Isolation and characterization of three subcomplexes of the mitochondrial NADH: ubiquinone oxidoreductase (complex I).
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Enzymically active subcomplexes were purified from bovine mitochondrial NADH:ubiquinone oxidoreductase (complex I) by sucrose-gradient centrifugation in the presence of detergents. These subcomplexes, named $I_2$, $I_3$, and $I_4$, catalyse ferricyanide and ubiquinone-1 (Q-1) reduction by NADH at similar rates to complex I, but do not catalyse the reduction of decylubiquinone. In addition, the Q-1 reductase activity of all the subcomplexes is insensitive to rotenone. Chemical and EPR analyses of the subcomplexes show that FMN and all the Fe-S clusters of complex I are present, but that the line shape of cluster 2 is modified. The smallest subcomplex, $I_3$, contains only approximately 13 subunits, as compared to approximately 22 in the previously described subcomplex $I_4$ [Finel, M., Skehel, J. M., Albracht, S. J. P., Fearnley, I. M. & Walker, J. E. (1992) Biochemistry 31, 11425–11434], but it retains the 75-, 51-, 49-, 30-, 24-, 23- (TYKY) and 20-kDa (PSST) subunits, which are suggested to form a functional core that comprises the EPR-detectable Fe-S clusters 1–4, and FMN. The structural and functional implications of such an arrangement are discussed.

NADH:ubiquinone oxidoreductase (complex I) is the largest enzyme of the mitochondrial respiratory chain. Complex I comprises of at least 41 different protein subunits (Arizmendi et al., 1992), FMN and several Fe-S clusters, and conserves energy by catalyzing proton translocation linked to the redox activity (Beinert and Albracht, 1982; Ragan, 1987; Weiss et al., 1991; Walker, 1992; Finel, 1993). The EPR-detectable Fe-S clusters are named in this study in the order of their discovery (cluster 1b, $g_{x=y} = 1.92, 1.94, 2.02$; cluster 2, $g_{x=y} = 1.92, 1.92, 2.05$; cluster 3, $g_{x=y} = 1.88, 1.94, 2.10$; cluster 4, $g_{x=y} = 1.86, 1.93, 2.04$; Beinert and Albracht, 1982). These clusters are also referred to in the literature as N-1b, N-2, N-4 and N-3, respectively. The midpoint potential of cluster 2 is pH dependent and higher than the midpoint potentials of the other clusters (Ingledew and Ohnishi, 1980). Therefore, cluster 2 has been assumed to serve as the electron donor to ubiquinone, and to be intimately linked to the proton translocation mechanism of the enzyme (de Jong et al., 1994).

One of the current goals in the study of complex I is to identify the binding sites of the individual Fe-S clusters. EPR measurements on the flavoprotein fragment of complex I indicated the presence of cluster 4 (N-3) and a binuclear cluster; Q-1, ubiquinone-1.

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Abbreviations. Decylubiquinone, 2,3-dimethoxy-5-methyl-6-n-decyl-1,4-benzoquinone; LDAO, N,N-dimethyldodecylamine N-oxide; Q-1, ubiquinone-1.

Enzyme. NADH:ubiquinone oxidoreductase (EC 1.6.5.3).
very remote from the other Fe-S clusters of complex I (Finel et al., 1992).

In this study, we describe the isolation and properties of three subcomplexes of the enzyme (Ia, IS and IAS), two of which contain substantially fewer subunits than the previously described subcomplex Ia (Finel et al., 1992). Subcomplex Ia has been previously briefly described in a different context (Arizmendi et al., 1992). In this study, we report on the enzymic activities, the EPR properties, and the subunit composition of these subcomplexes. We conclude that a catalytic core of seven predominantly hydrophilic subunits contains all the EPR-detectable Fe-S centres, and the FMN, and we discuss the structural and functional implications of this conclusion.

MATERIALS AND METHODS

Chemicals

N,N-Dimethyldecylamine N-oxide (LDAO) and lauroylsarcosine (microselect grade) were purchased from Fluka. Dodecysucrose was purchased from Nova Biochem. Ubiquinone (Q-1) was a generous gift of Hofmann-La Roche. 2,3-Dimethoxy-5-methyl-6-n-decyl-1,4-benzoquinone (decylubiquinone) was synthesised by Dr Hase in the Department of Organic Chemistry, University of Helsinki, according to Wan et al. (1975).

