Mitochondrial NADH:Ubiquinone Oxidoreductase (Complex I): Proximity of the Subunits of the Flavoprotein and the Iron–Sulfur Protein Subcomplexes†,‡

Mutsuo Yamaguchi and Youssef Hatefi*

Department of Molecular and Experimental Medicine, Division of Biochemistry, The Scripps Research Institute, La Jolla, California 92037

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ABSTRACT: The proximities of the three subunits (51, 24, and 9 kDa) of the flavoprotein subcomplex (FP) and five subunits (75, 49, 30, 18, and 13) of the iron–sulfur protein subcomplex (IP) of the bovine NADH: ubiquinone oxidoreductase (complex I) were investigated by cross-linking studies. The cross-linking reagents used were disuccinimidyl tartrate and ethylene glycol bis(succinimidyl succinate). The cross-linked products were identified by sodium dodecyl sulfate gel electrophoresis and immunoblotting with antibodies specific for each subunit. Results showed that the three FP subunits are juxtaposed to one another, and only the 51 kDa subunit of FP is in close proximity to only the 75-kDa subunit of IP. The 75-kDa subunit cross-linked to the 30- and the 13-kDa subunits, the 49-kDa subunit cross-linked to the 30-, 18-, and 13-kDa subunits, and the 30-kDa subunit cross-linked to the 18- and the 13-kDa subunits. No cross-linked products of 75+49-, 75+18-, or 18+13-kDa subunits were detected. These results are consistent with the occurrence of potential electron carriers in FP and IP subunits. These electron carriers are FMN and one iron–sulfur cluster in the 51-kDa subunit, one iron–sulfur cluster in the 24-kDa subunit, and apparently two iron–sulfur clusters in the 75-kDa subunit.

The bovine mitochondrial NADH:ubiquinone oxidoreductase (complex I) is composed of at least 40 unlike subunits plus phospholipids (Walker et al., 1992). It is located in the mitochondrial inner membrane, and it catalyzes the oxidation of NADH by ubiquinone -10 in a reaction that is linked to proton translocation from the mitochondrial matrix to the intermembrane space. The redox carriers of complex I are FMN, bound ubiquinone, four EPR[1]-visible iron–sulfur clusters (N1b, N2, N3, and N4) that are reducible by NADH, and possibly two or more iron–sulfur clusters that are either EPR-silent or not reducible by NADH (Hatefi, 1985). Seven of the subunits of complex I are encoded by the mitochondrial DNA (mtDNA) and synthesized within the mitochondrion (Chomyn et al., 1985); the remainder are cytoribosomal products and are imported. The amino acid sequences of most of the latter subunits have been recently determined (Fearnley et al., 1989; Pilkington & Walker, 1989; Runswick et al., 1989; Wakabayashi et al., 1990; Skehel et al., 1991; Pilkington et al., 1991; Masui et al., 1991; Dupuis et al., 1991a,b; Fearnley et al., 1991; Arizmendi et al., 1992; Walker et al., 1992). Point mutations in four of the mtDNA-encoded subunits have been shown to result in Leber's hereditary optic neuropathy (Wallace, 1992). Complex I deficiency has also been demonstrated in Parkinson's disease, in severe lactic acidosis, as well as in various neuromuscular myopathies (Wallace, 1992).

Resolution of complex I by chaotropic salts (Hatefi & Hanstein, 1975; Galante & Hatefi, 1978) results in solubilization of two subcomplexes, which can be separated by ammonium sulfate fractionation. These subcomplexes are a flavoprotein (FP) with three subunits of molecular masses 51, 24, and 9 kDa and an iron–sulfur protein (IP) with six or seven polypeptides of molecular masses 75, 49, 30, 18, 15, and 13 and often one of 20 kDa (Hatefi et al., 1985; Masui et al., 1991). NADH binds to the 51-kDa subunit, which also appears to house FMN plus a tetranuclear iron–sulfur cluster (Ohnishi et al., 1981, 1985). The 24-kDa subunit seems to house a binuclear iron–sulfur cluster (Ohnishi et al., 1981, 1985; Pilkington & Walker, 1989), and the 75-kDa subunit seems to house a tetranuclear and possibly a binuclear iron sulfur–cluster (Ohnishi et al., 1985; Runswick et al., 1990). The extraction of FP and IP leaves the remaining polypeptides of complex I (designated the hydrophobic protein fraction, HP) and the phospholipids as a highly insoluble aggregate. All the mtDNA-encoded subunits of complex I are in HP. In addition, HP contains a 23-kDa subunit, which contains two sets of cysteine motifs capable of participating in the attachment of two tetranuclear iron–sulfur clusters (Dupuis et al., 1991a).

