BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **227,** 135–139 (1996)
ARTICLE NO. 1479

Intersubunit Interactions in the Bovine Mitochondrial Complex I as Revealed by Ligand Blotting

Grigory I. Belogrudov and Youssef Hatefi[1]

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

Received August 21, 1996

Bovine mitochondrial complex I (NADH:ubiquinone oxidoreductase) is composed of 3 structural domains, designated FP (flavoprotein, 3 subunits), IP (iron-sulfur protein, 7-8 subunits) and HP (hydrophobic protein, $>$ 30 subunits). IP intervenes between FP and HP, and in complex I its 75 kDa subunit appears to interact with the 51 kDa subunit of FP. In this study, we show by the technique of ligand blotting that isolated IP binds (a) only to the 51 kDa subunit of FP, and (b) to the 42, 39, 23, 20 and 16 kDa subunits of HP. Because a 23 kDa and a 20 kDa subunit of complex I are potential iron-sulfur proteins, these and our previous results are consistent with the following possible path of electrons in complex I: NADH $\rightarrow$ 51 and 24 kDa subunit of FP $\rightarrow$ 75 kDa subunit of IP $\rightarrow$ 23 and 20 kDa subunits of HP $\rightarrow$ ubiquinone.

The bovine mitochondrial complex I (NADH:ubiquinone oxidoreductase) is composed of more than 40 unlike subunits (1). It was shown in 1967 that bovine complex I can be divided into 3 distinct fractions, subsequently termed the FP (flavoprotein), the IP (iron-sulfur protein) and the HP (hydrophobic protein) fractions or subcomplexes (2,3). Bovine FP is composed of 3 polypeptides with molecular masses of 51, 24 and 9 kDa. The 51 kDa subunit binds NAD(H) and contains FMN and a tetranuclear iron-sulfur cluster (3). The 24 kDa subunit contains a binuclear iron-sulfur cluster, and the 9 kDa subunit carries no redox centers (3). IP contains 7 major polypeptides with molecular masses of 75, 49, 30, 18, 15, 13 and 11 kDa, of which the 13 and the 11 kDa subunits comigrate upon SDS gel electrophoresis (4-6), and the 75 kDa subunit contains a tetranuclear and a binuclear iron-sulfur cluster (7-9). FP and IP are water-soluble, whereas HP is highly water-insoluble and contains the 7 subunits of complex I that are encoded by the mitochondrial DNA. This tripartite arrangement of the subunits of bovine complex I appears to be the architectural blueprint of complex I from *Neurospora crassa* and *Escherichia coli* (10,11), possibly of the enzyme from *Paracoccus denitrificans* as well (12), except that the *Neurospora* enzyme has $\geqslant$ 32 subunits (11), and those from *E. coli* and *P. denitrificans* 14 subunits each (10,12).

We have previously studied the near-neighbor relationships of the subunits of bovine complex I by cross-linking experiments and detection of cross-linked products by immunoblotting with subunit-specific antibodies. The results showed that in FP each of the 3 subunits can be cross-linked to the other two, that in IP the 75 kDa subunit cross-linked to the 30, the 18 and the 13 kDa subunits, and that the latter subunits also cross-linked to the 49 kDa subunit. The 18 and the 13 kDa subunits did not cross-link, nor did the 75 and the 49 kDa subunits. Furthermore, the only link between FP and IP involved the 51 kDa subunit of the former and the 75 kDa subunit of the latter, and IP appeared to intervene between FP and HP (13,14).

[1] To whom inquiries should be addressed. Fax: (619) 784-2054.
Abbreviation: SDS-PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis.

0006-291X/96 $18.00

This paper shows that the technique of ligand blotting (15) can be used to investigate intersubunit interactions in complex I. As will be seen, in an enzyme complex composed of more than 40 unlike subunits, ligand blotting is highly selective and informative.

MATERIALS AND METHODS

FP, IP (16) and complex I (17) were isolated as described. The IP fraction was used without further purification. [^{125}I]protein A was from Amersham, and Tricine from Sigma. The sources of other reagents were the same as before (14).

