Catalytic Sector of Complex I (NADH:Ubiquinone Oxidoreductase): Subunit Stoichiometry and Substrate-Induced Conformation Changes†,‡

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ABSTRACT: The electron carriers of the mitochondrial NADH:ubiquinone oxidoreductase (complex I) are contained predominately in two extramembranous subcomplexes, a flavoprotein (FP) and an iron–sulfur protein (IP). FP contains three subunits with molecular masses of 51, 24, and 9 kDa. The 51-kDa subunit carries the NADH binding site and contains FMN and a tetranuclear iron–sulfur cluster. The 24-kDa subunit contains a binuclear iron–sulfur cluster. IP contains seven subunits with molecular masses of 75, 49, 30, 18, 15, 13, and 11 kDa. It contains a tetranuclear and very likely a binuclear iron–sulfur cluster in the 75-kDa subunit. FP and IP make contact through the 51- and the 75-kDa subunits. The remainder of complex I (hydrophobic protein (HP), 31 subunits) is largely membrane-intercalated and contains two iron–sulfur clusters apparently in a 23-kDa subunit and possibly another in a 20-kDa subunit. In this study, the stoichiometries of the FP and IP subunits in complex I were determined by radioimmunoassay. Per mole of complex I, there are 2 mol of the 15-kDa subunit and 1 mol each of the FP and the four largest IP subunits. The stoichiometries of the 13- and the 11-kDa subunits could not be determined separately, because they comigrate upon gel electrophoresis. In addition, the effect of substrates (NADH, NADPH, NAD, and NADH plus potassium ferricyanide to rapidly oxidize NADH via FP) on the cross-linking patterns of FP and IP subunits was investigated, using three different cross-linking reagents of different molecular lengths. Results showed that treatment of complex I with NADH or NADPH, but not with NAD or NADH + $K_3Fe(CN)_6$, prior to cross-linking resulted in changes in the extent (decrease or increase) of cross-linking among the FP subunits, between the 75- and the 51-kDa subunits, among the IP subunits, and between the IP and the HP subunits. In other words, reduction of complex I by NAD(P)H appeared to cause conformational changes involving proximities among and between the FP, IP, and HP subunits. It is proposed that, by analogy to recent evidence regarding the mode of energy transfer in the ATP synthase complex, the extensive subunit proximity changes observed upon substrate reduction of complex I may be the manner in which energy coupling and transfer take place within this enzyme complex, i.e., via conformational energy transfer from FP and IP to HP, where proton translocation is effected.

Among the enzyme complexes that compose the mitochondrial electron transport/oxidative phosphorylation system (Hatefi, 1985), NADH:ubiquinone oxidoreductase (complex I) has the most complex structure and the least understood mechanism of electron transfer and proton translocation. This is partly because complex I has at least 40 unlike subunits (Fearnley & Walker, 1992; Walker *et al.*, 1992), and partly because the slowest electron-transfer step among its several redox components (FMN, binuclear and tetranuclear iron–sulfur clusters, and ubiquinone) is the initial step of hydride ion transfer from NADH to the enzyme. However, from a biomedical standpoint, complex I might be considered the most important of the respiratory chain enzyme complexes, because many human mitochondrial diseases result from complex I deficiencies. These diseases include Leber's hereditary optic neuropathy, severe and fatal lactic acidosis, various neuromuscular myopathies, and possibly Parkinson's disease (Wallace, 1992; Robinson, 1993).

Early in the study of complex I, it was found that most of the electron carriers of complex I can be isolated as two water-soluble subcomplexes, FP and IP[1] (Hatefi & Stempel, 1967, 1969). FP is composed of three subunits with molecular masses of 51, 24, and 9 kDa (Hatefi *et al.*, 1985; Ragan & Hatefi, 1986). It contains FMN and a tetranuclear iron–sulfur cluster in the 51-kDa subunit and a binuclear iron–sulfur cluster in the 24-kDa subunit (Ohnishi *et al.*, 1981, 1985). NADH reacts with the 51- kDa subunit (Deng *et al.*, 1990). IP is composed of seven subunits with molecular masses of 75, 49, 30, 18, 15, 13, and 11 kDa (Hatefi *et al.*, 1985; Ragan & Hatefi, 1986; Masui *et al.*, 1991). The latter two subunits comigrate on SDS gels at a position corresponding to a 13-kDa polypeptide. Therefore, they are referred to as the 13B and 13A subunits, respectively (Masui *et al.*, 1991). IP contains a tetranuclear and very likely a binuclear iron–sulfur cluster in the 75-kDa subunit (Ohnishi *et al.*, 1985; Runswick *et al.*, 1989). The remaining ≥30 subunits of complex I and phospholipids (~20% by dry weight) are separated from FP and IP as a highly water-insoluble aggregate (designated HP) (Ohnishi *et al.*, 1985), which contains the seven mtDNA-

