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Minireview

The Proton-Translocating NADH : Ubiquinone Oxidoreductase: A Discussion of Selected Topics

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The proton-translocating NADH : ubiquinone oxidoreductase (complex I) is a large, multi-subunit and multi-redox centre enzyme which is found in the mitochondrial inner membrane and plasma membrane of some bacteria. In this minireview an attempt has been made to critically discuss selected topics in the structure and function of this enzyme. A special emphasis is given to the iron-sulphur cluster and to the proteins that may bind them. Previous suggestions for the mechanism of proton-translocation by complex I are discussed. Subcomplexes that contain several but not all of the subunits of the intact mitochrondrial enzyme are described and analysed in order to identify the functional core of the enzyme. The data on the trans-membrane organisation of several subunits is examined. It is hoped that most of the suggestions as well as the questions raised here could be experimentally tested in the near future.

KEY WORDS: NADH dehydrogenase; iron–sulphur clusters; proton pumping; subomplexes;

INTRODUCTION

The mitochondrial NADH : ubiquinone oxidoreductase (complex I) is a large and complex membrane enzyme. It catalyses the reduction of ubiquinone by two electrons donated from NADH, and this reaction provides the substrate for the other proton-translocating enzymes of the respiratory chain. In yeast and bacteria, ubiquinone reduction by NADH is also catalyzed by simpler enzymes (De Vries and Marres, 1987; Jaworowski *et al.*, 1981). The important difference between such enzymes and complex I is that only the latter couples electron transfer to proton translocation across the membrane. This proton translocation activity transduces a significant portion of the redox energy to an electrochemical gradient of protons across the membrane, which can be used to synthesise ATP (Mitchell, 1961).

Mitochondrial complex I is located in the inner membrane of organelle and is composed of about 40 polypeptide subunits. The redox active centres that participate in the electron transfer reactions are one FMN and at least four different Fe-S clusters per monomeric complex. Some bacteria have a homologous enzyme, called NDH1, which is located in the cytoplasmic membrane (Yagi, 1991). NDH1 of *Paracoccus denitrificans* is presently the best characterized bacterial complex I, and it will be further discussed below.

Several reviews on complex I have been published in recent years (Ragan, 1987; Weiss *et al.*, 1991; Walker, 1992). This article does not attempt to cover the entire literature on the enzyme, but to discuss a few topics, and to examine them in the light of recent developments in the field.

COMPOSITION AND POTENTIALS OF THE Fe-S CLUSTERS

Four different iron–sulfur clusters are reproducibly observed by EPR spectroscopy in mitochondrial complex I (Table I). Clusters 1a and 5 were not included in this table since at present it is not certain that they are integral components of complex I.

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Table I. EPR-Detectable Iron-Sulfur Clusters of Complex I[a]

Name[b]	g values			Type
	g_z	g_y	g_x	
Cluster 1b (N-1b)	2.02	1.94	1.92	2Fe–2S
Cluster 2 (N-2)	2.05	1.92	1.92	4Fe–4S
Cluster 3 (N-4)	2.10	1.93	1.88	4Fe–4S
Cluster 4 (N-3)	2.04	1.93	1.86	4Fe–4S

[a] Data from Ohnishi (1979) and Beinert and Albracht (1982).
[b] The nomenclature of Albracht is used in this article (Beinert and Albracht, 1982). The nomenclature of Ohnishi (1979) is given in parentheses.

Cluster 1b is detectable by EPR spectroscopy in NADH-reduced enzyme, already at 77°K (Orme-Johnson *et al.*, 1974). In bovine complex I the spectral symmetry of this cluster is rhombic, but it has an axial symmetry in *Paracoccus* (Meinhardt *et al.*, 1987) and *Neurospora* (Wang *et al.*, 1991). The midpoint potential of cluster 1b in all these systems is in the range of −245 to −335 mV (Ingledew and Ohnishi, 1980; Meinhardt *et al.*, 1987; Wang *et al.*, 1991).

There is a disagreement concerning the content of cluster 1b in bovine complex I. Albracht and co-workers find that the ratio of cluster 1b to FMN (and the other Fe-S clusters) is 0.5 (Van Belzen *et al.*, 1992), while others reported a ratio of nearly 1.0 (Orme-Johnson *et al.*, 1974; Ohnishi, 1979; Kowal *et al.*, 1986). However, in the *Paracoccus* cytoplasmic membrane both Albracht's and Ohnishi's laboratories find that cluster 1b is present in the same concentration as the other clusters (Albracht *et al.*, 1980; Meinhardt *et al.*, 1987).