SDS-PAGE (12%) was done according to Laemmli (18). The protein bands were either stained with Coomassie Brilliant Blue or electrotransferred onto nitrocellulose filters (0.22 μm) as described elsewhere (14). The nonspecific binding sites on nitrocellulose were blocked by incubation with 3% skim milk in PBS (50 mM sodium phosphate/150 mM NaCl, pH 7.4) for 1 h at 37° C. Then the filters were incubated for 2 h at 4° C in a PBS solution containing 20 μg of IP per ml and 1% skim milk. The filters were washed in PBS three times (10 min each), and fixed for 30 min in 0.5% formaldehyde in PBS, followed by 30 min incubation in 2% glycine in PBS and again in PBS alone (19). IP bound to nitrocellulose was detected with affinity-purified antibodies to the 13, 18 and 30 kDa subunits as detailed elsewhere (14). Briefly, the affinity-purified antibodies, diluted 100-1000 fold in PBS containing 1% skim milk and 0.3% Tween-20, were incubated with the nitrocellulose sheets for 1 h at 24° C. The sheets were washed several times in the PBS/skim milk/Tween buffer lacking antibodies, and incubated for 1 h with a solution of [^{125}I]protein A (200,000 cpm/ml) in the same buffer. They were washed as before, dried, and exposed to X-ray films for 1-2 days at $-70°$ C, using intensifying screen. In several experiments, the primary antibodies were visualized with 3,3′-diaminobenzidine after incubating the nitrocellulose sheets with goat horseradish peroxidase-conjugated anti-rabbit IgG (20). All the experiments were repeated at least 3 times, and representative results from each set are shown here.

The electroeluted 51 kDa subunit of FP was cleaved either with CNBr in 70% formic acid or at the Asp-Pro bond with formic acid, as described by Runswick et al. (8). The reactions were stopped by adding 9 volumes of distilled water and the mixtures were lyophilized. Peptides were dissolved in SDS sample buffer and separated by the SDS-PAGE method of Schägger and von Jagow (21). Protein concentration was determined according to Lowry et al. (22), using bovine serum albumin as standard.

RESULTS AND DISCUSSION

As indicated above, cross-linking studies on purified bovine complex I had shown that within FP and IP there were multiple cross-linked products of their respective subunits, showing in each case the close proximities of subunits, but cross-linking between FP and IP involved only the 51 kDa subunit of the former and the 75 kDa subunit of the latter (13,14). The ligand blotting results shown in Fig. 1 agree with our previous finding. In this figure, lane 1 shows the 3 subunits of purified bovine FP displayed on an SDS gel stained with Coomassie Brilliant Blue. Lane 2 shows a ligand blot of the FP subunit transferred to nitrocellulose, incubated with a solution of IP, then treated with affinity-purified antibodies to the 13 kDa subunits of IP (see legend to Fig. 1), and finally with [^{125}I]protein A. It is seen that only the region of the 51 kDa subunit of FP became radioactive, indicating that IP, the antibodies, and [^{125}I]protein A were bound only to this region of the nitrocellulose sheet. Lane 3 shows the same experiment as in lane 2, except that the treatment of the nitrocellulose sheet with IP was omitted. It is clear that under these conditions the antibodies and [^{125}I]protein A did not bind to the nitrocellulose. Lane 4 shows an immunoblot of complex I treated with the affinity-purified antibodies to the 13 kDa subunits of IP, followed by [^{125}I]protein A. It is seen that the only complex I subunits that reacted with our subunit-specific antibodies were the comigrating 13 kDa subunits of IP. It may be added here that the staining of a nitrocellulose strip identical to that shown in lane 2 of Fig. 1 with Ponseau S revealed that all 3 FP subunits had been efficiently transferred onto nitrocellulose. IP binding to the 51 kDa subunit of FP could also be visualized when goat horseradish peroxidase-conjugated anti-rabbit IgG was used instead of [I^{125}]protein A for the detection of bound primary antibodies. Furthermore, preincubation of IP in 6 M urea resulted in the loss of IP binding to the 51 kDa subunit of FP (data not shown). The data of Fig. 1 do not show which subunit(s) of IP interact with the 51 kDa subunit of FP. However,