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[1] Abbreviations: FP, IP, and HP, the flavoprotein, iron–sulfur protein, and hydrophobic protein fractions of complex I, respectively; mtDNA, mitochondrial DNA; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DST, disuccinimidyl tartarate; EGS, ethylene glycol bis(succinimidyl succinate); EEDQ, *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline.

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encoded subunits of complex I. A 23-kDa subunit of HP has two cysteine cluster motifs for housing two tetranuclear iron–sulfur clusters (Dupis *et al.*, 1991). In addition, a 20-kDa subunit of HP contains three conserved cysteine residues, which could be involved in coordinating with iron atoms in a third iron–sulfur cluster (Arizmendi *et al.*, 1992b).

In *Neurospora crassa* treated with chloramphenicol to inhibit mitochondrial protein synthesis, a low molecular weight NADH dehydrogenase is elaborated, which contains all of the redox components and the corresponding polypeptides found in bovine FP + IP (Weiss & Friedrich, 1991; Weiss *et al.*, 1991; Friedrich *et al.*, 1993). In the absence of chloramphenicol, this low molecular weight dehydrogenase becomes associated as a peripheral sector with a membrane-intercalated sector, which includes the mtDNA-encoded subunits as well as various nuclearly encoded hydrophobic polypeptides of the *Neurospora* complex I [Weiss & Friedrich, 1991; Weiss *et al.*, 1991; also see Nehls *et al.* (1991), for similar conclusions reached by gene disruption experiments]. Thus, it appears that bovine FP and IP and the corresponding *Neurospora* low molecular weight dehydrogenase compose, in each organism, the principal catalytic sector of complex I, whereas the act of proton translocation by complex I is accomplished by bovine HP or its membrane sector analog in *Neurospora*.

This perception has focused attention on FP + IP as an important sector of complex I deserving of more in-depth structural and functional studies than had been accorded it previously. We have recently investigated, with the use of cross-linking reagents, the proximities of FP and IP subunits in these isolated preparations as well as in complex I (Yamaguchi & Hatefi, 1993). Here, we report the stoichiometry of FP and IP subunits in complex I and the effect of substrates on their physical relationships.

MATERIALS AND METHODS

Materials. Disuccinimidyl tartarate (DST) and ethylene glycol bis(succinimidyl succinate) (EGS) were obtained from Pierce, and *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (EEDQ) was from Sigma. Nitrocellulose membrane (0.2-μm pore size) was obtained from Schleicher & Schuell. Anti-rabbit IgG–peroxidase conjugate, NADH, NAD, and NADPH were from Calbiochem. [^{125}I]Protein A, labeled by the method of Bolton and Hunter, and enhanced chemiluminescence (ECL) reagents were from Amersham. SDS and acrylamide were from Bio-Rad. Complex I (Hatefi, 1978) and FP and IP (Galante & Hatefi, 1978) were prepared according to the references cited. Antibodies against the FP and IP subunits were raised and affinity-purified as reported previously (Han *et al.*, 1988, 1989).