Hatefi and Hanstein (1973) found that cluster 1b is reduced by NADH but not by NADPH even though this substrate drives rotenone-sensitive oxidative phosphorylation. This finding was later followed by pre-steady-state kinetic examination of the reducibility of the different clusters by both these electron donors (Van Belzen and Albracht, 1989). The slow reduction of cluster 1b by NADPH and its low content relative to the other clusters were taken to suggest that complex I is a heterodimer, and that only one of its protomers contains cluster 1b (Van Belzen *et al.*, 1990).

A different debate has been on the presence of a very low potential binuclear cluster that is not reduced by NADH. Ohnishi and co-workers observed such a redox centre, the midpoint potential of which is −380 mV or lower, and named it cluster N-1a (Ohnishi and Salerno, 1982). On the other hand, Albracht *et al.* (1977) could not detect such a cluster. It might be added that even if the presence of a very low potential cluster will be verified, it does not necessarily mean that it participates in the electron transfer activity of the enzyme.

Two binuclear clusters were detected in *Paracoccus*, but neither has a very low midpoint potential (Meinhardt *et al.*, 1987). Cluster N-1b has a similar potential to the mitochondrial cluster 1b, and the other cluster, N-1a, has a higher potential than 1b, and not much lower as reported in mitochondria. The similarity between the *Paracoccus* and mitochondrial clusters N-1a is the pH dependence of their midpoint potential. Interestingly, in *Paracoccus* the potential of cluster N-1a is very close to that of cluster 2, the only other cluster in the enzyme whose midpoint potential is dependent on pH.

The EPR studies on *Paracoccus* complex I have thus far been conducted with cytoplasmic membranes. EPR spectroscopy of purified and fully active complex I from *Paracoccus* could help to clarify the content and properties of the binuclear, as well as tetranuclear, clusters in this enzyme.

A second binuclear cluster, in addition to cluster 1b, has not been found in *Neurospora crassa* complex I in the redox potential range measured so far (Wang *et al.*, 1991).

The tetranuclear Fe-S clusters are visible in EPR spectroscopy of NADH-reduced enzyme below 20 °K (Ohnishi, 1979; Beinert and Albracht, 1982). The spectral symmetry of cluster 2 is axial, while clusters 3 and 4 are rhombic. The midpoint potentials of clusters 3 and 4 are in the same range as that of cluster 1b, i.e., −245 to −330 mV, and independent of pH (Ingledew and Ohnishi, 1980; Meinhardt *et al.*, 1987; Wang *et al.*, 1991).

The midpoint potential of cluster 2 is high, but its accurate value is not yet clear. Ingledew and Ohnishi (1980) reported that in particles prepared from both pigeon and bovine heart mitochondria the potential of cluster 2 is high, and pH-dependent with 60 mV per pH unit. In pigeon heart mitochondria at pH 7.0 it was −20 mV, and in bovine −150 mV (Fig. 1 of Ingledew and Ohnishi, 1980). In the summary of that work, however, only the pigeon heart mitochondrial value was given (Table I of that article) and it has later been cited in many cases as the potential of cluster 2 of the bovine enzyme as well.

Later studies reported that the midpoint potential of cluster 2 in bovine, *Neurospora*, and *Paracoccus*

is around −150 mV at pH 7–7.5 (Van Belzen, 1991; Wang *et al.*, 1991; Meinhardt *et al.*, 1987).

At very low temperature and high radiation power a fourth tetranuclear cluster is visible in complex I, called cluster 5 (Ohnishi, 1979; Beinert and Albracht, 1982). At present it is unclear if this cluster is a true component of complex I or a contaminant since its spin concentration is low. In addition, in submitochondrial particles of the fungus *Candida crusei* it was not detected at all (Albracht *et al.*, 1977). However, if cluster 5 is a contaminant, it must be a tightly bound one since subcomplex Iα contains cluster 5 even though it lacks many subunits of the intact enzyme (Finel *et al.*, 1992).

The total number of Fe-S clusters in complex I is not known with certainty. Determination of nonheme iron and acid-labile sulfur indicated in many cases that there are more clusters than seen by EPR (Ragan, 1987). If so, the additional clusters must be either diamagnetic or magnetically coupled to another paramagnetic center. The latter possibility was tested in a low-temperature magnetic circular dichroism study, which yielded no evidence for paramagnetic Fe-S clusters in complex I that are EPR invisible (Kowal *et al.*, 1986). These authors also discuss possible overestimations in the quantitation of the nonheme iron content in complex I.

The enzyme used by Kowal *et al.* (1986) was not intact complex I but a subcomplex called "high-moleculear-weight NADH dehydrogenase." This preparation has all the EPR-detectable Fe-S clusters (Kowal *et al.*, 1986), but the lineshapes of some of them are slightly modified (Finel *et al.*, 1992). This enzyme lacks several subunits of complex I, and it does not reduce quinone analogues (Paech *et al.*, 1982). Thus, the possibility that some EPR-invisible Fe-S clusters were lost during its preparation cannot be ruled out.

