23 h against 1000 volumes of a medium containing 0.2 M NaCl, 0.2 mM EDTA, 10 mM Tris · HCl buffer (pH 8), 1 mM dithiothreitol, 0.1 mM ATP and 10% (v/v) methanol. The effect of

TABLE II

EFFECT OF CHOLATE AND RECONSTITUTIVE DIALYSIS ON THE ATPase ACTIVITY OF OLIGOMYCIN-SENSITIVE ATPase

Preparation	ATPase activity (μmol/min per mg)					
	Before dialysis	Time (h) after addition of cholate and start of dialysis				
		0	2	4	6	22
Fresh	14.4	8.7	7.8		7.0	6.0
Three times frozen and thawed	8.0	4.9	3.8	3.8	3.8	3.6

the cholate/asolectin medium and the dialysis on the ATPase activity is shown in Table II. The rapid loss of activity in the presence of cholate is not reversed by dialysis. In Fig. 2 it is shown that the resulting vesicles are capable of storing energy derived from the ATPase reaction, as monitored by the quenching of the fluorescence of 9-amino-6-chloro-2-methoxyacridine [25]. The quenching is reversed by oligomycin and uncouplers (the latter is not shown) very slowly. Mg^{2+} alone causes some quenching of the fluorescence, so that the final level of fluorescence is slightly lower than the original level. The quenching caused by energization was 80% of the fluorescence obtained in the presence of Mg-ATP and oligomycin or uncoupler. Addition of the coupling factors OSCP and F_1

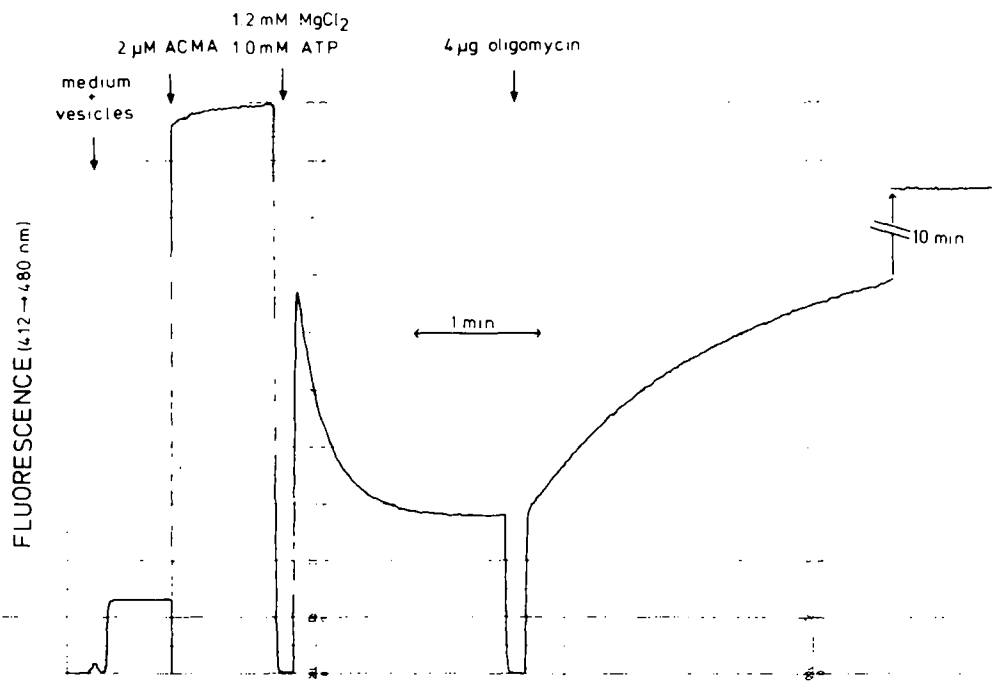

Fig. 2. Quenching of the fluorescence of 9-amino-6-chloro-2-methoxyacridine (ACMA) to ATPase vesicles, prepared as described in the text, and diluted till 0.15 mg ATPase protein/ml with the dialysis medium, 2 μM dye was added. Further additions and the wavelengths for emission and excitation are indicated in the figure.

