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On the Relationship Between the Oligomycin-Sensitivity Conferring Protein and other Mitochondrial Coupling Factors

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Abstract

1. The oligomycin-sensitivity conferring protein (OSCP) has been further investigated and by modifying the purification procedure, the protein has been obtained free of minor contaminants. The single protein has the properties of both an energy-transfer factor and an oligomycin-sensitivity conferring factor when assayed with suitable depleted submitochondrial particles.

2. Several physical and chemical properties of OSCP have been examined. The protein has an isoelectric point of around 9·3 and is not inhibited by sulfhydryl reagents such as iodoacetate, iodoacetamide or parachloromeruriphenyl sulphonate, or by high concentrations of iodine.

3. Knowledge of those and other physical properties of OSCP allows comparison of OSCP with several other previously described coupling factors.

Introduction

The organization of the proteins which make up the mitochondrial ATP-synthesizing system has been and continues to be an extremely important problem confronting investigators. There is general agreement, based on reports from many laboratories (notably those led by Green and Racker) that the electron-transport complexes and the ATPase enzyme (Racker's F_1*) represent respectively the sites of oxido-reduction and ATP-synthesis. In addition there are other proteins, variously called "coupling" factors or "energy-transfer" factors which appear to be responsible for the integration ("coupling") of the enzymic events occurring in the electron transport complexes and in the ATPase enzyme complex.

In a previous report from this laboratory MacLennan and Tzagoloff[1] described the preparation of a highly purified "oligomycin-sensitivity conferring protein" (OSCP). This soluble protein preparation was a coupling factor for mitochondrial energy-requiring processes and oxidative phosphorylation. When added to A-particles it increased P/O ratios and increased the rates of ATP-P_i exchange, ATP-energized NAD^+ reduction by succinate and ATP-energized pyridine nucleotide transhydro-

* Abbreviations: OSCP, oligomycin-sensitivity conferring protein; DTT, dithiothreitol; A-particles, depleted submitochondrial particles prepared as described in "Methods"; F_1 coupling factor one (oligomycin-insensitive ATPase), prepared as described in "Methods"; CMPS, parachloromercuriphenylsulfonic acid.

genation. The protein also facilitated the binding of F_1 to depleted mitochondrial membrane fragments.[1,2] The resultant bound ATPase activity was partly inhibited by oligomycin, hence the designation "oligomycin-sensitivity conferring protein". Further studies suggested that the OSCP formed the "stalk" or connecting link between the spherical bound F_1 and the membrane.[2]

The purification method previously described yields a preparation of OSCP which contains some contaminants.[1] In this report a modified purification method is described which yields a protein free of minor contaminants. Several physical and chemical features of OSCP have been examined and are described. Knowledge of these features have enabled us to make a useful comparison of OSCP with several other previously described coupling factors and to make some suggestions as to the organization of the various "coupling factors" in mitochondria.

Materials and Methods

Purification of OSCP from sub-mitochondrial particles. Sub-mitochondrial particles were prepared as described previously.[3] These particles were then used directly as a starting source for the purification of OSCP.

The particles (500 ml) were extracted twice with 3·5 M NaBr solution in the presence of DTT, following the procedure described previously.[1] The extracted particles were suspended in a solution of 0·2 M KCl – 1 mM EDTA in a final volume of 500 ml.

The particles were then extracted twice with ammonia solution (final concentration of $NH_3 = 0{\cdot}4$ N) and the particles were separated from the soluble supernatants as described previously.[1]