LOCATION OF THE Fe-S CLUSTERS IN COMPLEX I

This topic was revolutionized by the primary structure determination of subunits of bovine complex I, and of the *Neurospora* and *Paracoccus* enzymes (reviewed by Fearnley and Walker, 1992). Until then, identification of subunits that bind Fe-S clusters was done by EPR spectroscopy of fractions prepared from intact complex I by treatment with chaotrophic agents, mainly perchlorate (for a review, see Ragan, 1987). However, these proteins might have been partly denatured (see discussion below), and the analyses were hampered by the lack of typical line shapes of the known clusters in those fractions. In addition, the signals that were detected were present at lower spin concentration than expected from the amount of iron found in the fractions (Ohnishi *et al.*, 1985; Ragan *et al.*, 1986). Due to these difficulties, the conclusion derived from such experiments should be treated with caution. For example, it was determined that the 49, 30, and 13 kDa subunits bind between them one tetra- and one binuclear clusters (Ohnishi *et al.*, 1985). However, the primary structure of these polypeptides does not reveal any potential Fe-S cluster binding site (Fearnley and Walker, 1992). Similarly, it was previously thought that the 70 kDa subunit of succinate dehydrogenase binds two binuclear clusters, since after purification using chaotrophic agents, it contained sufficient amounts of nonheme iron and acid-labile sulfur (summarized in Ohnishi and Salerno, 1982). However, the primary structure indicates that all the Fe-S clusters of complex II are within the 27 kDa protein, and none in the 70 kDa subunit (Ohnishi, 1987).

The treatment with chaotrophic agents probably denatures a certain population of the enzyme with concatenate release of Fe-S clusters. These clusters might then dissociate or be converted to smaller ones and bind to the protein, but not necessarily in their native binding sites. Such a possibility would be in line with the observation that following extraction from complex I the majority of the Fe-S clusters were binuclear and not tetranuclear as expected from the EPR data (Paech *et al.*, 1981).

The 24 kDa subunit was suggested to bind cluster 1b (Ohnishi *et al.*, 1985), and it was reported to have a weak homology to other Fe-S binding proteins (Pilkington and Walker, 1989). However, that homology is even weaker if the *Paracoccus* counterpart is taken into account (not shown), and the EPR data may not be sufficient since the protein was treated by perchlorate (see discussion above).

Four cysteines in 24 kDa protein are conserved in mammals and *Paracoccus* (Xu *et al.*, 1992). Two out of these four are also conserved in the NAD-reducing hydrogenase of *Alcaligenes eutrophus*, an enzyme thought to be homologous to the NADH dehydrogenase section of complex I (Pilkington *et al.*, 1991a). However, since only two out of the four cysteines are conserved in the hydrogenase, and four are needed to bind such a cluster, this homology does

not suggest that cluster 1b is bound by the 24 kDa subunit.

One could argue that the hydrogenase binuclear cluster is not homologous to cluster 1b, and is bound by another domain of that enzyme. In this case the idea that cluster 1b is part of the NADH dehydrogenase "module" common to both complex I and the NAD-reducing hydrogenase (Walker, 1992) will have to be modified.

What might be the role of the two strictly conserved cysteines in the 24 kDa subunit? It has recently been shown that although there are enough highly conserved cysteines in the Rieske subunit of the bc_1 complex of *Rhodobacter capsulatus*, not all of them are involved in binding the Fe-S cluster of this enzyme (Davidson *et al.*, 1992). It was suggested instead that two of the conserved cysteines form an S-S bridge, and this might also be the case with the 24 kDa subunit of complex I.

Which subunit binds cluster 1b if it is not the 24 kDa protein? One of the subunits of the NAD-reducing hydrogenase is homologous to the 51 plus 24 kDa subunits (α or Hox F), and another (γ or Hox U) to a segment of the 75 kDa subunit of complex I. These two hydrogenase subunits were reported to bind between them two tetranuclear and one or two binuclear Fe-S clusters (Schneider *et al.*, 1979, 1984). It was later suggested that subunit γ binds two tetranuclear clusters, while the α subunit binds the binuclear cluster (Tran-Betcke *et al.*, 1990). However, this suggestion was made before the homology to complex I was known, and it might now have to be amended. The arrangement of the fully conserved cysteines in the segment of the α subunit which is homologous to the 51 kDa subunit of complex I indicates that it binds a tetra- and not a binuclear cluster. This, in turn, suggests that the γ subunit binds one bi- and one tetranuclear clusters.

The cysteines of the 75 kDa subunit of complex I are fully conserved in the γ subunit of the hydrogenase (Pilkington *et al.*, 1991a). This holds for the cysteines that probably bind a tetranuclear cluster (cys 153, 156, 159, and 203 in the bovine sequence), as well as seven others. This conservation and the possibility that the γ subunit of the hydrogenase binds two Fe-S clusters suggest that the 75 kDa and not the 24 kDa subunit of complex I binds a binuclear cluster in this domain of the enzyme. The cluster is probably 1b, although this still needs confirmation.