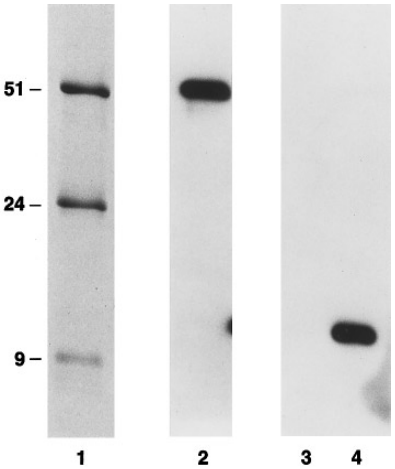

FIG. 1. Ligand blotting of FP with IP. Purified FP (5μg) and complex I (15 μg) were subjected to 12% SDS-PAGE, and the polypeptides were either stained with Coomassie Brilliant Blue (lane 1) or electrotransferred onto nitrocellulose (lanes 2-4). Lane 2, FP after ligand blotting with IP followed by immunodetection of IP with antibodies directed against the 13 kDa subunits of IP and [I^{125}]protein A. Lane 3, same as lane 2, except that incubation of the nitrocellulose sheet with IP was omitted. Lane 4, immunoblotting of complex I with antibodies directed against the 13 kDa subunits of IP. These antibodies were raised against the M_r 13,000 protein band electroeluted from SDS gels. This band, as mentioned in the text contains the comigrating 13 and 11 kDa subunits of bovine complex I, which for convenience are jointly referred to here as the 13 kDa subunits. The molecular masses in kDa of the FP subunits are indicated on the left of lane 1.

combined with our earlier cross-linking studies, it is possible that the isolated FP and IP also interact by way of their two largest subunits. In functional terms, this would make sense, because in IP the 75 kDa subunit is the only known component that contains redox carriers.

Fig. 2 shows data on the interaction of IP with subunits of the HP fraction of complex I. In panel A, lanes 1, 2 and 3 show, respectively, the SDS-PAGE of FP, IP and complex I. Lanes 1, 2 and 3 of panel B show ligand blotting of the same gels as in panel A after transfer of the protein bands to nitrocellulose, and incubation sequentially with IP, affinity-purified antibodies against the 30 kDa subunit of IP, and [^{125}I]protein A. It is seen that in lane 1 of panel B only the 51 kDa subunit reacted, as before. In lane 2 of panel B the prominent subunits that reacted with IP were the 75, and the 49 kDa subunits, which agrees with our previous cross-linking experiments showing cross-linked products of 75 + 30, 49 + 30, 75 + 18, 75 + 13, 49 + 18, 49 + 13, 30 + 18 and 30 + 13 kDa subunits (13,14). Lane 3 of panel B shows that IP appears to interact, in order of increasing mobility on the SDS gel, with the 75, 51 + 49, 42, 39, 23, 20 and 16 kDa subunits of complex I, among which the last 5 polypeptides are found mainly in the HP fraction. Lanes 4, 5 and 6 are controls lacking the treatment of the nitrocellulose sheet with IP. As expected, there were no reactive bands in lane 4 (FP), and only one reactive band (the 30 kDa subunit) in lane 5 (IP) and lane 6 (complex I). Panel C is the same as panel B, except that the affinity-purified antibodies used here were against the 13 kDa subunits of IP. Similar results were obtained when the antibodies used for the detection of bound IP were specific for the 18 kDa subunit (data not shown). In addition, preliminary results have indicated that a 15 kDa CNBr fragment of the 51 kDa subunit also binds IP. From the amino acid sequence of the 51 kDa subunit (1), one can surmise that this reactive region may be located between residues 131 and 272 of the 51 kDa subunit.