Purification of subcomplexes

All the purifications were carried out at 0–4°C. Complex I was isolated from bovine heart mitochondria according to Ragan et al. (1987). The subcomplexes were purified by sucrose-gradient centrifugation of complex I under different conditions. Complex I was diluted to 5–7 mg protein/ml containing either 1% (mass/vol.) LDAO and 100 mM potassium phosphate, pH 7.5 (1s), 1% lauroylsarcosine, and 50 mM Tris/HCl, pH 7.5 (IS), or 0.5% LDAO, 1% lauroylsarcosine and 100 mM potassium phosphate, pH 7.5 (Ia). Approximately 0.5 ml of this solution was layered on top of a 4.5-ml sucrose gradient that had been prepared in tubes of the SW 50.1 rotor (Beckman), and centrifuged at 200000×g for 18–20 h. The gradients were 5–15% (mass/vol.) sucrose in the presence of 0.5% LDAO and 0.5 M potassium phosphate, pH 7.5 (Ia), 20–50% sucrose in the presence of 1% lauroylsarcosine and 50 mM Tris/HCl, pH 7.5 (IS), or 5–15% sucrose in the presence of 0.5% LDAO, 1% lauroylsarcosine and 0.2 M potassium phosphate (ILs). Following centrifugation, the sharp yellow-brown band at the middle of the gradient was collected and concentrated by adding dodecysucrose (or laurylmaltoside) to 1% and sodium cholate to 2%, followed by the addition of cold, saturated, neutralised ammonium sulfate to a final concentration corresponding to 45% saturation. The suspension was centrifuged for 10 min at 12000×g and the dark-brown pellet was dissolved in a small volume of buffer containing 20 mM Tris/HCl, pH 7.5, 1 mM EDTA, 0.1% dodecysucrose, and 10% (by vol.) glycerol and stored at −70°C.

Activity assays

The enzymic activities of complex I and the subcomplexes were assayed spectrophotometrically at 30°C. The buffer contained 1 mM EDTA and 20 mM Tris/HCl. The rates of ferricyanide reduction by 4–6 N mM enzyme, deter-
mined by measuring the bound FMN, were followed at 420 nm minus 500 nm, in the presence of 0.8 mM ferricyanide and 0.1 mM NADH at pH 7.0. Cytochrome c reduction by 7–11 nM enzyme was measured at 550 nm minus 540 nm, in the presence of 0.2 mM NADH and 30 mM ferri-
cytochrome c at pH 8.0. The oxidation rate of NADH (0.2 mM) by 7–11 nM enzyme in the presence of sonicated phospholipid (0.5 mg/ml) and either Q-1 (0.2 mM) or decylubiquinone (0.2 mM) was assayed at 340 nm and pH 7.0.

EPR spectroscopy

EPR measurements for the X-band (9 GHz) were made with a Bruker ECS 106 EPR spectrometer at a field-modulation frequency of 100 kHz. Cooling of the sample was performed with an Oxford Instruments ESR 900 cryostat with an ITC4 temperature controller. The sample temperature indication from this instrument was correct from 4.2–100 K within 2% as ascertained from the Curie dependence of a copper standard (10 mM CuSO4 + 5 H2O, 2 M NaClO4, 10 mM HCl). The microwave power incident to the cavity was 260 mW, as measured with a HP432B power meter. The magnetic field was calibrated with an AEG magnetic field meter. The X-band frequency was measured with a HP 5350B microwave frequency counter. Quantification of EPR signals was carried out by direct double integration of the experimental spectra (Aasa and Vännärd, 1975; Albracht, 1984).

Analytical methods

The protein concentration was determined by the bicinchoninic acid method (Pierce), except during the purification of complex I where the Biuret method was employed. FMN was extracted and its concentration was determined according to Ragam and Racker (1973). SDS/PAGE was performed using the buffer system of Laemmli (1970), with an acrylamide gradient of 12–30% (mass/vol.). In addition, SDS/ PAGE, containing 16% acrylamide and 6 M urea, was performed as described by Schägger and von Jagow (1987), but without a spacer gel and using the acrylamide/bisacrylamide mixture that has 1.5% cross-linker. Protein sequence analyses after SDS/PAGE, electrophoresis to a poly(vinylidene difluoride) membrane and staining with Coomassie-brilliant-blue dye, were performed using a modified ABI 477A/120A sequenator in the liquid-phase mode (Baumann, 1990).