The 51 kDa subunit has a potential tetranuclear Fe-S cluster-binding site, in addition to the FMN and NADH binding sites (Pilkington *et al.*, 1991a). This was suggested to be cluster 4 (N-3, Ohnishi *et al.*, 1985), a cluster that was previously reported to interact magnetically with the semiquinone radical of FMN (Salerno *et al.*, 1977).

If the 51 kDa subunit binds cluster 4 (N-3), then the current candidate for the tetranuclear cluster in the 75 kDa subunit is cluster 3 (N-4). It is not likely that either the 51 kDa or the 75 kDa subunits bind cluster 2 since both these subunits are present in the small isoform of *Neurospora crassa* complex I which lacks cluster 2 (Wang *et al.*, 1991).

Krishnamoorthy and Hinkle (1988) have reported that cluster 3 (N-4) can be modified by *N*-bromosuccinimide without significant inhibition of the rotenone-sensitive quinone reduction activity. They have thus suggested that cluster 3 is not located on the main pathway of electrons from NADH to ubiquinone. An alternative explanation to their results is that the rates of reduction and oxidation of cluster 3 are not limiting in the quinone reduction activity under the conditions used. This possibility might gain some support from the result that modification of the last 20% of cluster 3 caused complete inhibition of quinone reduction, but only a small inhibition of the ferricyanide reduction activity (Fig. 3 in Krishnamoorthy and Hinkle, 1988).

The best candidate for binding cluster 2 is the 23 kDa subunit (Dupuis *et al.*, 1991), and this protein is present in subcomplex Iα that was shown to contain cluster 2 (Finel *et al.*, 1992). It is not yet known whether this subunit is present in the small form of complex I purified from mitochondria of *Neurospora crassa* grown in the presence of chloramphenicol, an enzyme that lacks cluster 2 (Wang *et al.*, 1991).

Cluster 2 is thought to be embedded in the membranous domain of complex I (e.g., Weiss *et al.*, 1991), while the primary structure of the 23 kDa subunit does not indicate the presence of any hydrophobic transmembrane helices (Dupuis *et al.*, 1991). On the other hand, this subunit was not observed among the hydrophilic fractions generated by chaotrophic treatments, except in one reported case (Masui *et al.*, 1991). Hence the 23 kDa subunit might still be in close contact with the membranous domain of complex I.

The 23 kDa subunit has two potential binding sites for tetranuclear clusters (Dupuis *et al.*, 1991). It is interesting to find out which of them binds cluster 2, and what the role of the other site is. Is it possible that two clusters 2 are bound by this polypeptide, but that

there is a strong anticooperativity between them so that only one can be reduced, and EPR-detectable, at any given moment? Such a system may stabilize a semiquinone radical at this site, which might be an important part of the proton-translocation machinery (see below). At present, however, this is just a speculation.

PROTON TRANSLOCATION

Complex I translocates two protons across the membrane per one electron transferred from NADH to ubiquinone (Wikström, 1984; Brown and Brand, 1988). A proton-translocation efficiency of less than one H^+/e^- was observed earlier (Ragan and Hinkle, 1975), but this could have been due to experimental difficulties.

In the absence of detailed knowledge of the electron-transfer pathway within complex I, one cannot fully understand the mechanism of proton translocation. Still, it is interesting to discuss several models that were suggested for this activity, since it might help to design future experiments.

In complex I, as in cytochrome oxidase, protons are taken up for both substrate reduction and vectorial translocation across the membrane. Some of the relevant questions in such a case are whether there are two different proton uptake sites or a single one for both these reactions. Does the pH dependence of the midpoint potential of cluster 2 reflect its involvement in the proton-translocation activity or merely in the scalar reaction of ubiquinone reduction?

In both cytochrome oxidase and the cytochrome bc_1 complex most of the pumping machinery is probably embedded in the membranous domains of the enzymes. Does this hold also for complex I where many redox centres are bound by hydrophilic polypeptides? A very important question is whether or not all the protons are pumped by the same mechanism and coupled to the same electron transfer step.

Ragan (1987) suggested a model for proton translocation by complex I that could account for pumping stoichiometries of either 2, 2.5, or even $3H^+/e^-$. The essence of that model is that electrons are transferred through two different pumps which are connected in series. The first one, a flavin cycle, operates at lower potentials, while the other pump, a Q cycle, operates at higher potentials. The flavin cycle requires the presence of a very low-potential Fe-S cluster that can reduce $FMNH^{\cdot}$ to $FMNH_2$. Such a cluster might be N-1a, but its presence in bovine complex I is questionable (Albracht *et al.*, 1977), and in *Paracoccus* its potential is too high (Meinhardt *et al.*, 1987).