The sequence of the electron carriers of complex I is not known, even those whose reduction by NADH can be monitored by EPR spectroscopy. The problem is that reducing equivalents appear to distribute among the redox carriers of complex I faster than they are donated to complex I by NADH. However, titration of complex I by NADH as well as potentiometric studies have indicated that the E_m order of the EPR-visible iron–sulfur clusters is N1b ≤ N4 < N3 < N2, with the $E_{m,7}$ of N1b being close to that of NADH/NAD and the $E_{m,7}$ of N2 being close to that of ubiquinol/ubiquinone (Hatefi, 1985).

Since most of the electron carriers of complex I are located in FP and IP, structural studies on these subcomplexes would be important in understanding the sequence of these redox centers and the mechanism of action of this enzyme complex. This paper presents the results of our studies on the near-neighbor relationships of the subunits of FP and IP in the isolated state as well as in complex I.

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* To whom correspondence should be addressed.

[1] Abbreviations: EPR, electron paramagnetic resonance; FP, flavoprotein subcomplex; IP, iron–sulfur protein subcomplex; HP, hydrophobic protein fraction of complex I; DST, disuccinimidyl tartrate; EGS, ethylene glycol bis(succinimidyl succinate); SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; PVDF, poly(vinylidene difluoride).

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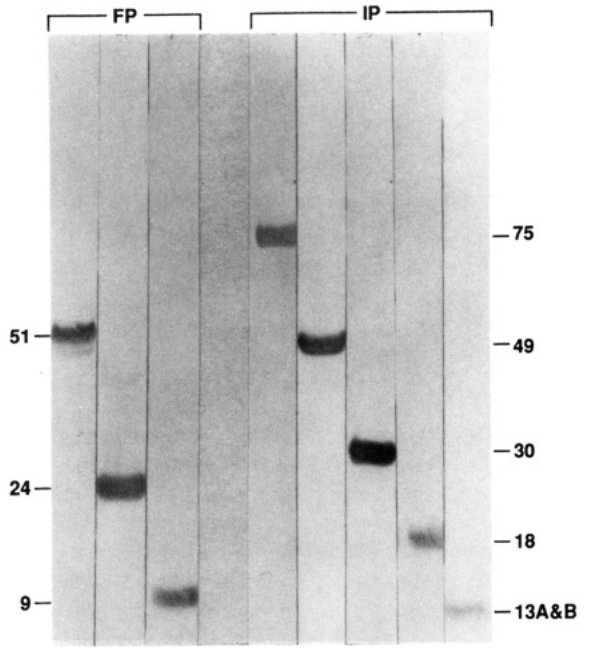

FIGURE 1: Immunoblots of complex I with affinity-purified antibodies to the FP and IP subunits. Complex I subunits were transferred from SDS–polyacrylamide gels to PVDF membranes as described under Materials and Methods. Twenty micrograms of complex I were loaded per lane. The PVDF membranes were incubated with affinity-purified antibodies to the 51-kDa (lane 1, left to right), 24-kDa (lane 2), 9-kDa (lane 3), 75-kDa (lane 4), 49-kDa (lane 5), 30-kDa (lane 6), 18-kDa (lane 7), and 13-kDa (lane 8) subunits, respectively. Then, membranes were washed with PBS, incubated with peroxidase-conjugated anti-rabbit IgG antibody, and washed with PBS again, and color was developed.

MATERIALS AND METHODS

Materials. Disuccinimidyl tartrate (DST) and ethylene glycol bis(succinimidyl succinate) (EGS) were obtained from Pierce. PVDF membrane was obtained from Millipore; anti-rabbit IgG–peroxidase conjugate was from Calbiochem, and 4-chloro-1-naphthol from Sigma. Complex I (Hatefi, 1978) and FP and IP (Galante & Hatefi, 1978) were prepared according to the references cited. Antibodies to FP and IP components were raised and affinity-purified as reported previously (Han et al., 1988, 1989).