Cross-Linking Conditions. Complex I used for cross-linking experiments with DST and EGS was dialyzed overnight at 4 °C against 50 mM triethanolamine hydrochloride (pH 8.0) containing 0.25 M sucrose and 0.1% Triton X-100. When complex I was cross-linked with EEDQ, the buffer was adjusted to pH 7.6. Freshly prepared solutions of DST or EGS in dimethyl sulfoxide or EEDQ in methanol were added to clarified solutions of complex I (2–4 mg/mL) to final concentrations of 1, 0.2, or 5 mM, respectively, and incubated at 23 °C for 1 h in the absence or presence of various ligands as detailed in the text. The final concentration of organic solvents did not exceed 2%. When the dependence of cross-linking patterns on the presence of different substrates (NAD, NADH, and NADPH) was studied, complex I was preincubated with the indicated ligand before the addition of cross-linkers. The concentrations of NAD, NADH, and NADPH used were 3 mM each in most experiments, and that of $K_3Fe(CN)_6$ was 10 mM. The high concentration of NAD(P)H used was primarily to ensure that complex I remained reduced during the prolonged (1 h) incubation with the cross-linking reagents, lest structural modification as a result of cross-linking should create electron leaks to oxygen. Also, since the K_m for NADPH is >500 μM (Hatefi & Hanstein, 1973), it was necessary to employ a NADPH concentration several times this value. However, when complex I was preincubated for 5 min with as little as 10 μM NADH and then cross-linked with DST, EGS, or EEDQ, the results were essentially the same as those where the NADH concentration was 3 mM. At the latter NAD(P)H concentrations, preincubation times of 5–40 min gave the same results. The cross-linking reaction with DST, EGS, or EEDQ was quenched by the addition of 50 mM ammonium acetate, 5 mM glycine, or an excess of β-mercaptoethanol, respectively.

Immunoblotting. Protein samples were denatured by the addition of equal volumes of SDS–PAGE sample buffer and subjected to SDS–PAGE in the Laemmli buffer system (Laemmli, 1970). Polypeptides were electroblotted from gels to nitrocellulose membranes in 25 mM Tris/192 mM glycine buffer (pH 8.3) containing 20% methanol according to Towbin *et al.* (1979), in Bio-Rad Transblot cells at 60 V for 1 h. Following transfer, the nonspecific binding sites on nitrocellulose membranes were blocked by incubation with 5% skim milk in Tris-buffered saline (25 mM Tris-HCl and 150 mM NaCl, pH 7.4) at 37 °C for 1 h. Affinity-purified antibodies diluted 100–5000-fold in Tris-buffered saline, containing 1% skim milk and 0.3% Tween-20 (TBS-T buffer), were incubated with sheets of membrane for 1 h at 23 °C. Sheets were thoroughly washed several times in the above buffer and incubated for 1 h with antirabbit IgG–peroxidase conjugate diluted 1000-fold in the same buffer. The membranes were washed with TBS-T buffer and then with TBS-T buffer containing 2 M NaCl, rinsed with TBS, and developed using the enhanced chemiluminescence (ECL) kit from Amersham, according to its instructions.

Quantitative immunoblotting was performed essentially as described by Hekman *et al.* (1991). The binding sites of primary antibodies were revealed with [^{125}I]protein A (250 000 cpm/mL) dissolved in TBS-T buffer after incubation with the nitrocellulose blots for 1 h at 23 °C. The nitrocellulose sheets were washed, dried in air, and exposed to Fuji medical X-ray film at −70 °C overnight. Radioactive bands were then excised from the nitrocellulose, and the radioactivity associated with each band was determined in a γ counter. A standard curve was constructed by plotting the counts per minute versus the nanograms of each purified subunit placed on the gels (Hekman *et al.*, 1991). The concentration of each IP and FP subunit per microgram of complex I electrophoresed on the same gel was then determined with reference to this standard curve. Samples of purified subunits used for the construction of calibration curves were obtained by electroelution from preparations of FP and IP as specified previously (Han *et al.*, 1988, 1989). Protein concentrations of purified subunits were determined according to Lowry *et al.* (1951). Conversion from mass to molarity was made using the published molecular weights (Walker, 1992). For complex I, a molecular weight of 907 000 was employed (Arizmendi *et al.*, 1992a; Walker, 1992).

RESULTS

FP and IP Subunit Stoichiometry. Knowledge of subunit stoichiometry is *sine qua non* for study of the structure of an

Table 1: Stoichiometry of FP and IP Subunits in Complex I

subunit (kDa)	n^b	SD	mol/mol of complex I
75	5	0.13	1.06
49	5	0.057	1.05
30	5	0.09	1.19
18	4	0.26	1.01
15	5	0.21	2.15
13A/B	4	0.106	0.89[a]
51	6	0.106	1.1
24	4	0.033	0.87
9	3	0.06	0.93

[a] This means 0.89 mol each of 13A and 13B per mole of complex I; see text. [b] n, number of separate determinations; SD, standard deviation.