The mixed supernatants were adjusted to pH 8·0 with 6 N acetic acid. DTT was added to 0·2 mM concentration. The solution was centrifuged (78,000g × 15 min) and the pellet was discarded. The volume of the supernatant fluid was measured and 5 volumes of cold water were added. The solution was stirred briskly while 45 ml of packed CM-Sephadex-C-25 (coarse) (previously equilibrated with a solution 20 mM in KCl, 40 mM in ammonium acetate, pH 8·0) was poured into the solution. Brisk stirring was maintained for 40 min. The suspension then stood 1 hour. The bulk of the liquid was removed by aspiration, leaving a slurry of ion-exchanger. The slurry was poured into a column 14 cm high × 4 cm diameter, fitted with a sintered glass disk at the base. The ion-exchanger was retained in the column and appeared bright pink at this stage. The column was washed with 45 ml of a solution 15 mM in Tris-SO_4, 0·1 mM in EDTA, 0·1 mM in DTT and 0·15 M in KCl, pH 7·5, at an elution rate of 2 ml/min. The effluent was discarded. The column was developed with a linear gradient between 100 ml of a solution 15 mM in Tris-SO_4, 0·1 mM in EDTA, 0·1 mM in DTT and 0·15 M in KCl, pH 7·5 in the reservoir nearest the column and 100 ml of a solution 15 mM in Tris-SO_4, 0·1 mM in EDTA, 0·1 mM in DTT and 0·28 M in KCl, pH 7·5 in the other reservoir. The elution rate was 30 ml/hr, fractions of 8 ml were collected, and the column was allowed to run dry. Fractions were assayed for activity by their ability to stimulate ATP-P_i exchange when added to A-particles.[1] Most of the pink material eluted in the first half of the gradient. All active fractions with no absorption at 550 nm were pooled. Solid ammonium sulfate was added to 42% saturation (23·8g/100 ml). The suspension stood 60 min and was centrifuged (78,000g × 20 min). The final pellet was dissolved in

20 mM Tris-SO_4, pH 8·0 and the total protein was assayed. The protein was stored as a suspension in 75% saturation ammonium sulfate solution at 4°. It was stable for several months.

This modified procedure yields 8·0–16·0 mg OSCP from 20 g of sub-mitochondrial particle suspension. The protein is soluble (up to 4 mg/ml) in 20 mM Tris-SO_4, pH 8·0 or 20 mM Tris-SO_4 – 1 mM EDTA, pH 8·0.

Assays. In all assays OSCP was added as a solution in 20 mM Tris-SO_4, pH 8·0 (1 mg/ml) and F_1 as a solution in 40 mM Tris-SO_4 – 0·1 mM EDTA – 2 mM ATP, pH 7·5 (10 mg/ml). The specific ATPase activity of the F_1 ranged from 80 to 120 μmoles/min/mg. ATP-$^{32}P_i$ exchange and ATP-driven reduction of NAD^+ by succinate were assayed essentially as described previously.[1] The reaction temperature for ATP-P_i exchange was 25°. Binding of F_1 to depleted membrane fragments by OSCP was assayed as described previously.[1]

Polyacrylamide disk-gel electrophoresis. Three buffer systems were used: (a) the phenol-urea-acetic acid system (pH $\simeq$ 3) of Takayama, MacLennan, Tzagoloff, and Stoner[4] as modified by Senior and MacLennan;[5] (b) the SDS-phosphate system (pH 7·5) as described previously;[3] (c) a Tris-glycine-urea system, pH 9·3. In this system the electrode reservoir buffer was 30 mM Tris-25 mM glycine adjusted to pH 9·3 with KOH. The gels were 5 cm long, 5% in acrylamide, and were polymerized in electrode reservoir buffer to which urea (7 M) was added. Samples of OSCP were dissolved in the same buffer containing urea (7 M), at 5 mg/ml, and dialysed for 2 days against this buffer. The protein concentration was adjusted to 3 mg/ml before electrophoresis. Pre-electrophoresis was carried out for 3 hr at 2 mA/tube. Both reservoir buffers were then replaced; the samples were applied and electrophoresis was carried out for 3 hr at 2 mA/tube. Cytochrome *c* was a useful marker, moving 1 cm/hr under these conditions. All gels were stained with Coomassie Blue as described previously.[3, 5]

Amino acid analyses. OSCP was dissolved in water and dialysed for 4 days at 0° against large volumes of water. The suspension was lyophilized. Hydrolysis of the protein, and amino acid analyses were carried out as described previously.[5] Here 2 mg samples of protein were hydrolysed for 22 hr.

Preparation of coupling factors F_c and F_3. Factor F_c was prepared and assayed as described by Bulos and Racker.[6] The pH fractionation was employed at Step 3B. Factor F_3 was prepared from frozen beef heart mitochondria essentially as described by Fessenden and Racker.[7] At the sonication stage a Branson sonicator was used. With the setting at full power, the mitochondria were sonicated for 3 one minute periods, with cooling on ice for two minutes between each sonication period. Factor F_3 was assayed by measuring the stimulation of ATP-driven NAD^+ reduction by succinate in A-particles. The activities of the F_c and F_3 preparations were very similar to those previously described.[6, 7]