Cross-Linking Conditions. The samples for cross-linking were dialyzed at 4 °C for 6 h against 50 mM triethanolamine hydrochloride, pH 8.0, containing 0.25 M sucrose. FP (1.0 mg/mL), IP (4.0 mg/mL), and complex I (4.0 mg/mL) were incubated at 23 °C for 1 h with 1 mM DST or 0.2 mM EGS. DST or EGS was dissolved in dimethyl sulfoxide, and the final concentration of dimethyl sulfoxide was 1% in the reaction mixture. In the case of complex I, prior to cross-linking, 0.1% Triton X-100 was also added to clarify the turbidity formed during dialysis. The cross-linking reaction with DST or EGS was quenched by addition of 50 mM ammonium acetate (Smith et al., 1978) or 5 mM glycine (Abdella et al., 1979), respectively.

Immunodetection of Peptides by Protein Blotting. Protein samples were denatured by addition of 0.1 volume of SDS-denaturation buffer (0.1 M sodium phosphate, pH 8.5, 0.1 M sodium acetate, 50% glycerol, 10% SDS, and 10% β-mercaptoethanol), and SDS–polyacrylamide gel electrophoresis was carried out on gels (14 × 0.15 × 10 cm) containing 7% (w/v) acrylamide with the buffer system of Davies and Stark (1970). Peptides on the gel were transferred onto PVDF membranes at 30 V for 2 h in 20 mM Tris–acetate, pH 8.3, containing 1 mM EDTA, using the Bio-Rad mini trans-blot apparatus. Membranes were incubated with 1% skim milk in PBS (50 mM sodium phosphate/150 mM NaCl, pH 7.4) at 37 °C for 1 h and then overnight at 4 °C with each affinity-purified antibody diluted 20–60-fold in 50 mM Tris-HCl, pH 8.0, containing 2 mM $CaCl_2$ and 3% bovine serum albumin. These sheets were washed three times with PBS and incubated for 2 h with anti-rabbit IgG–peroxidase conjugate diluted 2500-fold in the dilution buffer described above. They were then washed with PBS three times and stained by immersion in 25 mL of PBS mixed with 5 mL of ethanol containing 15 mg of 4-chloro-1-naphthol and 15 μL of 29% H_2O_2.

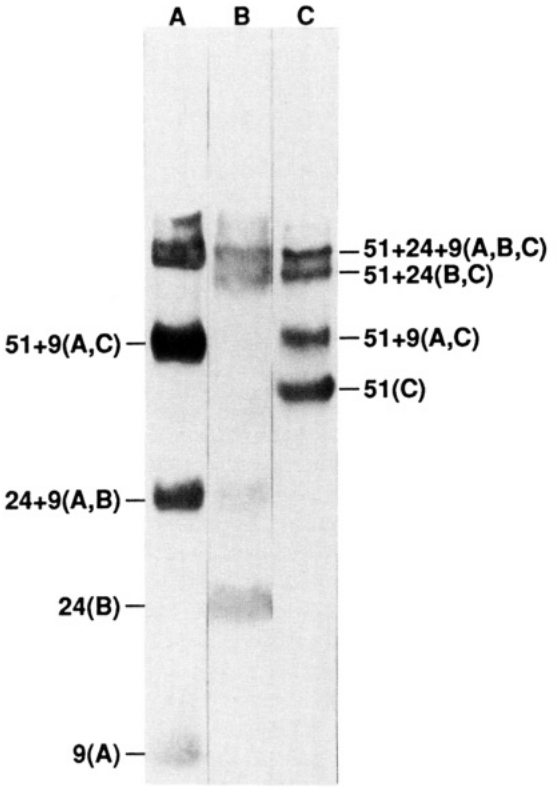

FIGURE 2: Immunoblotting of cross-linked FP with antibodies to the FP subunits. FP was cross-linked with 1 mM DST, analyzed by gel electrophoresis on a 7% acrylamide gel, and immunoblotted as described under Materials and Methods. Five micrograms of cross-linked FP were loaded per lane. Immunoblotting was done with affinity-purified antibodies to the 9-kDa subunit (lane A), the 24-kDa subunit (lane B), and the 51-kDa subunit (lane C), respectively. Here and in Figures 3–6, numbers refer to the kilodaltons of non-cross-linked or cross-linked subunits; letters in parentheses refer to the lanes where non-cross-linked and cross-linked subunits are located.