RESULTS

The subcomplexes were isolated from complex I using sucrose-gradient centrifugation under different conditions. Addition of 0.5 M potassium phosphate to the LDAO-containing sucrose gradient that was previously used to prepare subcomplex Ia (Finel, 1993), removed several of the approximately 22 subunits present in the subcomplex Ia preparation, and yielded the subcomplex Ia with 15 subunits [Fig. 1; compare Arizmendi et al. (1992)]. However, if LDAO was replaced by lauroylsarcosine, but in the absence of potassium phosphate, the resulting subcomplex (IS) contained approximately the same number of subunits as Ia, although with a different subunit composition (Fig. 1A). When LDAO was supplemented with both lauroylsarcosine and potassium phosphate, two more subunits were removed relative to subcomplex IIa, yielding IAS, the smallest subcomplex in this series isolated so far (Fig. 1A and B).
The SDS/PAGE analysis (Fig. 1) shows unequivocally that all the subcomplexes contain the 75-, 51-, 49- and 30-kDa subunits. In addition, protein sequencing verified that both subcomplexes IS and I2S also contain the 24-, 23-, 20- and 18-kDa subunits (GAGG, TYKY, PSST and AQDQ, respectively), previously also reported in subcomplex IA (Arizmendi et al., 1992). In addition to these complexes, subcomplexes I2S and I4 contain approximately five and seven smaller subunits, respectively (Fig. 1A and B). Subcomplex I2S lacks two subunits of subcomplex I4, as shown in Fig. 1B. Under the SDS/PAGE conditions of Fig. 1A, however, these proteins migrate together as a single band with an apparent molecular mass of 17 kDa. Both these subunits are also absent from subcomplex IS (Fig. 1B).

Subcomplex IS uniquely contains at least one mitochondrially encoded hydrophobic protein, ND4, which was previously found in the membranous subcomplex I4, but which is absent from I4 (Fig. 1A; Finel et al., 1992). It is also worth noticing that both subcomplexes I4 and IS contain traces of the mitochondrially encoded subunits ND1 and ND2 (Fig. 1A; Finel et al., 1992). These subunits are absent from I4 and IS, suggesting a more efficient purification of a mostly hydrophilic domain of the enzyme.

The enzymic activities were calculated/FMN in order to simplify comparisons between the different preparations (Table 1). The high rate of ferricyanide reduction, and the very low cytochrome c reductase activity are similar to those of complex I (Table 1). In contrast, a large difference in activity was found with the hydrophobic ubiquinone analogue decylubiquinone as electron acceptor. The decylubiquinone reductase activity of complex I was high and fully sensitive to rotenone, but the subcomplexes exhibited only negligible activity in the presence of this acceptor (Table 1, compare the results for Q-1), and their Q-1 reductase activity, although similar in rate to that of complex I, was completely insensitive to rotenone (data not shown).

EPR spectra of NADH-reduced complex I, and of the subcomplexes I4, I2S and IS, are presented in Fig. 2. Whilst the subcomplexes have similar spectra, the most significant difference relative to the parent complex I is that the g 2.05 line (gJ of cluster 2, is absent from the subcomplexes; instead a line at g = 2.04 is observed. The g 2 of cluster 2 (an extensive trough at g = 1.92) is detectable, however, in all the spectra. The overall line shape in the g = 1.90–1.95 re-
2.2 2.1 2.0 1.9 1.8
G-VALUE

Fig. 2. EPR spectra of subcomplexes I₁, I₂S and IS compared to the spectrum of complex I. (A) complex I; (B) subcomplex I₁; (C) subcomplex I₂S; (D) subcomplex IS. The EPR conditions were as follows: microwave frequency, 9425 MHz; microwave power incident to the cavity, 0.26 mW; modulation amplitude, 0.64 mT; temperature, 10 K. The spectra were normalised to the same microwave frequency, and plotted with the same amplitude in the g 1.92–1.94 region.

The g-ion is virtually the same in complex I (Fig. 2, trace A) and in subcomplex I₁ (Fig. 2, trace B). This indicates that the g value of cluster 2 has shifted from 2.05 in complex I to 2.04 in subcomplex I₁. The gₓ line of cluster 3 is found at g ≈ 1.883 in both complex I and subcomplex I₁ (Fig. 2, traces A and B, respectively). In the other two subcomplexes, however, this line has noticeably shifted to higher field (g ≈ 1.877), although the position of the gₓ line of cluster 3 has not changed.