CM-sephadex chromatography of factor F_c. The protein was precipitated by addition of solid ammonium sulfate to 80% saturation. After centrifugation the protein was dissolved in a solution 15 mM in Tris-SO_4, 0·1 mM in EDTA and 0·1 mM in DTT, pH 7·5, at 2 mg/ml. This solution was applied to a column (20 cm high × 1·5 cm diameter) of CM-sephadex-C25 (coarse) equilibrated in the same buffer. The column was developed by washing first with 50 ml of the buffer solution containing 0·15 M KCl and then with 50 ml of the buffer solution containing 0·28 M KCl. The second eluate was collected. Solid ammonium sulfate was added to give 45% saturation and the precipitated

protein ("F_c-CM eluate") was collected. These chromatography conditions were chosen to closely resemble those used above to purify OSCP.

Other procedures. Protein was estimated by Miller's method[8] standardized with bovine serum albumin. F_1 was prepared by the method of Senior and Brooks.[3] A-particles were prepared as described by Fessenden and Racker.[9]

Results

Polyacrylamide gel electrophoresis of the purified OSCP. The behavior of the protein in each of the three systems used is shown in Fig. 1A-C. When densitometric tracings of these gels were made[5] only one absorbing peak was seen in each gel. It should be noted that the protein migrated slowly (2 mm/hr) towards the cathode in the high pH system (Fig. 1C). Thus the isoelectric point of the protein under these conditions was slightly higher

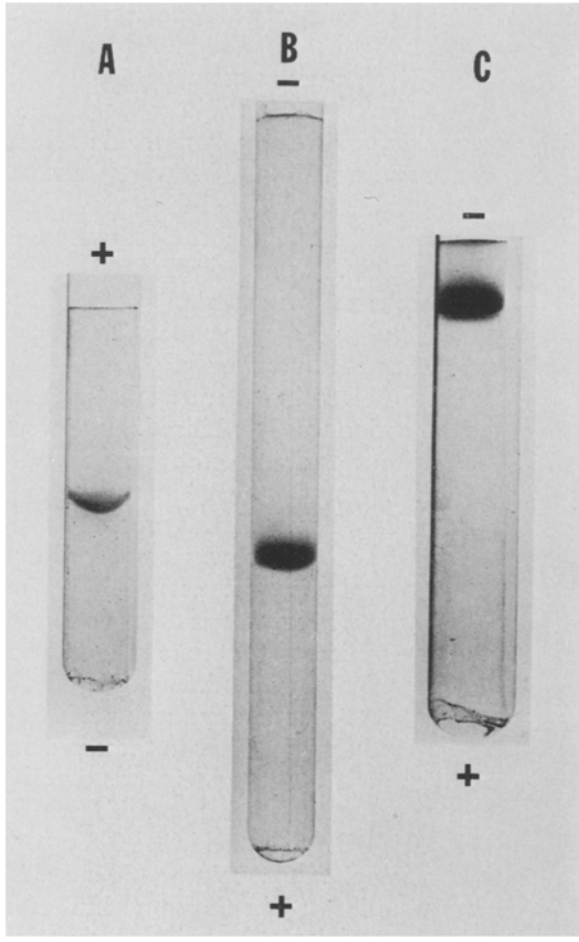

Figure 1. Polyacrylamide gel electrophoresis of OSCP. A, in phenol-urea-acetic acid (62·5 μg). B, in SDS-phosphate (40 μg). C, in Tris-glycine-urea (40 μg).

than pH 9·3. When the electrodes were reversed in the high pH system, no protein entered the gel. No stained material was observed on the origins of gels run in SDS or Tris-glycine-buffer. Only one band was seen in each system, therefore the purification procedure described here yields a homogeneous protein. The molecular weight obtained from gel-electrophoresis in the SDS-buffer was 17,900 ± 500 (mean of four experiments). This is in agreement with the value (18,000) obtained previously by gel filtration in dilute aqueous buffer.[1]

Amino acid analysis of OSCP. Recoveries were 85% to 100% on the basis of weights of amino acids recovered per dry weight of protein hydrolyzed. Variation in recovery of cysteic acid was very large and the value for cysteic acid shown in Table I cannot be considered reliable. The results are shown in Table I. Calculation indicates there is one histidine residue per molecule.