Protein Assay. Protein concentration was measured by the method of Peterson (1977) with bovine serum albumin as a standard.

RESULTS

Figure 1 shows immunoblots of complex I with immunopurified antibodies to the individual subunits of FP (51, 24, and 9 kDa) and five subunits of IP (75, 49, 30, 18, and 13A and 13B). Antibody to the 15-kDa polypeptide was not used in these studies, because the 15-kDa polypeptide appeared to be present in relatively low amounts in complex I and showed inconsistent patterns in the cross-linking experiments. In previous studies, we had also considered the possibility that the 15-kDa polypeptide may be a loosely bound component, because it distributed indistinctly during fractionation of complex I (Han et al., 1989). However, as seen in Figure 1, the antibodies used were each specific for the subunits indicated and reacted only with a single polypeptide of complex I. The

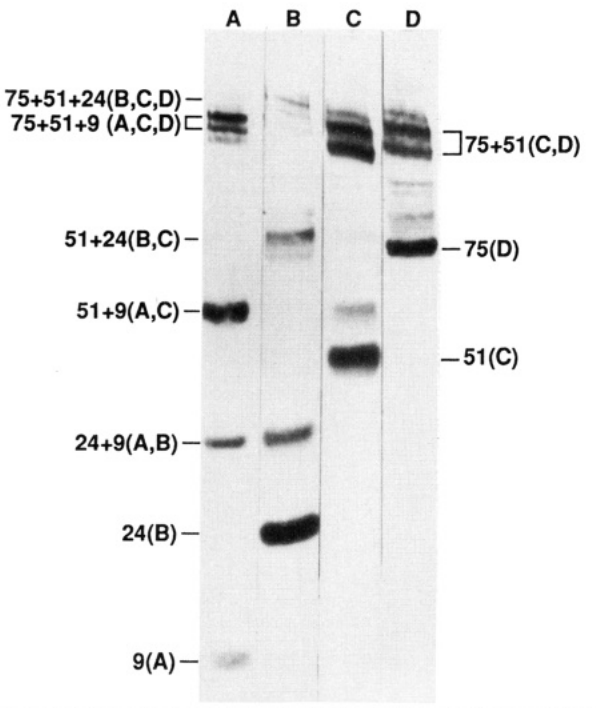

FIGURE 3: Immunoblotting of cross-linked complex I with affinity-purified antibodies to the three subunits of FP and the 75-kDa subunit of IP. Complex I was cross-linked with 0.2 mM EGS and analyzed by immunoblotting as described under Materials and Methods. Twenty micrograms of cross-linked complex I were loaded per lane. Immunoblotting was done with affinity-purified antibody to the 9-kDa subunit (lane A), the 24-kDa subunit (lane B), and the 51-kDa subunit (lane C) of FP and to the 75-kDa subunit of IP (lane D).

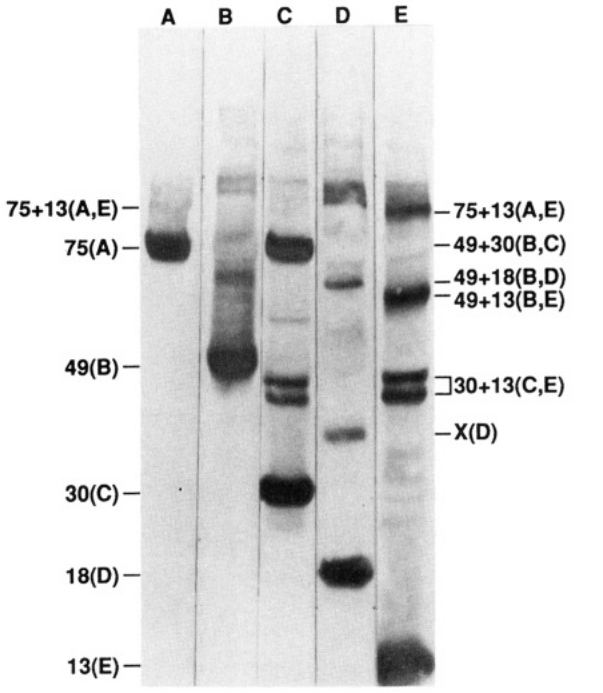

FIGURE 4: Immunoblotting of IP cross-linked with DST. IP was cross-linked with 1 mM DST and analyzed by immunoblotting as described under Materials and Methods. Twenty micrograms of protein were loaded per lane. Immunoblotting was done with affinity-purified antibody to the 75-kDa subunit (lane A), the 49-kDa subunit (lane B), the 30-kDa subunit (lane C), the 18-kDa subunit (lane D), and the 13-kDa subunit (lane E), respectively. For other details, see text.