enzyme complex. The importance of this information is readily apparent from the composition of the ATP synthase complex, in which certain subunits are present in duplicate and triplicate copies, and one subunit is present in as many as 10–12 copies (Fillingame, 1990; Hekman *et al.*, 1991; Penefsky & Cross, 1991). On the basis of Coomassie blue stain intensities of protein bands on SDS gels, several subunits of complex I were suggested to be present as single copies (Ragan, 1976, 1980). However, since proteins are known to differ in their ability to retain the dye, this method of relative stoichiometry estimation is unreliable. A much more reliable procedure, which we have applied satisfactorily to the ATP synthase complex, is the estimation of subunit concentration by radioimmunoblotting, using affinity-purified antibodies for each subunit and purified subunits for the determination of standard curves. The procedure involves the determination, via a standard curve, of the antibody titer of each purified antigen as estimated by the binding of ^{125}I-labeled protein A from *Staphylococcus aureus*. Then the concentration of that antigen in a given amount of sample (e.g., complex I) is determined after immunoblotting, treatment with ^{125}I-labeled protein A, counting the bound radioactivity, and reading the amount of the antigen from the standard curve [for details, see Materials and Methods and Hekman *et al.* (1991)]. Thus, as seen in Table 1, it was possible, with the use of this procedure, to show that, except for the 15-kDa subunit, all other subunits of FP and IP are present in complex I as a single copy per mole of complex I. As indicated elsewhere (Han *et al.*, 1989), the 15-kDa band, as recognized by our antibody, behaves anomalously. It appears to be loosely bound in IP and to distribute during the resolution of complex I among various fractions. According to Finel *et al.* (1992), their complex I-like preparation contains two subunits (15 and B15) with similar mobilities on SDS gels. Therefore, it is possible that our antibody recognizes more than one 15-kDa species, even though, according to Finel *et al.* (1992), IP, from which we isolated the 15-kDa band and raised antibody to it, does not contain the B15 species.

The stoichiometry given in Table 1 for the 13A and 13B subunits also requires an explanation because, as mentioned above, these subunits of IP comigrate on SDS gels and are difficult to separate (Masui *et al.*, 1991). We do not know whether our antibody, which was raised to the M_r = 13 000 protein band isolated from SDS gels, recognizes both the 13A and 13B subunits or only one of them. The value given in Table 1 is based on the assumption that the 13A and 13B subunits were present in our standard curve samples and in complex I at comparable concentrations and that our antibody recognized both. If we assume, however, that our standard curve samples contained equal amounts of the 13A and 13B subunits, but our antibody recognized only one of them, then complex I would contain 2 mol of that subunit per mole.

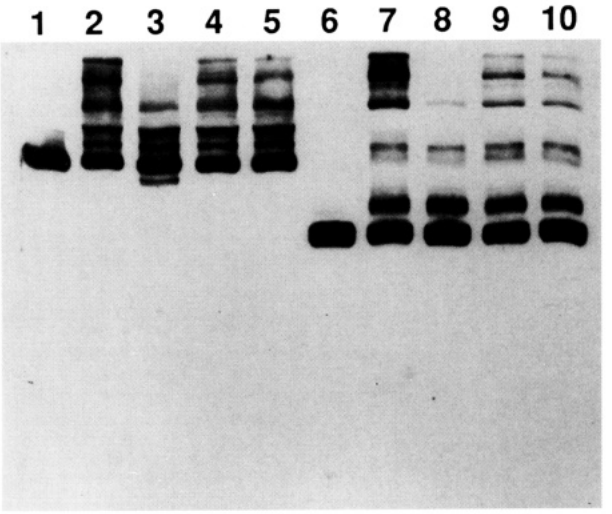

FIGURE 1: Effect of NADH on cross-linking between the 75- and 51-kDa subunits. Complex I was cross-linked with 1 mM DST in the absence (lanes 2 and 7) or presence of NADH (lanes 3 and 8), NADH + $K_3Fe(CN)_6$ (lanes 4 and 9), or NAD (lanes 5 and 10). Lanes 1 and 5 show un-cross-linked complex I. Samples (20 μg) were separated on 12% SDS–PAGE, electrotransferred to nitrocellulose membrane, and immunoblotted with affinity-purified antibodies to the 75-kDa subunit (lanes 1–5) and the 51 kDa subunit (lanes 6–10), as described under Materials and Methods. The immunoreative bands were visualized with the enhanced chemiluminescence (ECL) detection system.