TABLE I. Amino acid composition of OSCP

Amino Acid	Composition* (μmoles per 100 μmoles recovered)
Cysteic acid	0·6
Aspartic acid	4·6
Threonine	5·6
Serine	6·7
Glutamic acid	12·4
Proline	4·8
Glycine	6·3
Alanine	9·6
Valine	9·8
Methionine	3·3
Isoleucine	5·9
Leucine	12·5
Tyrosine	2·25
Phenylalanine	2·2
Lysine	8·9
Histidine	0·7
Arginine	4·5

* Values given are means obtained from hydrolyses of four protein samples. Variation was not more than 5% above or below these mean values except in the case of cysteic acid (see text).

Catalytic functions of OSCP. The protein prepared as described here had all the catalytic functions of the previously described preparation.[1] These included the stimulation of ATP-P_i exchange in A-particles, the ATP-driven NAD^+, reduction by succinate in A-particles, and the conferral of oligomycin-sensitivity (up to 60%) to F_1 bound to ammonia-extracted sub-mitochondrial particles or membrane fragments. Thus this single protein species acts as both an oligomycin-sensitivity conferring factor and as an energy-transfer factor.

Effect of chloromercuriphenylsulfonate and iodine on OSCP. Sulfhydryl reagents and iodine have previously been used as diagnostic inhibitors for mitochondrial "coupling factors". It was of great interest therefore to examine the effects of these inhibitors on OSCP, thereby making possible an examination of the relationship of OSCP to other "coupling factors".

OSCP was incubated with chloromercuriphenylsulfonate (CMPS) at varying concentrations, separated from unreacted CMPS by gel filtration and then reconstituted with A-particles in the presence of F_1. The details of the technique are given in the legend to Fig. 2. As seen in Fig. 2, none of the concentrations of CMPS used inactivated OSCP to any degree. In other experiments it was found that iodoacetate and iodoacetamide did not activate OSCP to any degree. In similar experiments I_2 (4 mM solution in 40 mM KI) was allowed to react for 10 min at 0° with OSCP. The unreacted iodine was then removed (technique exactly as in legend to Fig. 2). At levels of 735 nmoles and 1·4 μmoles I_2/mg OSCP, the activity of OSCP was unaffected.

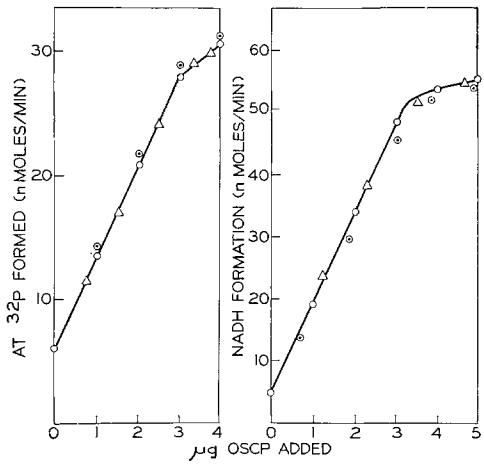

Figure 2. Effect of chloromercuriphenylsulonate on catalytic functions of OSCP. 0·3 mg of OSCP in 0·3 ml 20 mM Tris-SO_4 (pH 8·0) was incubated with varying amounts of CMPS for 40 min at 0°. Then the protein was applied to a column (60 cm high × 0·9 cm diam.) of Sephadex G-25 equilibrated with 20 mM Tris-SO_4, pH 8·0. The void volume peak containing OSCP was collected. After estimation of the protein concentration various amounts of OSCP were incubated with 0·5 mg of A-particles and 100 μg of F_1. ATP-P_i exchange (left) and ATP-driven NAD-reduction by succinate (right) were measured as described under "Methods." ○, untreated OSCP; △, OSCP pre-incubated with 1·55 μmoles CMPS/mg OSCP; ⊙ OSCP pre-incubated with 16·7 μmoles CMPS/mg OSCP.

Effect of preincubation of A-particles with chloromercuriphenylsulfonate on reconstitution with OSCP. It has previously been shown that CMPS at low concentration inhibits ATP-P_i exchange and ATP-driven NAD^+ reduction by succinate in submitochondrial particles.[10] This was found to be true also in the A-particle reconstituted with OSCP and F_1 (data not shown). The ATPase activity of submitochondrial particles was found to be insensitive to CMPS at the low levels required to inhibit ATP-P_i exchange,[10,11] and OSCP is insensitive to CMPS (above). Thus, it was likely that the functional-SH group(s) attacked by CMPS resided in the A-particle itself. This was demonstrated by incubating A-particles with CMPS prior to reconstituting with OSCP and F_1. As seen in Table II, preincubation of A-particles with CMPS completely abolished the ATP-P_i exchange usually noted after reconstitution with OSCP and F_1.