band marked 13A&B in the extreme right lane represents two separate polypeptides, 13A and 13B, which comigrate on SDS gels (Wakabayashi et al., 1990). The antibodies used were elicited to a mixture of these two polypeptides. This point should be kept in mind in interpreting the results of our cross-linking experiments. Two cross-linking reagents were used in these studies, DST and EGS with molecular lengths, respectively, of 0.64 and 1.61 nm. The cross-linking patterns were essentially the same, however, despite the considerable difference in the lengths of the reagents.

Figure 2 shows the results of the incubation of isolated FP with DST. The gels A, B, and C were blotted, respectively, with antibodies to the 9-, 24-, and 51-kDa subunits of FP. It is seen that each subunit cross-linked with the other two. The same cross-linking pattern was seen when complex I was treated with EGS (Figure 3). In addition, however, the 51-kDa subunit of FP was cross-linked to the 75-kDa subunit of IP (Figure 3, lanes C and D; the double band here and elsewhere may mean cross-linking involving different amino acid residues). There were also trimeric cross-links involving 75+51+9-kDa (Figure 3, lanes A, C, and D) and possibly 75+51+24-kDa subunits (Figure 3, lane B and very faint bands in lanes C and D), but there was no evidence of dimeric cross-linking between 75- and 24-kDa or 75- and 9-kDa subunits. As will be seen below, none of the FP subunits cross-linked with any other complex I subunit, which suggests that FP binds to the remainder of complex I by way of association between the 51-kDa subunit of FP and the 75-kDa subunit of IP. These results agree with the earlier findings of Chen and Guillory (1981), who demonstrated that photoaffinity labeling of complex I with radioactive arylazido-β-alanyl-NAD resulted in label incorporation not only in the 51-kDa subunit of FP, which bears the NAD binding domain, but also in the 75-kDa subunit of IP.

Figure 4 shows the immunoblots of isolated IP treated with DST and then blotted with antibodies to the 75-, 49-, 30-, 18-, and 13-kDa subunits, respectively, in lanes A, B, C, D, and E. The cross-linked products marked in Figure 4 indicate dimers of 75+13-, 49+30-, 49+18-, 49+13-, and 30+13-kDa subunits. Similar products are shown in Figure 5 as a result of treatment of isolated IP with EGS. In Figure 4 a double band is seen in lanes C and E, representing cross-linking of the 30- and the 13-kDa subunits. As mentioned above, one possibility is cross-linking between the subunits involving different amino acid residues. The other is that one band represents a cross-link between 30- and 13A-kDa subunits and the other between 30- and 13B-kDa subunits. The bands marked X in lanes D of Figures 4 and 5 and the band marked Y in lane E of Figure 5 have not been identified. They could represent dimers, respectively, of 18+18- and 13+13- (A and/or B) kDa subunits.

Figure 6 shows the immunoblots of complex I treated with EGS and blotted with antibodies to the 75-, 49-, 30-, 18-, and 13-kDa subunits, respectively, in lanes A, B, C, D, and E. The cross-linked products identified are 75+51- (see lane D of Figure 3), 75+30-, 75+13-, 49+30-, (a doublet), 49+13-, and 30+13- (a doublet) kDa subunits. There are also additional bands, which are absent from Figures 4 and 5. These are likely the products of cross-linking between the IP and various HP subunits. Immunoblots of complex I treated with DST showed similar cross-links as in Figure 6, plus the following: 75+30+18, 49+30+18, 49+18 (see also Figure 4), and 30+18. Absent from all the immunoblots were dimeric cross-linked products of the 24- or 9-kDa subunit of FP with any subunit of IP, of the 51-kDa subunit of FP with any IP subunit except the 75 kDa, and of 75+49- and 75+18-kDa subunits of IP.