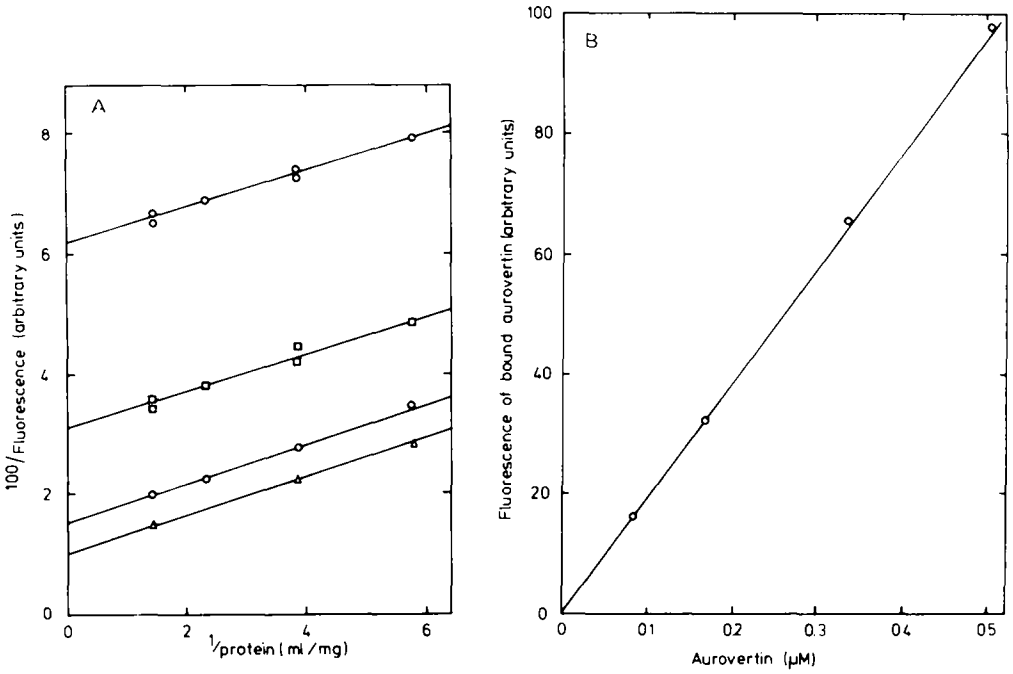

Fig. 3. Binding of aurovertin to the oligomycin-sensitive ATPase complex. (A) Various concentrations of the ATPase complex were titrated with aurovertin. Because of the long time needed to reach a constant fluorescence, only four concentrations of aurovertin were used. The inverse of the fluorescence is plotted against the inverse of the protein concentration. The four curves represent from top to bottom four aurovertin concentrations, 0.084, 0.168, 0.336 and 0.504 μM, respectively. (B) Extrapolation of the curves in Fig. 3A till infinite protein concentration (1/protein = 0) yields the inverse of the fluorescence of bound aurovertin. The fluorescence of bound aurovertin is plotted against the aurovertin concentration.

had no effect, either on the fluorescence quenching or on the oligomycin-sensitive ATPase activity, indicating either that F_1 could not be rebound to the complex to reconstitute an oligomycin-sensitive ATPase, or, more likely, that the loss of ATPase activity, relative to that of the original preparation, was not due to dissociation of F_1 from the complex. The ATPase activity of the vesicles was enhanced by 30% on addition of uncoupler (not shown).

We have not been able to reconstitute similar vesicles using the oligomycin-insensitive ATPase complex. On the contrary, vesicles containing the latter complex uncoupled vesicles containing the oligomycin-sensitive complex, possibly by the presence of residual Triton.

Binding of aurovertin to the ATPase complex

Since aurovertin is a specific ligand for the F_1 part of the ATPase complex [26], we can use the binding of aurovertin to the complex as a measure for the concentration of F_1 in the complex. To find the value for the fluorescence of bound aurovertin we measured the fluorescence with constant aurovertin concentrations at various protein concentrations (Fig. 3) Extrapolation of the curves, relating the inverse of the fluorescence with the inverse of the protein concentration, to infinite protein concentration yields the inverse of the fluorescence of bound aurovertin. Knowing the values for the fluorescence of