Effects of NADH and NAD on the Cross-Linking of FP Subunits. Early in our studies on the resolution of complex I by chaotropic salts, we found that complex I is structurally stabilized when treated with NADH, resulting in a slower rate of resolution by a given concentration of a chaotrope [Davis & Hatefi, 1969; also see Rossi *et al.* (1965)]. Similar results had been reported previously for the resolution of reduced versus oxidized complex III (ubiquinol:cytochrome *c* oxidoreductase) in the presence of 6.0 M guanidine hydrochloride (Rieske *et al.*, 1967). These data suggested that the stability change of these complexes upon reduction may be reflected in the proximities of their subunits and may be detectable by cross-linking experiments.

Using the cross-linking reagents DST and EGS with respective molecular lengths of 0.64 and 1.61 nm, we have shown recently that the three subunits of FP (51, 24, and 9 kDa) cross-link to one another and that the 51-kDa subunit only cross-links to the 75-kDa subunit of IP. Among the subunits of IP, the following cross-linked products, as indicated by their molecular masses in kilodaltons, were produced and identified: 75 + 30, 75 + 13, 49 + 30, 49 + 18, 49 + 13, 30 + 18, and 30 + 13 [Yamaguchi & Hatefi, 1993; also see Cleeter *et al.* (1985) and Gondal and Anderson (1985)]. The effects of substrates on these cross-linking patterns are described below.

Figure 1 shows the results of incubation of complex I with DST in the absence of substrates (lanes 2 and 7) and the presence of NADH (lanes 3 and 8), NADH plus $K_3Fe(CN)_6$ (lanes 4 and 9), or NAD (lanes 5 and 10). Lanes 1 and 5 show un-cross-linked complex I. After incubation, the mixtures were subjected to SDS gel electrophoresis, and the protein bands were transferred to nitrocellulose sheets. Lanes 1–5 were blotted with affinity-purified antibody to the 75-kDa subunit, and lanes 6–10 were blotted with affinity-purified antibody to the 51-kDa subunit. It is seen that, in the presence of NADH, but not NADH + $K_3Fe(CN)_6$ or NAD, the upper 75 + 51-kDa cross-linked products did not form or formed in lesser amounts. Furthermore, a band moving slightly faster

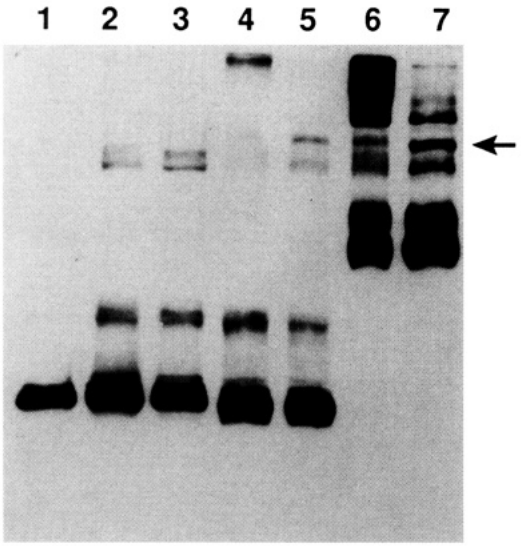

FIGURE 2: Effect of NADH on cross-linking between the 51- and the 24-kDa subunits. Complex I was cross-linked with 1 mM DST (lanes 2 and 3) or 0.2 mM EGS (lanes 4–7) in the absence (lanes 2, 4, and 6) or presence (lanes 3, 5, and 7) of NADH. Lane 1 shows complex I before cross-linking. Complex I (20 μg) was then subjected to 12% SDS–PAGE and immunoblotted with affinity-purified antibodies to the 24-kDa (lanes 1–5) and 51-kDa (lanes 6 and 7) subunits as described under Materials and Methods. Other details were as described for Figure 1.