The presence of cluster 2 in the subcomplexes was further verified by examining the temperature dependence of the new g = 2.04 line, and the g = 1.92 line (Fig. 3). Upon raising the temperature from 17 K to 27 K, the lines at g = 2.10, 1.88 and 1.86 become undetectable in complex I due to relaxation broadening. The g = 2.05 line of cluster 2 is, however, still partly visible at 27 K, along with its gₓ, line at g = 1.92 (Fig. 3A). The temperature dependence of the modified cluster 2 signal in subcomplex I₂S is very similar. At 25 K, and even at 30 K, the remnants of the broadened gₓ at g = 2.04 and the gₓ at g = 1.92 can still be observed (Fig. 3B). The line shapes of cluster 1b, as observed at 50 K, are virtually identical in complex I and subcomplex I₂S (Fig. 3A and B).

In addition to the Fe-S clusters 1b, 2–4, low levels of cluster 5 (gₓₓₓ ≈ 1.90, 1.93, 2.06; Albracht, 1974; Ohnishi, 1975) were also detected in NADH-reduced samples of the subcomplexes.

Cluster 2 is the only Fe-S centre in complex I that can be fully reduced by dithionite in the absence of NADH and redox mediators (Albracht et al., 1977). However, no Fe-S cluster was detected upon reduction of subcomplex I₁ with dithionite at pH 7 (dithionite dissolved in 1 M Tris/HCl, pH 7.4). The possibility that cluster 2 was partially converted into a [3Fe-4S] cluster was tested by EPR spectroscopy of aerobic subcomplex I₁ in the absence of added reductant, but no indication of this conversion was found.

The overall content of the NADH-reducible Fe-S centres was determined in the subcomplexes from the total concentration of spin-bound FMN (Table 2). The spin concentrations were calculated both at 4.3 K and at 50 K, under non-saturating radiation. The 4.3 K value includes the contribution of all Fe-S clusters. At 50 K, the concentration represents mainly the spin concentration of cluster 1b, but since the spin concentration of cluster 1b was obtained by direct...
double integration, it may be somewhat overestimated (van Belzen et al., 1992). These measurements show that the overall amount of EPR-detectable Fe-S clusters/FMN is lowered in the subcomplexes. However, comparison of the total spin concentrations at 4.3 K and 50 K indicates no specific loss of individual Fe-S clusters, but rather that the average Fe-S cluster content is lowered. This is supported by the EPR spectra, which showed that all Fe-S clusters are present, and that there are no significant changes in the relative amounts of the four clusters (Fig. 2). Since, nevertheless, the Q-1 reductase activity remained the same/bound FMN, it appears that rotenone-insensitive Q-1 reduction is not rate-limited by electron transfer through the Fe-S clusters.

**DISCUSSION**

Subcomplexes of the mitochondrial NADH-ubiquinone oxidoreductase have in the past provided information about the structural arrangement and functional properties of this very complicated enzyme. The subcomplexes described in this study relate to the previously described subcomplex Ia from bovine heart (Finel et al., 1992), and to the small isoform of complex I from *N. crassa* (Friedrich et al., 1989; Weiss et al., 1991). Both have been described as an assembly of predominantly hydrophilic subunits that contain all, or most, of the redox centres of complex I. The subcomplex IaS is thus far the smallest complex, containing approximately 13 subunits.

All subcomplexes retain an NADH-oxidizing activity with ferricyanide or Q-1 as acceptors that is comparable to that of complex I. However, the activity with decylubiquinone as acceptor is lost concomitant with the loss of rotenone-sensitivity of Q-1 reductase. The correlation between these findings suggests that the Q-1 reductase activity of the hydrophilic subcomplexes is not analogous to the normal ubiquinone-10 reductase activity of complex I. Altogether, the results of the activity measurements (Table 1) and the line shape of clusters 1b, 3 and 4 in EPR spectroscopy (Fig. 2) suggest that the electron-input domain in subcomplexes Ia, IS and IaS is similar to complex I. In contrast, the flavoprotein fragment isolated by Galante and Hatefi (1979) exhibited a high rate of antimycin-insensitive cytochrome c reduction and had altered EPR spectra (Ohnishi et al., 1981, 1985), indicating considerable modification of the electron-input domain.