Polyacrylamide gel electrophoresis of factors F_c and F_3. Beechey and co-workers[12] have recently suggested that factors F_3 and F_c contain OSCP as the active principle, since their activities were enhanced after chromatography on CM-cellulose. It was interesting therefore to pursue this suggestion by determining whether F_c and F_3 contained a

TABLE II. Effect of prior treatment of A-particles with CMPS on subsequent reconstitution with OSCP and F_1*

Particle	Additions	ATP-$^{32}P_i$ exchange rate (nmoles/min)
A-particle, untreated	F_1	5·0
	1 μg OSCP + F_1	8·2
	2 μg OSCP + F_1	14·4
	5 μg OSCP + F_1	25·1
A-particle pre-treated with 1 μmole CMPS/mg protein	F_1	1·7
	1 μg OSCP + F_1	0·8
	2 μg OSCP + F_1	1·7
	5 μg OSCP + F_1	1·6

* A-particles (2 ml; 50 mg) in 0·25 M-sucrose–10 mM Tris Cl, pH 7·5 were incubated with CMPS for 40 min at 0°. The particles were separated from unreacted CMPS by gel filtration on Sephadex G-25 in 20 mM Tris-SO_4 pH 8·0. For reconstitution 500 μg of particles were incubated with 100 μg F_1 and amounts of OSCP as described.

protein of MW = 18,000, and whether purification of this protein was achieved by chromatography on CM-ion exchangers. Samples of factor F_c and F_3 were examined by the SDS-gel method. Densitometric tracings of the gels are shown in Figs. 3A and 3B. Both preparations were extremely heterogeneous, and both contained a component which migrated with the same mobility as OSCP (i.e. of MW = 18,000). The protein fraction obtained by chromatography of factor F_c on CM-sephadex ("F_c-CM eluate") is shown in Fig. 3C. It is seen that the proportion of protein of MW = 18,000 was

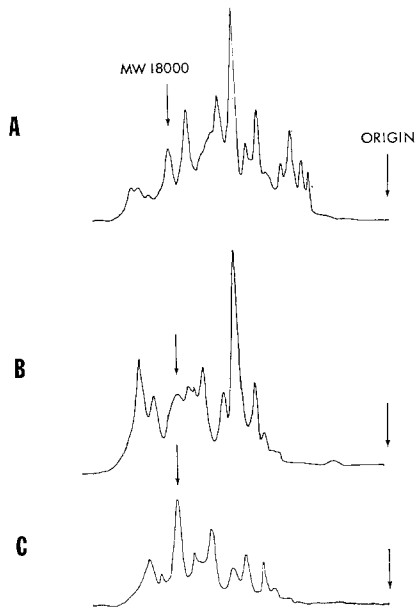

Figure 3. Polyacrylamide gel electrophoresis of F_c and F_3 in SDS-phosphate buffer. A, F_3 (60 μg); B, F_c (60 μg); C, F_c-CM eluate (60 μg). Migration was from the origin at the right (arrow) towards the anode at the left. The position of the component of MW 18,000 was calculated from a standard curve.[3] Pure OSCP moved to this position when co-electrophoresed with the factors in A, B, and C (not shown).

considerably increased by this procedure. Concomitantly, I noted that the oligomycin-sensitivity conferring activity of the "F_c-CM eluate" was increased 6-fold over the crude F_c, and that "F_c-CM eluate" was active as an energy-transfer-coupling factor, having a specific activity of 0·33 as defined by Beechey *et al.*[12] in the ATP-driven NAD^+ reduction assay.

I attempted to purify OSCP from F_3 by a similar chromatographic procedure but was unable to adsorb sufficient amounts of protein on CM-Sephadex. Neither the coupling factor activity nor the protein in F_3 of 18,000 MW were adsorbed in those experiments.