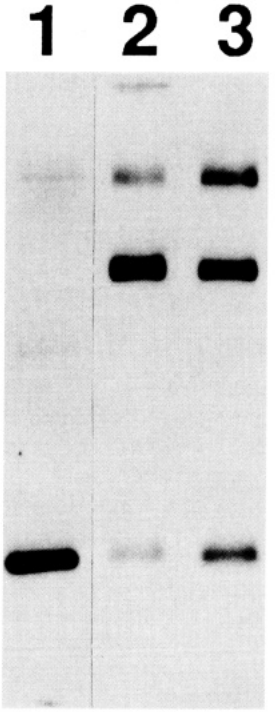

FIGURE 3: Effect of NADH on cross-linking of the 9-kDa subunit to the 24- and the 51-kDa subunits. Complex I cross-linked with 0.2 mM EGS in the absence (lane 2) or presence (lane 3) of NADH was subjected to 16% SDS–PAGE and immunoblotted with antiserum to the 9-kDa subunit (1:1000 dilution). Other details were as described for Figure 1.

than the 75-kDa subunit and recognized by the anti-75-kDa-IgG appeared under the 75-kDa band when complex I was treated with DST (Figure 1, lane 3) or EGS (data not shown) in the presence of NADH, which may be the result of internal cross-linking of the reduced 75-kDa subunit. As indicated in Materials and Methods, the amount of $K_3Fe(CN)_6$ added was more than sufficient to oxidize, via FP, all of the added NADH in the experiments of lanes 4 and 9. Therefore, the changes seen in the cross-linking patterns in Figure 1 could not be due merely to the presence of NAD(H) as a ligand, but rather they are a consequence of complex I reduction.

NADH treatment of complex I also affected the proximities of the 24-kDa and 9-kDa subunits to the 51-kDa subunit. Figure 2 shows, in lanes 2 and 3, the DST cross-linked products of the 24-kDa subunit in the absence and presence of NADH, respectively. The upper two bands are the products of 51 + 24-kDa cross-linking, which appear slightly more intense in the presence of NADH. Lanes 4–7 show the EGS cross-linked products of the 24-kDa subunit (lanes 4 and 5) and the 51-kDa subunit (lanes 6 and 7) in the absence (lanes 4 and 6) and presence (lanes 5 and 7) of NADH. It is seen that there is a new 51 + 24-kDa product of EGS cross-linking (marked by arrow), which is considerably intensified in the presence of NADH. Data for the 9-kDa subunit are shown in Figure 3. It is seen that two major EGS-induced cross-linked products of the 9-kDa subunit were formed, one with the 51-kDa and another with the 24-kDa subunit of FP, and that the former was favored in the presence of NADH. When DST was the cross-linking agent, the formation of both the 51 + 9-kDa and the 24 + 9-kDa products was favored in the presence of NADH (data not shown). When complex I was treated with NADPH instead of NADH, the changes in the cross-linking patterns of FP subunits were essentially the same as those shown above for the effect of NADH (data not shown).

Effects of NADH and NAD on the Cross-Linking of IP Subunits. In this work, a more effective cross-linking reagent proved to be EEDQ, which activates carboxyl groups, presumably via the formation of an unstable carbonic

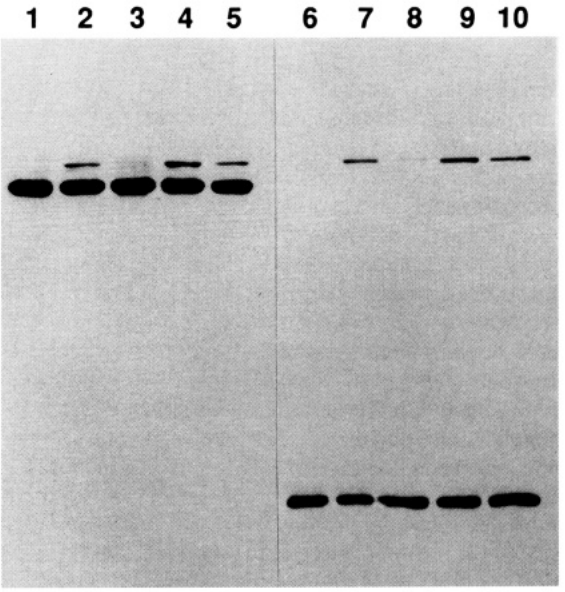

FIGURE 4: Effect of NADH on cross-linking of the 75-kDa subunit to the 18-kDa subunit. Complex I was cross-linked with 5 mM EEDQ in the absence (lanes 2 and 7) or presence of NADH (lanes 3 and 8), NAD (lanes 4 and 9), or NADH + $K_3Fe(CN)_6$ (lanes 5 and 10). Lanes 1 and 6 show un-cross-linked complex I. Samples (20 μg) were subjected to 12% SDS–PAGE and then immunoblotted with affinity-purified antibodies to the 75-kDa (lanes 1–5) and 18-kDa (lanes 6–10) subunits as described under Materials and Methods. Other details were as described for Figure 1.

anhydride. The latter can then react with water to regenerate the free carboxyl group or with a nearby nucleophilic amino acid side chain to yield, for example, an amide or an ester. This means zero-length cross-linking, which can be expected to be susceptible to small changes in the proximity of a nucleophile to the EEDQ-activated carboxyls.