The Q cycle pump suggested by Ragan (1987) requires the presence of two Fe-S clusters whose redox potential is high enough to be reduced by ubiquinol. Cluster 2 might be suitable if its potential is as high as in the enzyme from pigeon heart mitochondria (Ingledew and Ohnishi, 1980). However, in most other systems the midpoint potential of cluster 2 was found to be lower (see Section 2), and thus less likely to play such a role.

Krishnamoorthy and Hinkle (1988) have suggested two other versions of a flavin cycle as the core of the proton pump in complex I. In both models a direct electron transfer from FMN to cluster 2 is proposed. In these models the problem of a high-potential single electron acceptor is solved in agreement with a "b-cycle" mechanism (Wikström *et al.*, 1981). However, it might be difficult to incorporate either of these suggestions into the current ideas about the structure of the enzyme. The FMN appears to be bound by the 51 kDa subunit and to have in close proximity to it three Fe-S clusters; none of them is cluster 2 (see Section 3 and Pilkington *et al.*, 1991a). Hence, it is difficult to envisage direct electron transfer from it to cluster 2.

A model for proton translocation that is coupled to electron transfer from cluster 2 to ubiquinone has recently been suggested (Kotlyar *et al.*, 1990). This is an interesting version of a Q cycle that agrees well with the observation that the midpoint potential of cluster 2 is lower than -120 mV. However, the stoichiometry of this model is only $0.5\,H^+/e^-$, and thus it can account for only a quarter of the protons pumped by complex I (Kotlyar *et al.*, 1990).

SUBCOMPLEXES WITHIN COMPLEX I

The huge size of complex I is among the reasons that its structure and function are much less studied than those of the other large complexes of the respiratory chain. One way to overcome this difficulty is to isolate subcomplexes of the intact enzyme, and to characterize them. Several laboratories prepared different types of small complexes by various means, and provided important information on the structure of complex I.

A soluble high-molecular-weight NADH dehydrogenase was prepared by Singer and co-workers by a phospholipase treatment followed by incubation at alkaline pH (Paech *et al.*, 1982). This subcomplex had a high ferricyanide reductase activity but did not reduce Q_1. The reason for the lack of the latter activity is not clear, nor is the subunit composition of that enzyme known. The EPR spectrum of this subcomplex is similar to that of intact complex I (Kowal *et al.*, 1986).

The laboratories of Hatefi and Ragan developed the method to fractionate complex I by chaotrophic agents, such as perchlorate, in combination with ammonium sulfate precipitation (Ragan, 1987). This treatment breaks down the enzyme into three main fractions called flavoprotein (FP), iron-protein (P), and hydrophobic protein (HP). The FP contains FMN, the 51, 24, and 10 kDa subunits, and some nonheme iron and acid-labile sulfur. The IP contains the 75, 49, 30, 18, 15, and 13 kDa subunits as well as nonheme iron and acid-labile sulfur. The HP is a pellet that contains the rest of the subunits and some iron and sulfur.

The chaotrophic fractionation of complex I contributed to the identification of the 51 kDa subunit as the binding site for both NADH and its probable primary electron acceptor, FMN (Galante and Hatefi, 1979; Chen and Guillory, 1981). In addition, it facilitated the purification of several individual subunits for antibodies production and later for protein sequencing. However, as discussed above, there are difficulties in trying to assign specific Fe-S clusters to different fractions, let alone to smaller ones prepared by further treatment of the FP and IP fractions (Ohnishi *et al.*, 1985).

The FP fraction has an NADH oxidase activity with a variety of electron acceptors (Pagani and Galante, 1983). However, its activity is unstable, and the ferricyanide reduction activity, calculated per bound FMN, is much lower than that of the intact enzyme. On the other hand, the activities of the FP fraction with Q_1 or cytochrome *c* as electron acceptors are much higher than such activities of complex I (Galante and Hatefi, 1979).

It is not known whether or not Fe-S cluster(s) are needed for the ferricyanide reduction activity by either complex I or the FP fraction. The stimulation of this activity by guanidine HCl (Galante and Hatefi, 1979) does not indicate a requirement for specific Fe-S clusters. On the other hand, a correlation between activity and the amount of acid-extractable sulfur was reported for the FP fraction, and interpreted as involvement of Fe-S cluster(s) in this activity (Pagani and Galante, 1983).

