The polypeptide composition of the mitochondrial NADH:ubiquinone reductase complex from several mammalian species

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The polypeptide composition of isolated mitochondrial NADH:ubiquinone reductase (NADH dehydrogenase) is very similar to that of material immunoprecipitated from detergent-solubilized bovine heart submitochondrial particles by antisera to the holoenzyme. The specificity of the antisera for dehydrogenase polypeptides was determined by immunoblotting, which showed that antisera reacting with only a few proteins were able to immunoprecipitate all others in parallel. The polypeptide compositions of rat, rabbit and human NADH dehydrogenase were determined by immunoprecipitation of the enzyme from solubilized submitochondrial particles and proved to be very similar to that of the bovine heart enzyme, particularly in the high- M_r region. Further homologies in these and other species were explored by immunoblotting with antisera to the holoenzyme and monospecific antisera raised against iron-sulphur-protein subunits of the enzyme.

Analysis of the structure and function of mitochondrial respiratory-chain complexes has benefited from comparative studies of the enzymes from different species. The NADH dehydrogenase complex (EC 1.6.99.3), however, has been fully purified from bovine heart only (Complex I in the nomenclature of Hatefi *et al.*, 1962), although functionally very similar enzymes are present in mitochondria from other mammals and also from plants (Rich & Bonner, 1978), certain yeasts (Albracht & Subramanian, 1977) and *Paracoccus denitrificans* (Albracht *et al.*, 1980), for example. NADH dehydrogenases of simpler structure and function have been isolated from bacteria [e.g. *Escherichia coli* (Jaworowski *et al.*, 1981) and *Bacillus subtilis* (Bergsma *et al.*, 1982)] and from the yeast *Saccharomyces cerevisiae* (Duncan & Mackler, 1966). However, the differences between these enzymes and the mammalian type are so great that the comparative approach is of little use.

In the present paper we describe the use of antibodies to the bovine heart enzyme and its subunits to study the subunit composition of NADH dehydrogenase in other species. Direct immunoprecipitation of the complex from detergent-solubilized submitochondrial particles by

antibodies to the holoenzyme can be achieved in closely related mammalian species. We also report the use of immunoblotting to explore subunit homologies in a wider range of organisms.

Methods

Complex I was purified from bovine heart mitochondria by the method of Hatefi & Rieske (1967). Subunits were purified from the 'iron-protein fragment' of Complex I by preparative SDS/polyacrylamide-gel electrophoresis (Cleeter *et al.*, 1985). Rabbits were immunized with Complex I, Complex I solubilized in 1% (w/v) SDS or isolated subunits as generally described by Cleeter *et al.* (1985). Human liver, kidney and heart submitochondrial particles were prepared by the method of Moreadith *et al.* (1984), and were kindly provided by Dr. R. W. Moreadith. Liver mitochondria from other species were prepared as described by Pederson *et al.* (1978), and submitochondrial particles were obtained by ultrasonic disruption of mitochondria essentially by the procedure of Racker (1962) except that unbuffered 0.25M-sucrose was used as isolation medium.

Immunoprecipitation of the NADH: $K_3Fe(CN)_6$ reductase activity of detergent-solubilized submitochondrial particles was done as described by Smith & Ragan (1980). Larger-scale

Abbreviation used: SDS, sodium dodecyl sulphate

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production of immunoprecipitates for analysis by SDS/polyacrylamide-gel electrophoresis was carried out on between 2 and 4 mg of submitochondrial-particle protein (Smith & Ragan, 1980).

SDS/polyacrylamide-gel electrophoresis was carried out on 12–16%-(w/v)-acrylamide concave-gradient gels (Earley & Ragan, 1981) with the discontinuous buffer system of Laemmli (1970). Samples (1–2 mg of protein/ml) were dissociated in 2% (w/v) SDS and 2% (w/v) 2-mercaptoethanol. To inhibit endogenous proteinase activity, 5 mM-*p*-aminobenzamide was also included. Marker proteins of known M_r were obtained from Sigma Chemical Co., Poole, Dorset, U.K.

Immunoblotting on to nitrocellulose was done as described by Towbin *et al.* (1979). Antigen-antibody-complex formation was detected either by binding of 125 I-labelled protein A and autoradiography (Cleeter *et al.*, 1985) or by using an enzyme-linked assay. In the latter, the bound antibody was treated sequentially with biotinylated anti-(rabbit IgG) antibodies and a complex containing streptavidin linked to biotinylated peroxidase (Amersham International, Amersham, Bucks., U.K.). The peroxidase substrate was obtained from Bio-Rad Laboratories, Watford, Herts., U.K.

Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin (fraction V; Sigma Chemical Co.) as standard.