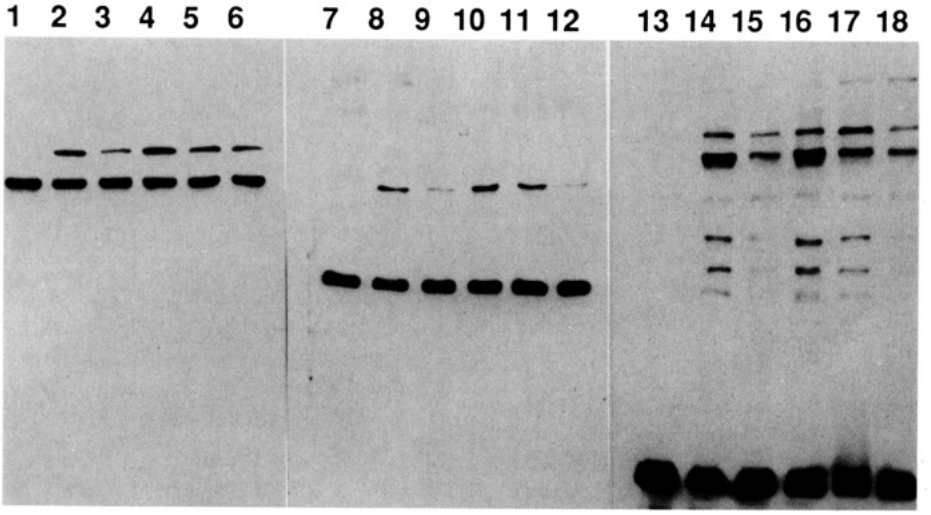

FIGURE 5: Effects of NADH and NADPH on cross-linking of the 49-, 30-, and 13-kDa subunits of IP to the subunits of HP. Complex I was cross-linked with 5 mM EEDQ in the absence (lanes 2, 8, and 14) or presence of NADH (lanes 3, 9, and 15), NAD (lanes 4, 10, and 16), NADH + $K_3Fe(CN)_6$ (lanes 5, 11, and 17), or NADPH (lanes 6, 12, and 18). Samples (20 μg) were separated on 12% SDS–PAGE followed by electrotransfer to nitrocellulose membrane and immunoblotting with affinity-purified antibodies to the 49-kDa (lanes 1–6), 30-kDa (lanes 7–12), and 13-kDa (lanes 13–18) subunits as described under Materials and Methods. Other details were as described for Figure 1.

Thus, using EEDQ, it was possible to detect cross-linked products involving the 75-, 49-, 30-, 18-, and 13-kDa subunits of IP,[2] all of which were altered in extent upon treatment of complex I with NADH or NADPH, but not with NAD or NADH + $K_3Fe(CN)_6$. Figure 4 shows the results for the 75- and 18-kDa subunits. These polypeptides do not cross-link in the presence of DST or EGS (Yamaguchi & Hatefi, 1993), but they do cross-link in the presence of EEDQ, giving a single cross-linked product that is greatly diminished in the presence of NADH (Figure 4, lanes 3 and 8) but not NAD (Figure 4, lanes 4 and 9) or NADH + $K_3Fe(CN)_6$ (Figure 4, lanes 5 and 10). Figure 5 shows the results of EEDQ-induced cross-linkings involving the 49-, 30-, and 13-kDa subunits. In all cases, the cross-linked products involved polypeptides of the HP fraction of complex I, because they were not recognized by antibodies to any of the FP or other IP subunits. However, it is clear that, in all instances, the cross-linking of the 49-, 30-, and 13-kDa subunits decreased in extent when complex I was treated with NADH or NADPH, but not when it was treated with NAD or NADH + $K_3Fe(CN)_6$. It should be added here that Patel and Ragan (1988) previously reported the effect of NADH on the cross-linking of the 75- and 51-kDa subunits. However, these authors did not check the effects of NAD and NADH + $K_3Fe(CN)_6$ as controls to distinguish between ligand binding and enzyme reduction, nor did they observe the effect of NADH on the cross-linking patterns of other subunits of complex I.