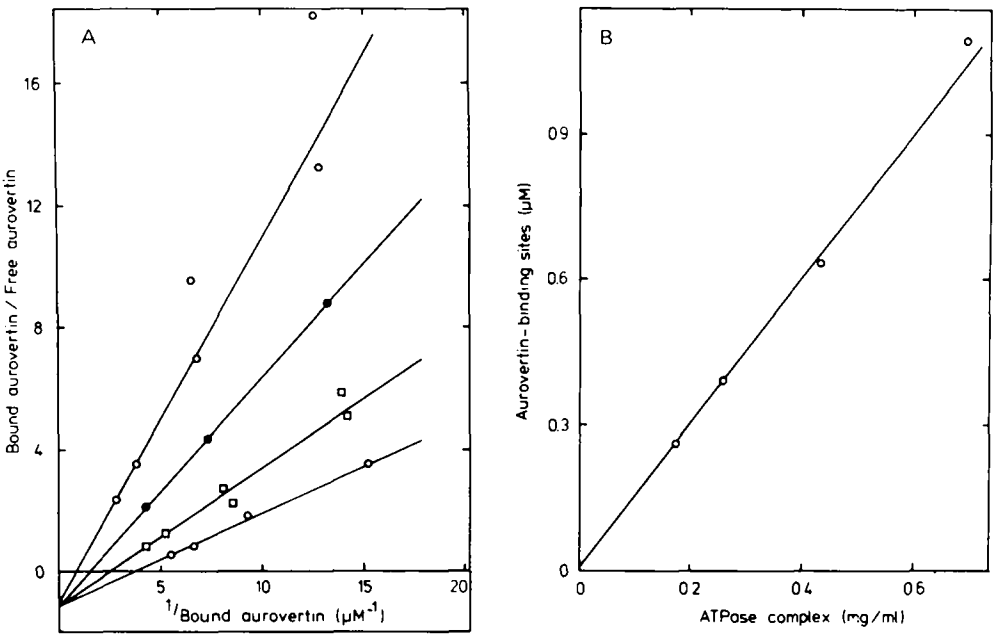

Fig. 4. Binding of aurovertin to the oligomycin-sensitive ATPase complex. (A) From the values for the fluorescence of bound aurovertin (Fig. 3B) and free aurovertin (determined separately) the concentrations of bound and free aurovertin are calculated for each point in this figure. The values for bound/free are plotted against the values for 1/bound. The different curves represent different protein concentrations. The intersection with the abscissa yields [binding sites]$^{-1}$ and the intersection with the ordinate $-K$ [17]. (B) The concentration of binding sites derived from A, is plotted against the protein concentation. Since the slope of the resulting curve equals 1.5, the concentration of binding sites is 1.5 nmol/mg protein.

bound and free aurovertin, for each point of a titration the concentrations of bound and free aurovertin can be calculated when the total concentration is known. As shown by Muller et al. [17], the Scatchard plots for the binding of aurovertin to isolated F_1 are not straight but hyperbolic, whereas plotting bound ligand/free ligand against 1/bound ligand gives straight lines, cutting the abscissa at the inverse of the concentration of binding sites. Such plots are shown for the ATPase complex in Fig. 4A for four different protein concentrations. Fig. 4B shows that the values found for the concentration of binding sites are proportional to the protein concentration and amount to 1.5 nmol/mg protein. The intersection with the ordinate gives the value of $-K$ (in this experiment 1.1) for the four-component equilibrium [17]. In various experiments with different preparations the concentration of binding sites varied only within 10%, and the value of K for the assumed four-component equilibrium varied between 0.6 and 1.5.

If the concentration of aurovertin-binding sites equals the concentration of F_1 in the complex (see Discussion), the apparent molecular weight is 666 000. This value is certainly an over-estimate, since the preparation still contains impurities. The oligomycin-insensitive form of the complex, isolated as described before, contained 2.2 nmol aurovertin-binding sites per mg protein, corresponding to a molecular weight of 450 000.

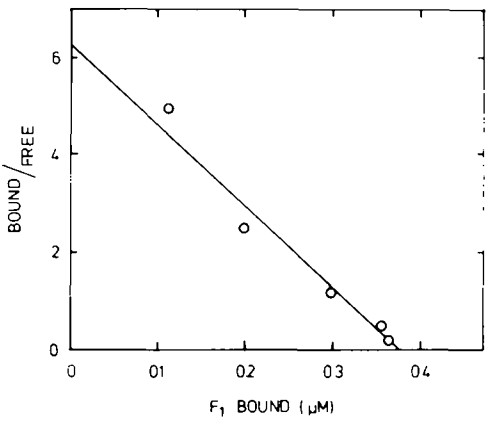

Fig. 5. Binding of F_1 to NaBr-treated ATPase complex. ATPase complex was twice treated with 3 M NaBr and the pellet suspended in 0.25 M sucrose/10 mM Tris · HCl buffer (pH 8)/0.2% cholate. The binding of F_1 to the NaBr-treated complex was studied by incubating various amounts of F_1 with 0.25 mg of the complex in a total volume of 0.5 ml in the presence of 2 mM $MgCl_2$/0.25 M sucrose/10 mM Tris · HCl (buffer (pH 8)/0.2% cholate. After 20 min incubation at room temperature the mixtures were centrifuged and the F_1 in the supernatant was determined according to ref. 27. From these determinations the bound F_1 was calculated and a Scatchard plot constructed. K_D = 0.06 μM, n = 0.375 μM = 0.75 nmol/mg NaBr-treated complex.

Rebinding of F_1 to stripped ATPase complex

Treatment of submitochondrial particles or the oligomycin-sensitive ATPase complex with urea or 3.5 M NaBr is known to extract the F_1, and F_1 may be rebound to the stripped particles. The rebinding of F_1 to F_1-depleted ATPase complex is a good test for its functioning as F_0.

Treatment of the oligomycin-sensitive complex with 3.5 M NaBr did not result in a separation of the F_0 part of the complex from the F_1 subunits on centrifugation. Also treatment with urea had disadvantages: a considerable amount of the F_0 part of the complex remained in the supernatant after centrifugation as will be described later. A better separation of the F_0 and F_1 part of the complex was obtained when the preparation was first dialyzed to remove residual cholate and then treated with NaBr. The resultant F_0 preparation, however, was not able to bind F_1. Since aggregation of F_0 was a possible explanation for this behaviour, the precipitate obtained after NaBr treatment of the complex was suspended in a medium containing 0.2% cholate (cf. ref. 9). Under these conditions F_1 could be bound with a K_D of 0.06 μM (Fig. 5). The concentration of binding sites was 0.75 nmol/mg protein, indicating that also under these conditions a large fraction of F_1-binding sites is not available for added F_1.