Subcomplex Ia lacks the cysteine-rich 19-kDa subunit (PGIV; Arizmendi et al., 1992), which is present in subcomplex Ia (Finel et al., 1992), and we did not detect this subunit in IaS or IS either. This subunit has been suggested to bind an Fe-S cluster (Dupuis et al., 1991b; Walker, 1992), but since Ia, IS and IaS contain all the EPR-detectable Fe-S clusters of complex I, we conclude that the 19-kDa subunit does not bind such a cluster. This is in agreement with the absence of a homologue of PGIV in the complex I operons of both *P. denitrificans* and *E. coli* (Yagi et al., 1993; Weidner et al., 1993). The 20.8-kDa subunit of *N. crassa* complex I, a homologue of PGIV, was recently reported to be a component of the membrane arm of that enzyme (Azevedo and Videira, 1994).

The fact that the high-potential cluster 2 is present in Ia and IaS, though structurally modified, is of particular interest. High-potential cluster 2 is lacking from the small isoform of complex I of *N. crassa*, which contains clusters 1, 3 and 4 (Wang et al., 1991). This isoform also apparently lacks the ferredoxin-type 23-kDa subunit (TYKY, Dupuis et al., 1991a), whereas analogues of the 75-, 51-, 49-, 30-, 24- and 18-kDa subunits are present (Weiss et al., 1991). Since the 23-kDa subunit (TYKY) is present in all the subcomplexes in this study, this subunit may comprise the binding domain of cluster 2. Binding of cluster 2 with the hydrophobic membranous ND5 subunit (Weiss et al., 1991; Wang et al., 1991) is not consistent with our data. ND5 is found in the hydrophobic subcomplex Ib, which has no EPR features, but it is absent from Ia (Finel et al., 1992), and from Ia and IaS, which all exhibit the cluster 2 EPR signal.

Genes encoding proteins homologous to the 75-, 51-, 49-, 30-, 24-, 23- and 20-kDa subunits of bovine complex I (TATA, SGDT, ARQW, ESSA, GAGG, TYKY and PSST, respectively) were recently found in both *P. denitrificans* (Yagi et al., 1993) and *E. coli* (Weidner et al., 1993). These bacterial NDH1 operons lack homologues of the other nuclear-encoded subunits of complex I, but they do contain homologues of all the mitochondrially encoded subunits (ND subunits). Interestingly, all the nuclear-encoded subunits, the homologues of which are present in these bacterial gene clusters, are also present in the subcomplexes described in this study, but none of the ND subunits were found in either Ia or IaS (Arizmendi et al., 1992; Fig. 1). The Fe-S cluster composition of the *Paracoccus* NDH1 is very similar to that of mitochondrial complex I (Albracht et al., 1980; Meinhardt et al., 1987). Together, all this information suggests that subcomplexes Ia and IaS comprise a largely hydrophilic domain of complex I, which contains the 75-, 51-, 49-, 30-, 24-, 23- and 20-kDa subunits, and which binds FMN and all the EPR-detectable Fe-S clusters. Cluster 2, however, may be bound near an interface between a hydrophilic domain and hydrophobic subunits of a membrane domain, explaining the susceptibility of this cluster to treatment with strong detergents. Thus, at least most of the redox chemistry of complex I occurs in a hydrophilic domain on the inside (mitochondrial matrix or bacterial cytoplasmic side) of the membrane, and this would require special structural arrangements to account for proton translocation. However, it should be mentioned that Patel et al. (1988) reported that the 49-kDa and 30-kDa subunits are exposed on the cytoplasmic side of the mitochondrial membrane, and Han et al. (1989) detected the 75-kDa subunit on both sides of the membrane.

Mitochondrial complex I translocates two protons and two electrical charge equivalents across the membrane/electron transferred between NADH and ubiquinone (Wikström, 1984). It seems reasonable to assume that translocation of at least one of these protons is directly linked to the redox chemistry, i.e. the oxidation of FMNH2 (proton release) and reduction of ubiquinone (proton uptake). If so,
the apparent localization of the redox reactions to an aqueous domain on the inside of the membrane would require transmembrane proton-conducting structures in contact with the FMNH\textsubscript{2}-oxidizing centre, and possibly also in contact with the ubiquinone reductase site. Such proton-conducting structures may be required in association with the electron-transferring centre(s) that catalyze translocation of the second proton, whether it is associated with oxidoreduction of a tightly bound quinone (Weiss et al., 1991), or an Fe-S centre. The mitochondrially encoded subunits might play a major role in the formation of such proton-conducting structures.

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