Discussion

Relationship of OSCP to other coupling factors. Coupling factors fall into two groups, those with overt or latent with ATPase activity (F_1 and Factor A) and those devoid of ATPase activity ("energy-transfer" factors). OSCP is without ATPase activity under any conditions and falls into the second group. Also in the second group may be placed Factors B, C, and D,[10, 13, 14] Factor X[15] and several factors of the "F" series (F_c, F_2, F_3, F_4, F_5, F_6) described by Racker and co-workers [references given in (16)]. Of the factors in this group only Factor B has previously been satisfactorily purified and shown to be a homogeneous protein preparation.

The great multiplicity of factors in this group, and the fact that only one factor had previously been purified, has recently stimulated some consolidation in this area. Thus Beechey[17] has suggested that there may be really only one coupling factor of the non-ATPase variety (i.e., that F_c, F_2, F_3, F_4, F_5, X, B are in effect all preparations of OSCP or "basic coupling factor" in greater or lesser purity), and recently Racker, Sanadi and co-workers have collaborated to show that B and F_2 are in fact the same protein.[18] The availability of a homogeneous preparation of OSCP and knowledge of some of its chemical and physical properties enables us now to make further suggestions regarding the number and arrangement of coupling factors in mitochondria.

OSCP and B differ in many chemical and physical properties (molecular weight, amino acid composition, susceptibility to inhibition by CMPS and iodine). Therefore B and OSCP are clearly distinct proteins.

Factor F_5 is inactivated by iodine (at much lower levels than are required to inactivate B), although not by the sulfhydryl-reagents parahydroxymercuribenzoate, iodoacetate and mersalyl.[19] Therefore it appears the active principle of F_5 is definitely not OSCP, and probably not B, and the purification to homogeneity of F_5 will be awaited keenly.

Like OSCP, neither F_c nor F_3 are inhibited by iodine, and both are insoluble in low concentrations (<40%) of ammonium sulfate solutions (Ref. 19, and A. E. Senior unpublished studies). Beechey and co-workers have shown[12] that both F_c and F_3 contain an active principle which behaves chromatographically like OSCP. Further, both F_c and F_3 give rise after further chromatographic resolution on CM ion exchangers to an active principle having both oligomycin-sensitivity conferring activity and energy-transfer activity.[12] Here, it was found that such chromatographic resolution gave marked resolution of a protein of MW = 18,000 when F_c was the source. Polyacrylamide gel electrophoresis in SDS indicated that F_3 contained a protein of MW = 18,000, but the active principle was not adsorbed to CM-Sephadex in these studies. The available

evidence therefore shows that F_c contains OSCP as the active principle and strongly suggests that F_3 contains OSCP as the active principle. Factor X is thought to contain OSCP as the active principle,[15] and it has already been shown that F_4 contains OSCP as an active principle.[1]

In conclusion, therefore, the presently available evidence indicates that OSCP and B are definitely separate components of the mitochondrial ATP-synthesizing system, that F_5 is very probably another distinct component (although it has not yet been purified to homogeneity), that F_c, F_3, F_4, and X all contain OSCP as the active principle, and that F_2 contains B as active principle. Data currently available on Factors F_6, C, and D do not allow comparisons of physical and chemical properties with those of OSCP, B, or F_5, so they remain "in chancery".

Organization of the mitochondrial ATP-synthesizing system. Although it seems clear that B, OSCP, and F_5 (and other factors which may "prove" themselves in the future) act as transducing links between the mitochondrial "headpieces" (F_1) and the electron transfer complexes, and that they are all involved at each phosphorylation site, it is extremely difficult to distinguish the order of interaction. The order of additions during assay appears to have little effect on the outcome of the experiments, even when particles are centrifuged and washed between additions. (A. E. Senior unpublished studies.) However some circumstantial evidence is available, It has been proposed[2] that OSCP makes up the stalk which connects the "headpiece" to the membrane and it has been shown here that prior treatment of A-particles with CMPS prevents the emergence of coupling (ATP-P_i exchange) normally seen when the particles are incubated with added OSCP and F_1. Factor B has been tentatively localized in the inner membrane continuum.[10] These observations suggest that the order of interaction might be: F_1 (headpiece) $\rightarrow$ B, F_5 (in the inner membrane) $\rightarrow$ electron transfer complex. Clearly this is a speculative suggestion, other and hitherto uncharacterized protein components are also probably involved. The interaction of these coupling proteins with the ATPase (F_1), with the electron transfer components, and with each other is however a problem of central importance to the understanding of oxidative phosphorylation and future work will no doubt be involved in examining this type of model.

Acknowledgements

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