Composition of the ATPase complex

A typical band pattern for the oligomycin-sensitive ATPase complex on 13% acrylamide-SDS gels stained with Coomassie Blue is shown in Fig. 6. It is relatively easy to identify the bands of the five subunits of F_1, numbered 2, 3, 6, 10 and 14. After extraction with 6 M urea at high pH these bands have disappeared (Fig. 6C) while they are relatively increased in the extract (Fig. 6B). Another band that is specifically removed is band 9 which comigrates with

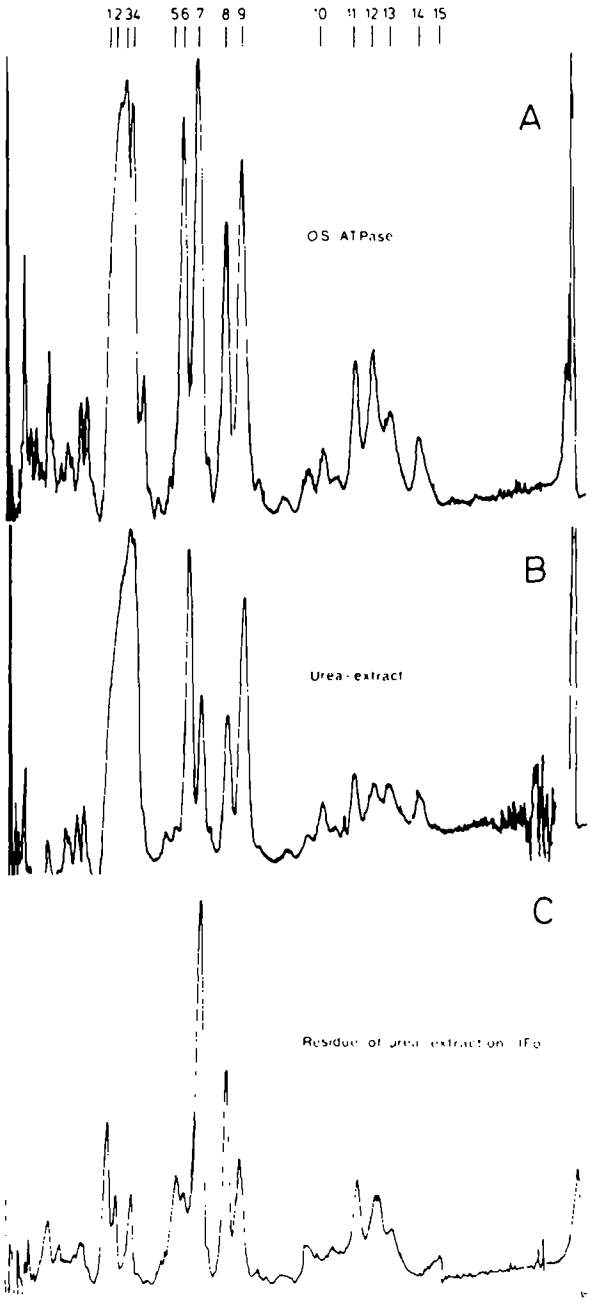

Fig. 6. SDS-polyacrylamide gel electrophoresis of the ATPase complex. For each disc about 75 μg protein was used. (A) The oligomycin-sensitive ATPase complex. (B) The urea extract of the complex, obtained by incubating the complex for 30 min at 0°C with 6 M urea at pH 9.0, and spinning down the residue. Because of the presence of residual cholate part of the F_0 is not precipitated. (C) The residue obtained after the extraction with urea (F_0).