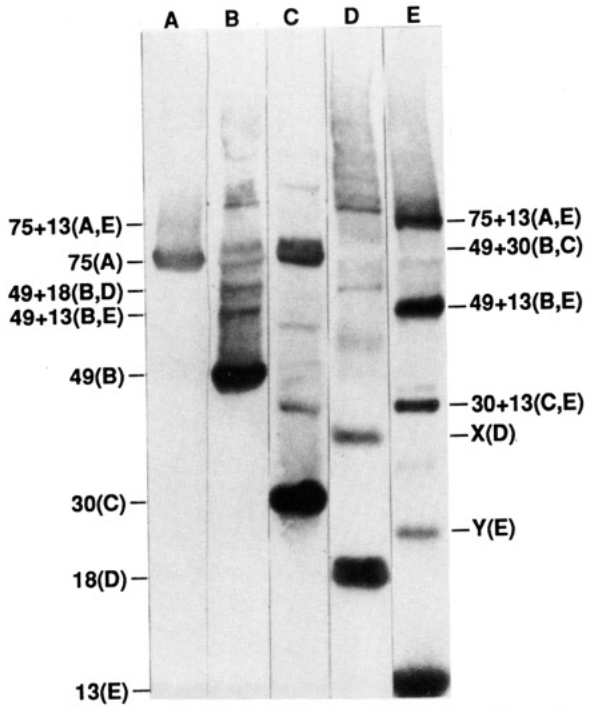

FIGURE 5: Immunoblotting of IP cross-linked with EGS. IP was cross-linked with 0.2 mM EGS and analyzed by immunoblotting as described under Materials and Methods. Twenty micrograms of protein were loaded per lane. Immunoblotting was done with affinity-purified antibody to the 75-kDa subunit (lane A), the 49-kDa subunit (lane B), the 30-kDa subunit (lane C), the 18-kDa subunit (lane D), and the 13-kDa subunit (lane E), respectively. For other details, see text.

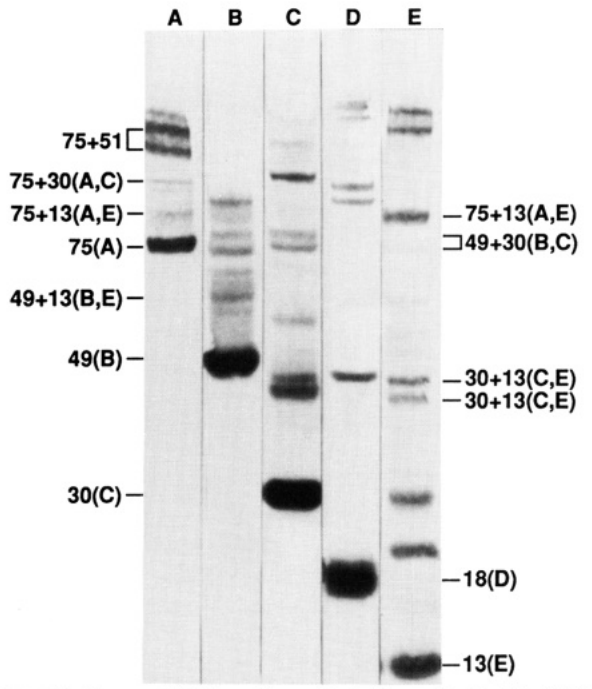

FIGURE 6: Immunoblotting of cross-linked complex I with affinity-purified antibodies to the IP subunits. Complex I was cross-linked with 0.2 mM EGS and analyzed by immunoblotting as described under Materials and Methods. Twenty micrograms of cross-linked complex I were loaded per lane. Immunoblotting was done with affinity-purified antibody to the 75-kDa subunit (lane A), the 49-kDa subunit (lane B), the 30-kDa subunit (lane C), the 18-kDa subunit (lane D), and the 13-kDa subunit (lane E), respectively.