Two other points of interest may be added here. First, the results indicate that the IP fraction of complex I is not simply a collection of polypeptides, but rather a structured domain or

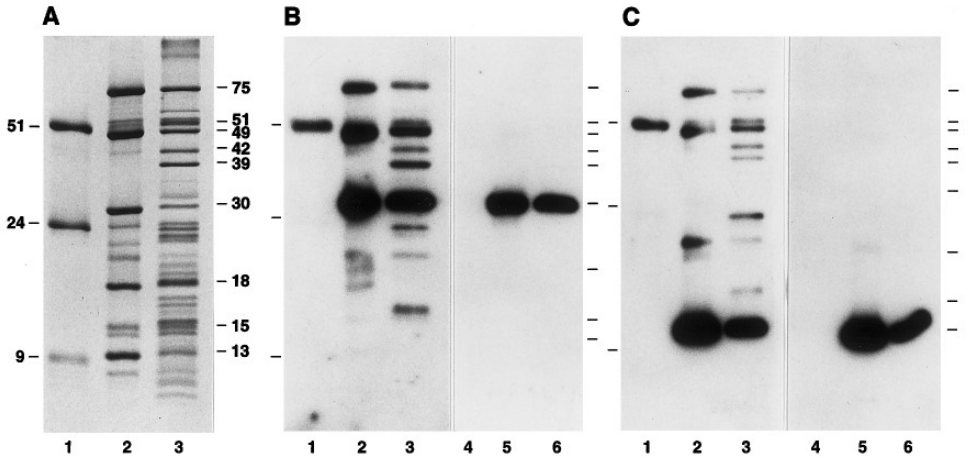

FIG. 2. Ligand blotting of FP, IP and complex I with IP. FP (5 μg), IP (10 μg) and complex I (15 μg) were subjected to 12% SDS-PAGE, and the polypeptides were either stained with Coomassie Brilliant Blue (panel A: lane 1, FP; lane 2, IP; lane 3, complex I) or electrotransferred onto nitrocellulose (panels B and C). Panel B, lanes 1-3: nitrocellulose sheets containing samples identical to those of panel A after incubation with IP followed by immunodetection with antibodies against the 30 kDa subunit of IP and [I^{125}]protein A. Panel B, lanes 4-6: immunoblots of duplicate nitrocellulose sheets with antibodies against the 30 kDa subunit of IP. Panel C, lanes 1-3: same as lanes 1-3 of panel B, except that the antibodies used for detection of bound IP were against the 13 kDa subunits. Panel C, lanes 4-6: immunoblots of duplicate nitrocellulose sheets with antibodies against the 13 kDa subunits of IP. The molecular masses in kDa of relevant subunits of complex I are indicated in panel A. The bars in panels B and C indicate in descending order the same molecular masses as marked in panel A.

sector of complex I, in which the subunits are held together. This conclusion derives from the fact that regardless of the IP subunit-specific antibodies used (i.e., specific for the 30, 18 or 13 kDa subunit of IP), the binding of IP to the 51 kDa subunit of FP could be observed. The same is true for the reactive HP subunits. Second, in addition to the 75, 51 and 24 kDa subunits which house iron-sulfur clusters, there are in complex I two other subunits containing cysteine motifs found in iron-sulfur proteins. These are a 23 kDa subunit, with 2 such motifs, and a 20 kDa subunit (23,24). Whether the polypeptides with approximate M_r values of 23,000 and 20,000 visualized in lanes 3 of panels B and C of Fig. 2 are the same as the additional potential iron-sulfur proteins of complex I mentioned remains to be seen. However, from a functional viewpoint it would be reasonable if the appropriate IP subunits were to bind to those HP subunits that carry redox centers.

ACKNOWLEDGMENTS

This work was supported by the United States Public Health Service Grant DK08126. We thank Dr. Ai-Li Han for preparation of the IP subunit-specific antisera, and C. Munoz for the preparation of bovine-heart mitochondria. This is publication 10275-MEM from The Scripps Research Institute, La Jolla, California.

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