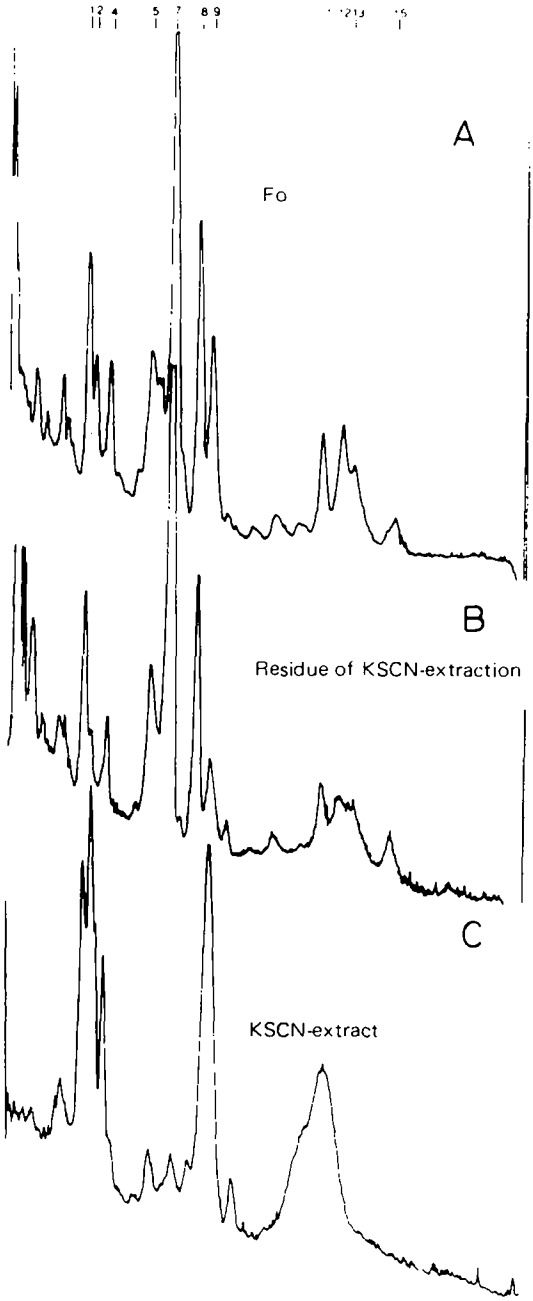

Fig. 7. SDS-polyacrylamide gel electrophoresis of the ATPase complex. (A) The residue of the urea extraction (the same as Fig. 6C), called F_0. (B) The F_0 was further treated with KSCN (see ref. 29), and the residue obtained after centrifugation was used for this experiment. (C) The KSCN extract of the F_0.