DISCUSSION

The results of the cross-linking experiments with FP, IP, and complex I are summarized in Figure 7, in which the areas of the circles represent the relative sizes of the FP and IP subunits. It is seen that the three FP subunits (the circles marked 51, 24, and 9 in Figure 7) are in close proximity to one another, and only the 51-kDa subunit is located close to the 75-kDa subunit of IP. This agrees with the distribution of the redox centers in the FP and IP subunits. The 51-kDa subunit of FP binds NADH (Deng et al., 1990) and apparently contains FMN and a tetranuclear iron–sulfur cluster. The 24-kDa subunit of FP also appears to house a binuclear iron–sulfur cluster (Ohnishi et al., 1981). However, the amino acid sequence data of Walker's laboratory indicate that among the IP subunits only the 75-kDa polypeptide contains arrangements of cysteine residues consistent with motifs for liganding a tetranuclear and possibly a binuclear iron–sulfur cluster (Runswick et al., 1989). Therefore, it is reasonable to assume that electrons from the redox centers of FP pass through those of the 75-kDa subunit on their way to ubiquinone. Interposed between the 75-kDa subunit and ubiquinone, there is at least one other iron–sulfur center, namely the high-potential tetranuclear center N2. This center may be housed in the 23-kDa subunit of HP, which according to the sequence data of Dupuis et al. (1991a) contains two CysXXCysXXCysXXXCysPro motifs found in ferredoxin-type tetranuclear iron–sulfur proteins. We have shown elsewhere that among the FP and IP subunits only the 75-kDa polypeptide appears to be transmembranous (Han et al., 1989). This too is consistent with the possibility of the 75-kDa subunit of IP interacting with the 23-kDa subunit of HP. It has been suggested that the hydropathy plot of the latter does not show a hydrophobic amino acid stretch of sufficient length to span

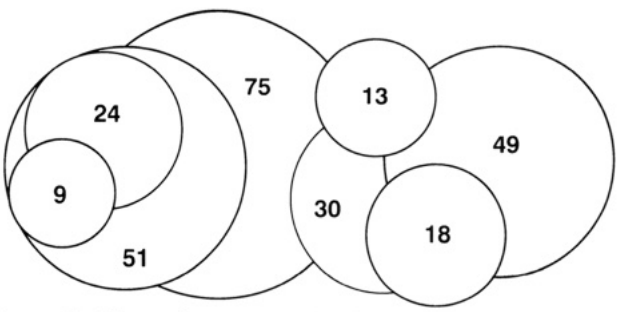

FIGURE 7: Schematic representation of the proximity of the FP and IP subunits in complex I. Circles represent the individual subunits, and the numbers inside the circles show their masses in kilodaltons. Cross-linked dimers of subunits were identified wherever two circles overlap. The scheme is not intended to convey any other structural information.

the membrane (Dupuis et al., 1991a). However, it is not impossible for the 23 kDa to be located within the membrane if it should be surrounded by other HP subunits, which have sufficient hydrophobic character for membrane intercalation.

Figure 7 also shows close proximity of the 75-kDa subunit to the 30- and the 13-kDa subunits, but not to the 49- and the 18-kDa subunits. This follows from our finding of 75+30- and 75+13-kDa dimers, and the absence of any indication of 75+49- and 75+18-kDa dimers.[2] Nor was there any indication of 18+13-kDa dimers, whereas other dimers as suggested by Figure 7 were seen, i.e., 49+30, 49+18, 49+13, 30+18, and 30+13 kDa. Essentially similar results were obtained by Cleeter et al. (1985) for the proximity of IP subunits in complex I. However, using the same cross-linking reagents as we have

[2] Even though the results of cross-linking of FP, IP, and complex I with either DST or EGS present a consistent pattern as depicted in Figure 7, we are cognizant of the fact that the absence of cross-linking evidence does not necessarily mean absence of subunit–subunit proximity.

done, Cleeter et al. concluded that the 51-kDa subunit of FP cross-links to both the 30- and the 49-kDa subunits of IP, for which we find no indication in our experiments. It should be pointed out that Cleeter et al. (1985) did not use an antibody to the 51-kDa subunit for direct identification. They isolated and cleaved the cross-linked products assumed on the basis of their mobility on SDS gels to be 51+49 and 51+30 dimers, subjected the cleaved products to SDS gel electrophoresis, and designated a protein band as the 51-kDa subunit of FP only on the basis of its mobility on the gels. In our case, no cross-linked bands were seen that blotted to the 51-kDa antibody as well as to the 49- or the 30-kDa antibody.

One other point deserve consideration here. In complex I, there is clear evidence of cross-linking between the 75- and the 30-kDa subunits (Figure 6), whereas no such evidence could be discerned in isolated IP cross-linked with either DST or EGS. Structural changes in the isolated IP resulting in separation of the 75- and 30-kDa subunits is, therefore, a possibility, and it is consistent with our previous findings regarding changes in the EPR signature of the iron–sulfur centers when FP and IP are removed from complex I (Ohnishi et al., 1981, 1985).

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