The homology between the NAD-reducing hydrogenase of *Alcaligenes eutrophus* and complex I prompted the idea of a "modular" NADH dehydrogenase unit in both enzymes (Walker, 1992). In the hydrogenase this domain is composed of two subunits; one is homologous to the 51 plus 24, and the other to a segment of the 75 kDa subunits of complex I (Pilkington *et al.*, 1991a). Why is the 75 kDa subunit missing from the FP fraction, while the 10 kDa subunit that has no counterpart in the hydrogenase is present? Is it a result of the functional differences between the enzymes or an outcome of a harsh treatment of complex I? Answers to such questions might clarify the subunit and redox-center compositions of the electron-input domain of complex I.

A different approach to subcomplex preparation was developed in the laboratory of Weiss working with the fungus *Neurospora crassa*. Inhibition of its mitochondrial protein synthesis by chloramphenicol gave rise to a subcomplex composed of only 13 nuclear-encoded subunits (Freidrich *et al.*, 1989). This enzyme is a rotenone-insensitive quinone reductase, called the small form of complex I.

EPR spectroscopy of the small form showed that it contains clusters 1b, 3, and 4, but not cluster 2 (Wang *et al.*, 1991). The concentration of cluster 3 (N-4) in the small form was, however, significantly lower than that of cluster 4 (N-3), in contrast to their 1 : 1 ratio in the large complex I (Wang *et al.*, 1991). This observation was explained by a loss or modification of cluster 3 during the isolation of the small form. Interestingly, EPR spectra of mitochondrial membranes from a mutant that contains only the small form indicate that already the membranous enzyme has less of cluster 3 (N-4) than of 4 (N-3) (Fig. 4 in Nehls *et al.*, 1982). Whether the change in the ratio of clusters 3 and 4 is directly related to the absence of cluster 2, and thus indicative of magnetic interactions between the latter cluster to one of the formers, remains to be studied.

The complement of the small form, the so called "membrane arm," was suggested to bind cluster 2 (Weiss *et al.*, 1991), and some evidence for that has recently been presented (Schmidt *et al.*, 1992). This evidence should, however, be substantiated by EPR spectroscopy of the immuno-precipitated "membrane arm," and not of mitochondrial membranes because the latter still contain some large complex I.

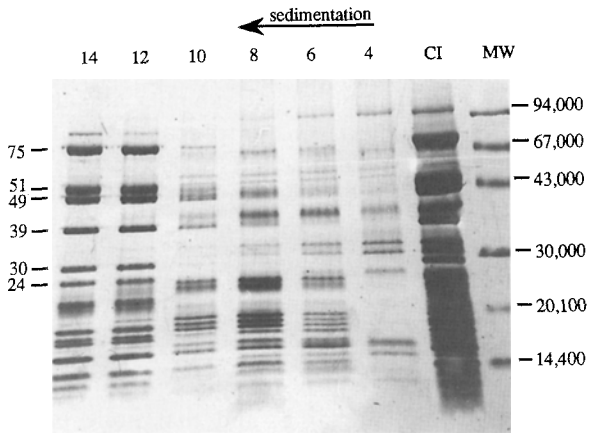

Fig. 1. Resolution of complex I by sucrose gradient centrifugation. Complex I (CI) was subjected to sucrose gradient centrifugation (20–50% sucrose) in the presence of 0.5% LDAO and 50 mM Tris-HCl, pH 7.5. After 18 h of centrifugation at 150,000 × *g*, fractions were collected from the top and analyzed by SDS-PAGE. The numbers above the lanes indicate the fraction in the gradient (24 altogether). Several subunits of subcomplex Iα are indicated on the left, and molecular mass markers are shown on the right.

New subcomplexes have recently been purified from bovine complex I by the use of a strong but not denaturing detergent (Finel *et al.*, 1992). Incubation with LDAO followed by anion exchange chromatography in the presence of this detergent resulted in splitting of complex I into two main subcomplexes, which were named Iα and Iβ. Subcomplex Iα is composed of about 20 subunits, has NADH : ferricyanide and NADH : Q_1 oxidoreductase activities, and contains all the EPR-detectable Fe-S clusters of intact complex I. Minor modifications of the line shapes of clusters 2 and 3 were detected, however. Subcomplex Iβ is composed of about 15 other subunits and does not contain any Fe-S cluster than can be reduced with either NADH or dithionite (Finel *et al.*, 1992). Since subcomplex Iα contains cluster 2 but lacks subunit ND5, it was concluded that ND5 is not the binding site of cluster 2, in contrast to an earlier suggestion (Weiss *et al.*, 1991). All the other subunits that were suggested thus far to bind Fe-S clusters are present in Iα.

Subcomplexes Iα and Iβ can also be prepared by sucrose gradient centrifugation in the presence of LDAO (Fig. 1). The results shown in Fig. 1 also raise the possibility that a third subcomplex is present, and contains some of the subunits that are not found in either Iα or Iβ, such as ND1 and ND2 (see fraction 4 in Fig. 1).