Results

Immunoprecipitation of bovine heart NADH dehydrogenase

None of the antiholoenzyme sera that we have examined caused any inhibition of the NADH: $K_3Fe(CN)_6$ reductase activity of isolated Complex I or submitochondrial particles from any species. This activity is therefore a convenient assay for immunoprecipitation of the enzyme, as shown in Fig. 1 for the reaction between bovine heart submitochondrial particles and a number of anti-(Complex I) sera. The residual, non-precipitable, activity is probably catalysed by another enzyme, since it has quite different kinetic characteristics (Smith *et al.*, 1980). The polypeptide compositions of the immunoprecipitates (Fig. 2, tracks 2 and 3) were identical for all precipitating antisera and differed very little from that of isolated Complex I (Fig. 2, track 1). Interference with the band profile by heavy chains and light chains of IgG can be minimized by dissociating samples in SDS and 2-mercaptoethanol at approx. 40°C or less. Under these conditions, the heavy chains and light chains do not dissociate and IgG appears as a series of bands of M_r greater than that of any Complex I subunit (track 2). Dissociation at 100°C caused

Fig. 1. *Immunoprecipitation of bovine heart NADH dehydrogenase from solubilized submitochondrial particles* Particles were dissolved in deoxycholate and Triton X-100, and NADH: $K_3Fe(CN)_6$ reductase activity was measured in the supernatant, after incubation with the indicated volume of antiserum and centrifugation. Antisera 2 (○) and 10 (●) were raised against native bovine heart Complex I. Antisera 3 (△) and 7 (▲) were raised against SDS-denatured Complex I. Total activity was unaffected by antiserum, and no activity was precipitated by control serum.

complete dissociation of IgG (track 3), but revealed that the immunoprecipitates were free from the most abundant impurity in Complex I preparations, the NAD(P) $^+$ transhydrogenase (apparent M_r 110000). The relative absence of a polypeptide of M_r 42000 from immunoprecipitates has been noted before (Heron *et al.*, 1979). However, solubilization in Triton X-100 causes loss of NADH: ubiquinone reductase activity, and so it cannot be concluded that this subunit is an impurity. Unfortunately, immunoprecipitation in detergents that do not cause loss of this activity (such as deoxycholate) has not given clear profiles.

The similarity in subunit composition between isolated Complex I and the immunoprecipitate is good evidence that these polypeptides are indeed constituents of the enzyme, particularly as different detergents were employed for the conventional and immunological isolations. However, since Complex I was itself used as the antigen, it could be argued that, even if several components of the preparation were impurities, antibodies raised to them would inevitably result in their appearance in the immunoprecipitate. One answer is to use an antiserum raised to a single subunit for immunoprecipitation, but, so far, our subunit-specific

Fig. 2. Polypeptide composition of Complex I and material immunoprecipitated from bovine heart submitochondrial particles with antiserum 10

The immunoprecipitate from 4 mg of particle protein was dissolved in 62.5 mM-Tris/HCl buffer, pH 6.8, containing 2% (w/v) SDS and 2% (v/v) 2-mercaptoethanol to a final volume of 50 μ l. One half was heated at 40°C for 1 min, the other at 100°C for 1 min. Complex I was dissolved in the same buffer (3 mg of protein/ml) and heated at 100°C for 1 min. Samples were electrophoresed as described in the Methods section and gels were stained with Brilliant Blue R. Track 1, Complex I (50 μ g of protein); track 2, immunoprecipitate from 1.2 mg of particle protein dissociated at 40°C; track 3, as track 2, but dissociated at 100°C. M_r values of well-resolved subunits are indicated to the left of track 1. Abbreviations: HC, IgG heavy chains; LC, IgG light chains; TH, NAD(P)⁺ transhydrogenase.

Fig. 3. Immunoblotting of Complex I with various anti-Complex I sera

Complex I was dissociated in SDS-containing buffer (see Fig. 2 legend) at 100°C for 1 min to give a final protein concentration of 2 mg/ml. The sample (0.1 ml) was loaded into a 7 cm-long well in the stacking gel. The gel was transferred on to nitrocellulose, which was then cut into 6 mm-wide strips, each corresponding to approx. 17 μ g of Complex I protein. Strips were incubated with the indicated antisera (at 1:100 dilution), and antigen-antibody-complex formation was detected by using the peroxidase-linked assay. The development time was not the same for each antiserum, and the absolute intensities of the stained bands are therefore not comparable. M_r values of antigenic polypeptides were determined by comparison with a strip of gel that had been stained for protein. Abbreviation: TH, NAD(P)⁺ transhydrogenase.

antisera (see below) have failed to agglutinate the holoenzyme. Alternatively, we can examine the specificity of the anti-holoenzyme sera by immunoblotting. As shown in Fig. 3, antisera raised in different rabbits differ greatly in their specificity for Complex I subunits. Antisera 9 and 10 react with many different subunits, presumably accounting for their potency in immunoprecipitating the enzyme (Fig. 1). However, antiserum 2, which precipitates the holoenzyme quite effectively, reacts with very few polypeptides of the enzyme. We therefore conclude that the polypeptides found in isolated Complex I are associated into a single structural unit. With the exception of the 42000- M_r polypeptide, this unit is not dissociated by non-denaturing detergents, and, by this criterion, all

the polypeptides may be regarded as genuine constituents of the enzyme.