DISCUSSION

As stated in the introduction, knowledge of the structure of complex I is very limited, and knowledge of its mechanisms of electron transfer and proton translocation is close to nil. Even the H^+/e stoichiometry of complex I is uncertain, and the values reported ($H^+/e \geq 2$) are too high to rationalize on the basis of a mechanism in which a single electron-transfer reaction would be coupled to the translocation of one proton. We have, therefore, begun to think about the coupling mechanism of complex I not by analogy to the Q-cycle hypothesis of complex III, in which ubiquinone is considered to be the agent that couples electron transfer to transmembrane proton translocation (Mitchell, 1976; Trumpower, 1990), but rather by analogy to the ATP synthase complex, in which there is no proton carrier and energy coupling between the catalytic and the proton-translocating sectors appears to take place via conformational changes of the subunits. Such a mechanism not only agrees with what we know about the structure of complex I but it also obviates the problem of having to rationalize the $H^+/e \geq 2$ stoichiometry on the basis of various hypothetical Q-cycle-type schemes, for which there is no experimental evidence (Mitchell, 1979; Ragan, 1987; Krishnamoorthy & Hinkle, 1988; Weiss & Friedrick, 1991; Vinogradov, 1993).

It is now certain that, in the proton-motive and sodium-motive ATP synthases, the translocated ions do not participate in ATP synthesis, nor do they originate in the reverse process at the site of ATP hydrolysis. Moreover, it is known that binding of the inhibitor oligomycin to the membrane sector F_o of the bovine ATP synthase complex greatly decreases the affinity of the catalytic sector F_1 for ATP at a distance of 20–90 Å (Matsuno-Yagi *et al.*, 1985; Penefsky, 1985; Hatefi, 1993). These results have suggested that the catalytic sites of F_1 and the region of oligomycin binding in F_o communicate via coupled conformational changes of ATP synthase subunits. Experiments in which the proximities of F_1 subunits of the *Escherichia coli* ATP synthase complex have been examined by cross-linking have also demonstrated that substrate binding by the β-subunits alters the spatial relationships among the α-, β-, γ-, and ϵ-subunits of F_1 (Capaldi *et al.*, 1992; Aggeler *et al.*, 1993).

The results described in this article show that the proximities of the three subunits of FP to one another, the proximity of the 51-kDa subunit of FP to the 75-kDa subunit of IP, and the proximities of all of the IP subunits (except the 15-kDa subunit, which was not studied for the reasons given above) to one another and to some of the HP subunits are altered when the catalytic sector of complex I is reduced by NADH or NADPH prior to the addition of the cross-linking reagent. Controls showed that these changes were not due merely to ligand binding, because the addition of NAD or NADH plus ferricyanide (which rapidly oxidizes NADH via FP) had essentially no effect. Preliminary studies, which will be

[2] Unlike DST and EGS, EEDQ did not effect any cross-linkings involving the 51-, 24-, and 9-kDa subunits of FP.

reported elsewhere when completed, have also shown a dramatic effect of NADH treatment on the susceptibility of the FP and IP subunits to proteolysis by trypsin.

It is noteworthy that the proximity changes that result from NAD(P)H treatment of complex I involve not only those subunits that contain electron carriers (i.e., the 75-, 51-, and 24-kDa subunits) but also those that are devoid of electron carriers. It is also noteworthy that among the latter there are subunits (e.g., the 49-, 30-, and 13-kDa subunits) that cross-link to subunits of the membrane sector HP and that the proximities between these IP and various HP subunits also change when complex I is reduced. In other words, one can envision a chain of conformational changes beginning with the reduction of FP and the 75-kDa subunit of IP, which is then transmitted from the 75-kDa subunit to the other IP subunits, and therefrom to the subunits of the membrane sector of complex I. These conformational changes, which alter the proximities of various complex I subunits to one another, may well be the device by which the energy derived from electron transfer through the catalytic components of complex I is transduced and conveyed to the subunits of the membrane sector, where pK_a changes of appropriate residues induced by these conformational changes would result in proton uptake and release on opposite sides of the membrane.

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