The splitting of complex I by sucrose gradient centrifugation at relatively low ionic strength indicates that the interaction between subcomplexes Iα and Iβ in the intact enzyme is mainly hydrophobic and not electrostatic or polar. This interaction must be rather strong since it was not broken by sucrose gradient centrifugation in the presence of Triton X-100 and 0.5 M potassium phosphate (not shown). Interestingly, a similar centrifugation of cytochrome oxidase in the presence of either Triton X-100 or LDAO monomerizes the dimeric enzyme but does not remove any subunit from the monomeric complex (Finel and Wikström, 1986).

An interesting question in studying the structure of complex I is whether different subcomplexes represent well-defined domains of the intact enzyme. But before such questions can be answered, one has to determine the subunit composition of each subcomplex. The list of subunits found in subcomplex Iα might indicate that it includes all the subunits of both FP and IP, as well as several other ones (Finel *et al.*, 1992). However, a few of these subunits were not reproducibly found in Iα, or appeared to be present at very low amounts. Among them are the 15 kDa subunit of the IP fraction, the 42 kDa subunit, and the hydrophobic subunit ND2. These polypeptides are probably impurities in subcomplex Iα and not its true components. This view is different from that shown in Fig. 9b of (Walker, 1992), but it is supported by the results shown in Fig. 1. The polypeptides with apparent *Mr* of 33 and 31 kDa, most probably the ND2 and ND1 subunits, respectively, sediment slowly in the sucrose gradient and differently from either subcomplex Iα or Iβ. They are mainly seen in fraction 4 of this gradient, while subcomplexes Iβ and Iα are in fractions 8 and 12–14, respectively (Fig. 1). The position of the 15 kDa subunit is not clear in this gel, but it is interesting that Ragan *et al.* (1986) already questioned it being part of the IP fraction, since it was not immuno-precipitated with the other polypeptides of this fraction.

We are currently characterizing new subcomplexes that have modified EPR spectra of cluster 2 and different subunit compositions (Finel *et al.*, in preparation). Two of these subcomplexes, called Iλ and IS, are shown in Fig. 2. Both of them were prepared by sucrose gradient centrifugation; Iλ in the presence of LDAO and high concentration of potassium phosphate, and IS in the presence of lauroylsarcosine. Subcomplex IS has more subunits, including the very hydrophobic ND4, but there are

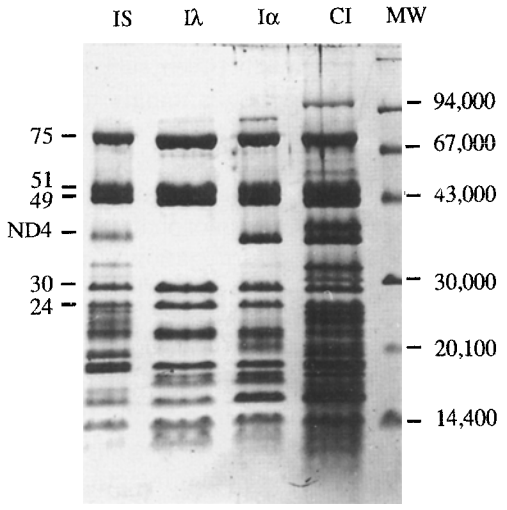

Fig. 2. SDS-PAGE analysis of three FMN-containing subcomplexes that were isolated from complex I by sucrose gradient centrifugation under different conditions. The names of the subcomplexes are given at the top of each lane, and complex I is marked as CI. Some subunits of subcomplex IS are indicated on the left, and molecular mass markers on the right. See Fig. 1 for subunits of subcomplex Iα.

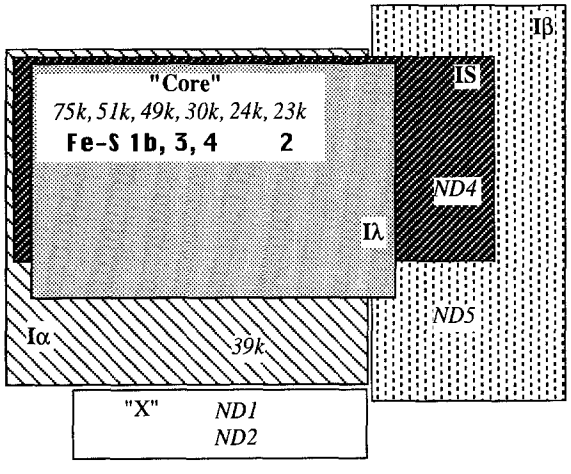

Fig. 3. A schematic illustration of the overlap in subunit composition between several different subcomplexes of complex I. Subcomplexes that contain identical subunits are drawn above each other. The "core" of complex I is an assembly of about 10 subunits, six of which are indicated in this scheme, as well as Fe–S clusters that are bound by core polypeptides. Only a few noncore subunits are indicated as examples for those that are present in some subcomplexes but absent from the others. Polypeptide subunits appear in *italic*. See Fig. 2 for SDS-PAGE analysis of the subcomplexes. The scheme does not show any distances between the proteins or the Fe–S clusters, nor their relation to the membrane.

also subunits that are present in subcomplex Iλ but are missing from IS (Fig. 2).