Immunoprecipitation of rat, rabbit and human NADH dehydrogenases

The ability of anti-(Complex I) sera to immunoprecipitate the enzyme from solubilized submitochondrial particles of other species was highly variable, and did not necessarily correlate with potency towards the bovine heart enzyme. As shown in Fig. 4, antiserum 2, for example, was quite unreactive towards the human enzyme, even though it was very effective against the bovine heart enzyme (Fig. 1). On the other hand, antiserum 10 was effective with the rabbit, rat and human enzymes (Fig. 4), causing maximal precipi-

Fig. 4. Immunoprecipitation of NADH dehydrogenase from solubilized submitochondrial particles of other species. Conditions were the same as in the legend to Fig. 1. ● and ○, Rat liver; ■, rabbit liver; ▲ and △, human liver. ○ and △, antiserum 2; ●, ■ and ▲, antiserum 10.

tation of between 60% and 70% of the NADH: $K_3Fe(CN)_6$ reductase activity. The non-precipitable activity is higher in these experiments than in those of Fig. 1, and is a consequence of using liver rather than heart tissue.

The polypeptide compositions of the immunoprecipitates produced with antiserum 10 are shown in Fig. 5. The rat enzyme (tracks 2 and 3) behaves identically with that from bovine heart (track 1) in the higher- M_r region, but differs substantially in the lower- M_r region. Interestingly, a protein of M_r 42000 is more prominent in rat enzyme immunoprecipitates, suggesting that it is a genuine subunit. Presumably it is less readily dissociated by detergent treatment than is the bovine heart enzyme subunit. The enzyme precipitated from rat heart behaved identically with that from rat liver (results not shown). The rabbit enzyme (track 5) and the human enzyme (track 7) again behaved identically with Complex I in the high- M_r region, but the low- M_r region is quite different for each species. The rabbit enzyme immunoprecipitate, but not the human, contains the 42000- M_r polypeptide, further suggesting a species-dependent variability in its ease of dissociation. The human enzyme immunoprecipitate, in particular, contains proteins that are unlikely to be NADH dehydrogenase constituents and presumably arise from non-specific precipitation. This was an even more serious problem with several other species tested, and we were unable to obtain precipitates from these species that unambiguously resembled Complex I, even in the high- M_r region of the gels.

The antisera that had limited ability to immunoprecipitate the human, rabbit or rat enzymes reacted very weakly in immunoblotting experiments with submitochondrial particles from these species. Antiserum 10, however, showed stronger reaction, but with a limited number of polypeptides (Fig. 6). In these experiments with whole membrane fractions, proteolysis of proteins was a problem and could not always be prevented by proteinase inhibitors. This was particularly marked for rabbit submitochondrial particles (track 2). In addition, comparison of the bovine heart enzyme sample (track 4) with Complex I (Fig. 3) shows that the 75000- M_r protein in the former has been degraded to a polypeptide of apparent M_r 70000. The sensitivity of the 75000- M_r protein to proteolysis is discussed in greater detail below.

Major antigenic polypeptides in all species tested co-migrated with the 51000- M_r and 24000- M_r proteins of Complex I. These are the two larger subunits of the 'flavoprotein fragment' of Complex I (Galante & Hatefi, 1979; Ragan *et al.*, 1982a), and the presence of antibodies to these proteins in antiserum 10 was confirmed by immunoblotting of the isolated flavoprotein fragment (Fig. 6, track 5). These two subunits are both iron-sulphur proteins (Ragan *et al.*, 1982a), and the 51000- M_r polypeptide contains the NADH-binding site (Chen & Guillory, 1981). The comparable potency of antiserum 10 in agglutinating the rat liver and bovine heart enzymes (Figs. 1 and 4) and the cross-reaction of the antiserum with these two polypeptides in rabbit and human tissue suggests that antibodies to these proteins may be important in immunoprecipitation. On the basis of chemical-modification studies we had previously concluded that the 51000- M_r and 24000- M_r proteins were inaccessible from the surface of the enzyme (Smith & Ragan, 1980). However, the binding of NADH to the larger polypeptide (Chen & Guillory, 1981), and recent chemical-cross-linking studies (Cleeter *et al.*, 1985), clearly indicated that the 51000- M_r protein is to some extent exposed, a view that is supported by these immunological experiments.