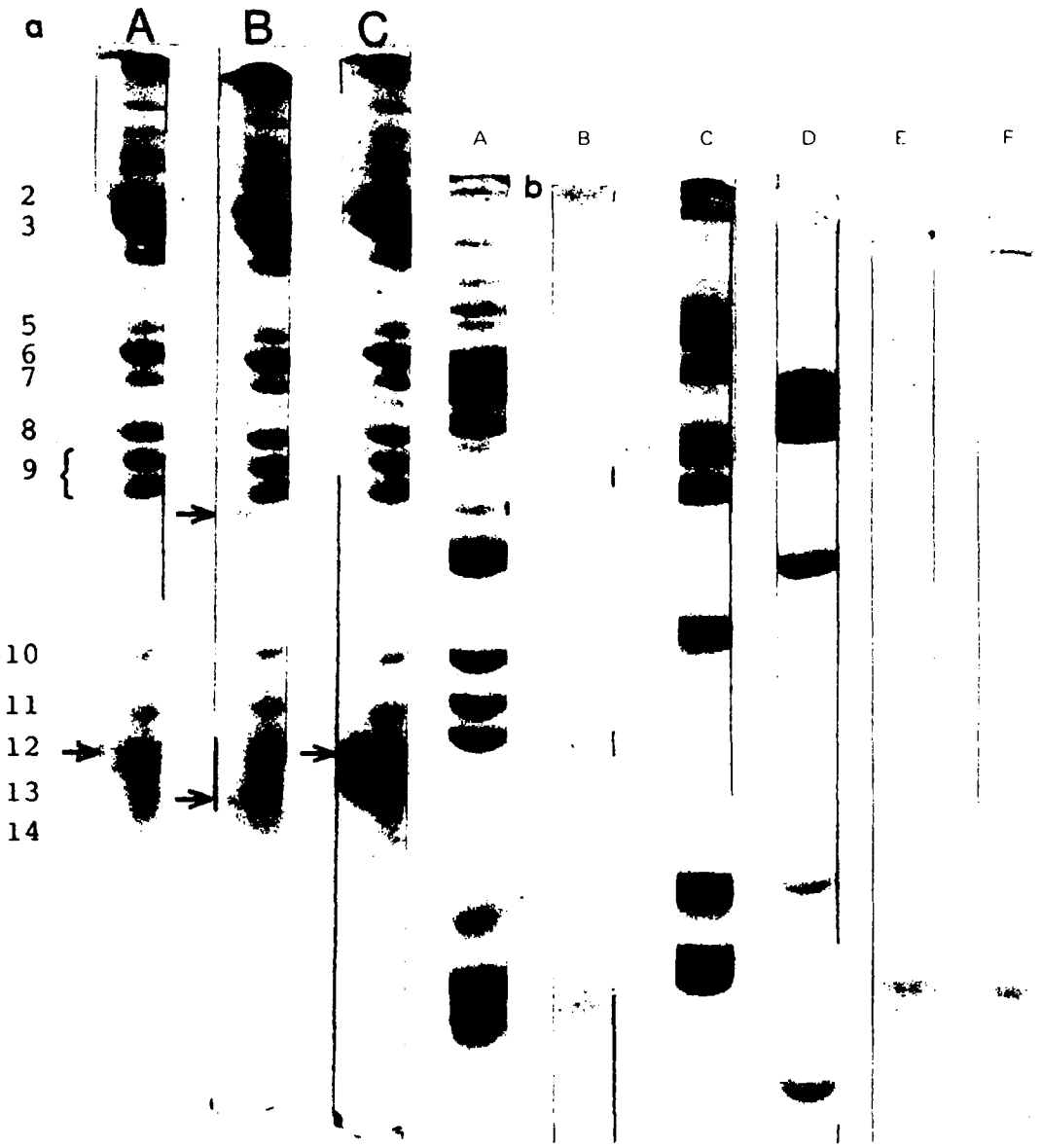

Fig. 8. SDS-polyacrylamide gel electrophoresis of subunits of the ATPase complex. After destaining the discs, they were ohorographed. (a) Split-gel electrophoresis of the ATPase complex and A, F_1 inhibitor; B, proteolipid fraction and C, Fc_2. (b) Separated discs were used for all preparations; A, ATPase complex; B, proteolipid fraction; C, standard proteins; D, F_1; E, F_1 inhibitor; F, Fc_2.

isolated OSCP, the molecular weight of which appears to be around 23 500 in our SDS gels. The molecular weights of the F_1 subunits are 53 000, 49 000, 34 000, 14 000 and 7500, respectively, in good agreement with the values reported in the literature [28]. The bands 1, 4 and 5 as well as the minor bands around band 10 represent impurities. As judged from the gel patterns in Fig. 6C, the bands 7, 8, 11, 12 and 15 could belong to F_0, the membranous part of

the complex. Band 13 represents a protein (M_r = 9000) that is much more easily extracted than the F_0 proteins, but not as fully as the F_1 subunits. This band probably is composite, one part being extractable (13a, impurity), the other part not (13b, possibly belonging to F_0).

For identification of the F_0 subunits we extracted the residue from the urea extraction (mainly F_0) with KSCN as described for submitochondrial particles [29]. In Fig. 7C it can be seen that apart from high molecular weight protein (around 50 000), material belonging to bands 9, 11 and 12 is extracted by this treatment. From Fig. 7B one may conclude that the protein corresponding to band 12 is more completely extracted than that of band 11 and this suggests that the former represents Fc_2 (F_6), since KSCN treatment is known to extract this coupling factor [29,30]. The fact that heat treatment leaves this band intact is consistent with this conclusion. Isolation of Fc_2 from the complex via heat treatment, followed by removal of the F_1 inhibitor by alcohol precipitation [30], confirms the identical migration of band 12 and the Fc_2, but also of band 12 and the F_1 inhibitor. This is shown in the split gels of Fig. 8. The F_1 inhibitor could also be isolated from F_1 preparations contaminated with inhibitor, by a simple heat treatment. The yield of inhibitor (about 1.4 nmol from 1 mg of ATPase complex) containing 1.5 nmol F_1, assuming a molecular weight of 10 000, suggests that the complex contains 1 mol of inhibitor per mol of F_1.

Using the chloroform/methanol extraction procedure described by Cattell et al. [31] the DCCD-binding protein can be isolated. The isolated lipoprotein fraction gives two bands on a SDS gel (Fig. 8), one diffuse band at a position around band 13 and one sharp band of varying intensity at a position between OSCP and the δ-subunit of F_1. These two bands are also the main peaks of radioactivity when the ATPase complex is treated with [^{14}C]DCCD. Since on urea-SDS gels (not shown) only the low molecular weight band is present, it seems most probable that the two bands on SDS gels represent the monomer and dimer of the DCCD-binding protein.

The oligomycin-insensitive ATPase complex gives a band pattern on SDS gels (Fig. 9) that is very similar to that from the oligomycin-sensitive preparation except in two respects: (i) bands ascribed to impurities are absent or less intense: (ii) band 7, with apparent molecular weight of 31 000, is missing. No definite conclusions on the identity of band 7 can be drawn, except that the content of cytochromes *b* and c_1 of the oligomycin-sensitive preparation is too low to account for this band. Possible candidates are the adenine nucleotide translocator protein [32,9] and the uncoupler-binding protein [6,33].

The function of the protein of band 8, with an apparent molecular weight of 26 000, which seems to be an integral part of the complex, is not known. Since a protein of similar molecular weight (about 22 000) is present in preparations of the ATPase complex from other sources, it could represent an essential structural protein.

The behaviour of the protein responsible for band 9 is rather variable. In Fig. 8 it appears double, as is often seen in SDS gels, but never in SDS-urea gels (not shown). Since after treatment with ammonia the upper of the two bands disappears, although not all of the OSCP has disappeared, we like to consider the possibility that bands 9a and 9b both represent OSCP. If we assume, how-