Examination of our FMN-containing subcomplexes suggests that about 10 subunits are always present (Fig. 2; Finel *et al.*, in preparation). These subunits probably form the core of the enzyme that bind the Fe-S clusters. This core is rather large and contains mainly hydrophilic polypeptides, the 75, 51, 49, 30, 24, and 23 kDa subunits, and some smaller ones.

The overlap between the different subcomplexes is shown schematically in Fig. 3. It should be emphasized that this cartoon does not represent a new structural model for complex I, and that none of the subcomplexes shown here is fully water-soluble.

Subcomplexes Iα and Iβ do not share any subunit or redox center. On the other hand, almost all the subunits of subcomplex Iλ are included in subcomplex Iα, while subcomplex IS contains subunits of Iα (and Iλ) as well as of subcomplex Iβ. All the core subunits and the EPR-detectable Fe-S clusters are present in each of the subcomplexes Iα, Iλ, and IS. In addition, subcomplex "X" represents a possible assembly of subunits which are not found in any of the subcomplexes described above. These subunit could be ND1, ND2, or some other units of the polypeptides seen in Fig. 1, fraction 4.

TRANS-MEMBRANE ORGANIZATION

The 3-D structure of complex I is not known, and it will probably be some time before we know it at high resolution. There are two models for the gross structure of the enzyme which were derived from low-resolution electron microscopy of 2-D crystals (Leonard *et al.*, 1987; Hofhaus *et al.*, 1991), but they do not resolve individual subunits. This leaves the studying of the trans-membrane organization of complex I to less direct methods.

Secondary structure prediction is currently a major tool in identifying trans-membrane segments of membrane proteins, but it is not certain that it also holds in a very large and multi-subunit protein like complex I. In such a case a polypeptide might be exposed to the aqueous phases on both sides of the membrane without much contact with the phospholipid bilayer.

The other approach to studying the location of a subunit in relation to the membrane is to label it by suitable reagents that either cannot cross biological membranes, or are very lipophilic.

The possibility that the 75 kDa, 49 kDa or 30 kDa subunits of complex I span the mitochondrial membrane is discussed below, because there are contradictions between their folding prediction and labeling results. The primary structure of the 75 kDa subunit gives no indication of trans-membrane α-helices, and it was suggested to be a peripheral membrane protein (Runswick *et al.*, 1989). It should be pointed out, however, that the C-terminal half of this polypeptide is rather hydrophobic, while the N-terminal half that contains the conserved cysteines is much more hydrophilic.

In the 49 kDa subunit a region of high hydrophobicity was found but it does not resemble typical trans-membrane helices because it contains several basic and acidic residues among the apolar ones (Fearnley *et al.*, 1989). The primary structure of the 30 kDa subunit does not contain any long hydrophobic region that might form a membrane-spanning α-helix (Pilkington *et al.*, 1991b).

Labeling results, on the other hand, implied that these subunits are trans-membrane. Patel *et al.* (1988) used iododiazobenzensulfonate to label mitochondria and submitochondrial particles, followed by immunoprecipitation of complex I. They found that the 49 kDa subunit is labeled mainly from the cytoplasmic side, the 30 kDa from both sides, and the 75 kDa subunit mainly, but not exclusively, from the matrix side. However, it is possible that the identification of the subunits was partly inaccurate so that the ND1 subunit was labeled from both sides and not (or in addition to) the 30 kDa protein. Since the enzyme was not purified after the labeling, it is also possible that unrelated polypeptides gave rise to the labeled bands at the position of the 49 kDa and 75 kDa subunits.

The approach taken by Han *et al.* (1989) is better in the sense that subunit-specific antibodies were used to detect them on both sides of the membrane. These authors found that the 75 kDa, but not the 49 kDa or the 30 kDa subunits, is transmembranous. This method, however, relies entirely on the purity and monospecificity of the antibodies, and those used were not monoclonal nor raised against a synthetic peptide with the correct sequence. This point is made because monospecific antibodies against the 75 kDa subunit wrontly identified this polypeptide as the major autoimmune antigen associated with primary biliary cirrhosis (summarized in Runswick *et al.*, 1989).

It thus appears that there are no sufficiently strong experimental evidence to either accept or reject the notion that one or more of the 75 kDa, 49 kDa, and 30 kDa subunits is trans-membrane, nor can we rely on the secondary structure prediction that they are not. This conclusion should be extended to include several other subunits such as the 23 kDa subunit about which there are no experimental results as yet.

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