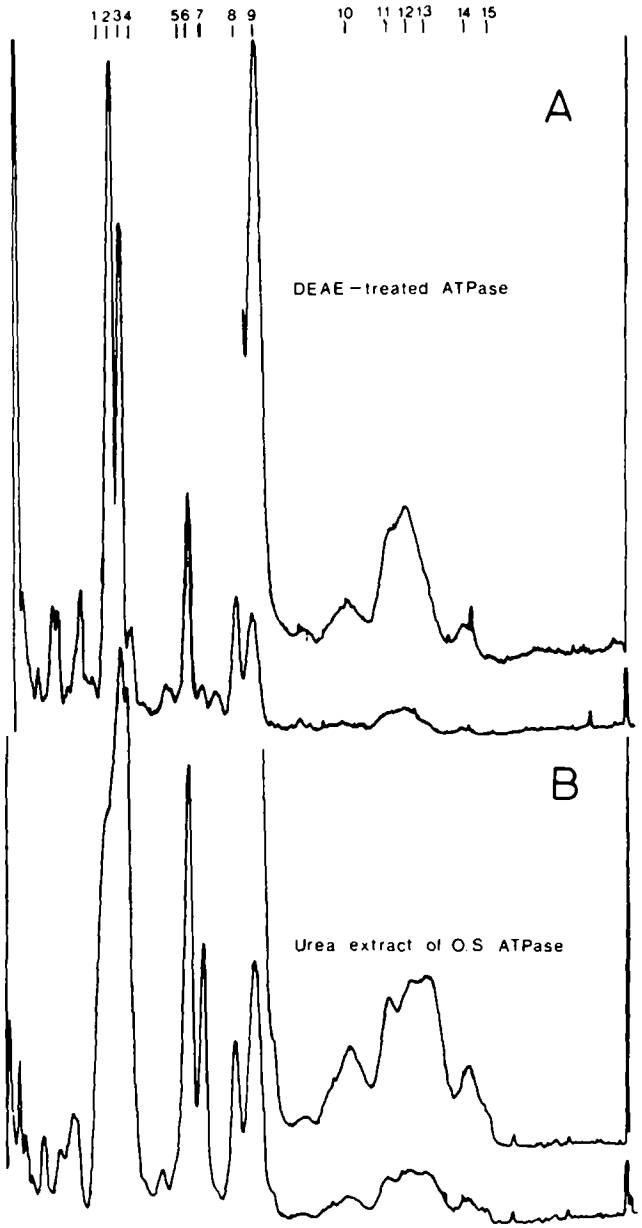

Fig. 9. SDS-polyacrylamide gel electrophoresis of the ATPase complex. (A) The oligomycin-insensitive ATPase complex. (B) The urea extract of the oligomycin-sensitive ATPase complex (Fig. 6B).

ever, that these bands represent different polypeptides we have to conclude that one of them (OSCP), is easily extracted with ammonia, and the other with thiocyanate, indicating that the latter is not a part of the F_0 (ATPase free from F_1 and OSCP), since, in particles at least, Fc_2 is the only polypeptide essential for F_0 activity that is removed by thiocyanate [29].

Discussion

The method described for the isolation of the oligomycin-sensitive ATPase is reproducible and the yield and purity do not depend critically on the concentration of the reagents used. The yield of oligomycin-insensitive complex is not very reproducible, due to the Triton-induced inactivation. From the polypeptide composition and the cold stability of its ATPase activity, we may conclude that we are dealing with a real ATPase complex and not with a form of isolated F_1.

From the finding that the apparent yield of both complexes is lower when the starting preparation has been freed from the ATPase inhibitor it is clear that during the isolation procedure this inhibitor is removed or inactivated. On the other hand, the yield of F_1 inhibitor, when isolated from the complex, suggests that a stoicheiometric amount of inhibitor is still present, although neither moderate heat treatment (65°C) nor trypsin can activate the complex. The most logical conclusion seems to be that during isolation of the complex the inhibitor is dislocated but not removed.

Calculations of the amounts of F_1 protein in the isolated complexes as compared with that present in the submitochondrial particles (based on the concentration of binding sites for aurovertin) indicate that half of the F_1 protein has been lost during the isolation procedure. Inactivation by Triton X-100 in the first step of the isolation is probably the reason for this loss.

The binding studies with aurovertin are a clear demonstration of the phenomena described by Muller et al. [17]. The fluorescence data reported indicate that the general equilibrium is $XY + A \rightleftharpoons XA + Y$, and that the K of the equilibrium is close to 1. This seems for the moment the most reasonable way of explaining the finding that the curves relating 1/fluorescence with 1/protein are completely linear and parallel for various aurovertin concentrations, and the Scatchard plots are hyperbolic. We can also confirm that this type of hyperbolic Scatchard plots is obtained for the binding of aurovertin to A particles [35]. A linear relationship is obtained when 1/[bound] is plotted on the abscissa, instead of [bound] as in the Scatchard plot. The proposed equilibrium (with $K \approx 1$) has as consequence that on dilution of a sample containing ATPase and aurovertin the relative proportion of bound aurovertin does not change. Within the range measured this was indeed the case.

Muller et al. [17] have reported that isolated F_1 binds 2 mol aurovertin per mol F_1 as measured with fluorescence. For submitochondrial particles, however, the concentration of aurovertin-binding sites agrees closely with the concentration of F_1 as determined via re-binding of added F_1 to stripped particles [27]. In the isolated ATPase complex also a binding of 1 mol aurovertin per mol of F_1 agrees closely with the amount of F_1 as estimated from gel electrophoresis and also with the amount of tightly bound adenine nucleotides, namely 2.2—3 per aurovertin-binding site (ATP/ADP = 2). Further studies on the F_1 content of the ATPase complex are in progress. Also on the identification and characterization of the subunits further work is done. The results on the DCCD-binding protein, reported here, indicate that the radioactive band, seen by Schuurmans-Stekhoven et al. [36] represents the dimer and not the monomer of the DCCD-binding polypeptide.

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References

1 Mitchell, P. (1966) Biol. Rev. 41, 445—502
2 Mitchell, P. (1972) J. Bioenerg. 3, 5—24
3 Tzagoloff, A., Byington, K.H. and MacLennan, D.H. (1968) J. Biol. Chem. 243, 2405—2412
4 Kagawa, Y. and Racker, E. (1971) J. Biol. Chem. 246, 5477—5487
5 Swanljung, P., Frigeri, L., Ohlson, K. and Ernster, L. (1973) Biochim. Biophys. Acta 305, 519—533
6 Hatefi, Y., Stiggall, D.L., Galante, Y. and Hanstein, W.G. (1974) Biochem. Biophys. Res. Commun. 61, 313—321
7 Kagawa, Y. and Racker, E. (1966) J. Biol. Chem. 241, 2467—2474
8 Bruni, A. and Bigon, E. (1974) Biochim. Biophys. Acta 357, 333—343
9 Serrano, R., Kanner, B.I. and Racker, E. (1976) J. Biol. Chem. 251, 2453—2461
10 Crane, F.L., Glenn, J.L. and Green, D.E. (1956) Biochim. Biophys. Acta 22, 475—487
11 Pullman, M.E., Penefsky, H.S., Datta, A. and Racker, E. (1960) J. Biol. Chem. 235, 3322—3329
12 Sumner, J.B. (1944) Science 100, 413—414
13 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412
14 Swank, R.T. and Munkres, K.D. (1971) Anal. Biochem. 39, 462—477
15 Knowles, A.F. and Penefsky, H.S. (1972) J. Biol. Chem. 247, 6617—6623
16 Bertina, R.M. (1972) Ph.D. thesis, University of Amsterdam, Gerja, Waarland
17 Muller, J.L.M., Rosing, J. and Slater, E.C. (1977) Biochim. Biophys. Acta, 462, 422—437
18 Litman, B.J. (1973) Biochemistry 12, 2545—2554
19 Cleland, K.W. and Slater, E.C. (1953) Biochem. J. 53, 547—556
20 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
21 Sone, N., Yoshida, M., Hirata, H. and Kagawa, Y. (1975) J. Biol. Chem. 250, 7917—7923
22 Holloway, P.W. (1973) Anal. Biochem. 53, 304—308
23 Bruni, A., Pilotti, A., Dabbebi-Sala, F. and Bigon, E. (1975) in Electron-Transfer Chains and Oxidative Phosphorylation (Quagliariello, E., Papa, S., Palmieri, F., Slater, E.C., and Siliprandi, N., eds.), pp. 179—184, North-Holland, Amsterdam
24 Kagawa, Y. (1974) in Methods in Membrane Biology (Korn, E.D., ed.), Vol. 1, pp. 201—269, Plenum Press, New York
25 Kraayenhof, R. (1970) FEBS Lett. 6, 161—165
26 Lardy, H. and Lin, C.H.C. (1969) in Inhibitors-Tools in Cell Research (Bücher, Th. and Sies, H., eds.), pp. 279—281, Springer Verlag, Berlin
27 Vadineanu, A., Berden, J.A. and Slater, E.C. (1976) Biochim. Biophys. Acta 449, 468—479
28 Senior, A.E. (1973) Biochim. Biophys. Acta 301, 249—277
29 Knowles, A.F., Guillory, R.J. and Racker, E. (1971) J. Biol. Chem. 246, 2672—2679
30 Kanner, B.I., Serrano, R., Kandrach, M.A. and Racker, E. (1976) Biochem. Biophys. Res. Commun. 69, 1050—1056
31 Cattell, K.J., Lindop, C.R., Knight, I.G. and Beechey, R.B. (1971) Biochem. J. 125, 169—177
32 Riccio, P., Aquila, H. and Klingenberg, M. (1975) FEBS Lett. 56, 133—138
33 Hanstein, W.G. and Hatefi, Y. (1974) J. Biol. Chem. 249, 1356—1362
34 Wagenvoord, R.J., van de Kraan, I. and Kemp, A. (1977) Biochim. Biophys. Acta 460, 17—24
35 van de Stadt, R.J., van Dam, K. and Slater, E.C. (1974) Biochim. Biophys. Acta 347, 224—239
36 Schuurmans-Stekhoven, F., Waitkers, R.F. and van Moerkerk, H.T.B. (1972) Biochemistry 11, 1144—1150