Immunoblotting with subunit-specific antisera

Fractionation of Complex I by $(NH_4)_2SO_4$ after treatment with chaotropic agents and detergents has led to the isolation of several iron-sulphur proteins (Ragan *et al.*, 1982a,b). The 'flavoprotein fragment', as already mentioned above, contains two iron-sulphur proteins of M_r 51000 and 24000, and it is clear from Fig. 6, for example, that subunits of the same M_r are present in the other mammalian species. The 'iron-protein fragment' of Complex I has iron-sulphur centres associated with the 75000- M_r , 49000- M_r and 30000- M_r or

Fig. 5. Polypeptide composition of immunoprecipitated rat, rabbit and human NADH dehydrogenases

Immunoprecipitates were obtained by using antiserum 10 as described in the Methods section and Fig. 2 legend. Track 1, immunoprecipitate from 1.2 mg of bovine heart particles dissociated at 100°C; track 2, immunoprecipitate from 1.2 mg of rat liver particles dissociated at 100°C; track 3, as track 2, but dissociated at 40°C; track 4: Complex I (50 µg of protein); track 5, immunoprecipitate from 1.5 mg of rabbit liver particles dissociated at 40°C; track 6, Complex I (50 µg of protein); track 7, immunoprecipitate from 1.5 mg of human kidney particles dissociated at 100°C. The experiments on the rat enzyme (tracks 1, 2 and 3), the rabbit enzyme (tracks 4, 5) and the human enzyme (tracks 6, 7) were performed with different gels.

13000- M_r polypeptides (Fig. 1) (Ragan *et al.*, 1982b). The latter two subunits cannot be separated without loss of the iron-sulphur chromophore, so which subunit carries the centre is not known. We have purified these four proteins by preparative SDS/polyacrylamide-gel electrophoresis and used them for production of subunit-specific antibodies (Cleeter *et al.*, 1985). Immunoblotting of submitochondrial particles is shown in Fig. 7, where we used a mixture of all four antisera. Experiments with single antisera established that they were monospecific. The apparent exception is the antiserum to the 75000- M_r polypeptide, which in some instances reacted additionally with polypeptides migrating just ahead of the 49000- M_r polypeptide (chicken, rabbit; see also Fig. 6, track 2), just ahead of the 30000- M_r polypeptide (human, quail) or, in bovine heart, with one polypeptide of M_r 70000 (see also Fig. 6, track 4) and one

migrating rather more slowly than the 13000- M_r polypeptide. The extent of these reactions depended on the state of purification of the 75000- M_r polypeptide (i.e. whether in mitochondria, in Complex I, in the iron-protein fragment or in isolation) and the length of storage of the material, and was generally increased by omitting proteinase inhibitors during dissociation in SDS. We attribute these additional bands on the blot to proteolytic degradation of the 75000- M_r polypeptide both before and after SDS treatment. Proteolysis was greater in cruder material or on increased length of storage, and was clearly very species-dependent.

Apart from chicken and quail preparations, where proteolysis could not be prevented, all species contain a polypeptide homologous with the 75000- M_r bovine heart protein and of the same apparent M_r . Comparison of Fig. 7 with Fig. 5 suggests that immunoprecipitates, particularly of

Fig. 6. Immunoblotting of submitochondrial particles from different species with antiserum 10

Particles from the indicated species were electrophoresed, transferred on to nitrocellulose and treated with antiserum 10 (1:100 dilution). Antigen-antibody-complex formation was detected by using the peroxidase-linked assay. Track 1, rat liver (38 μ g of protein); track 2, rabbit liver (80 μ g of protein); track 3, human liver (63 μ g of protein); track 4, bovine heart (35 μ g of protein); track 5, flavoprotein fragment of Complex I (10 μ g of protein). Abbreviation: TH, NAD(P)⁺ transhydrogenase.

the rabbit enzyme, are not prone to proteolysis of this polypeptide, presumably because the offending proteinase has been removed before dissociation with SDS. The antiserum to the 49000- M_r bovine heart protein also identifies an homologous polypeptide of the same M_r in all species tested. This protein can clearly be seen in the immunoprecipitates of rat and rabbit enzymes (Fig. 5) but not that of the human kidney enzyme, since in the last-mentioned case this region of the gel is obscured by IgG heavy chains. Satisfactory gel profiles for the human enzyme were not obtained under the milder dissociation conditions employed for the other species.

The antisera to the 30000- M_r and 13000- M_r bovine heart proteins also identify homologous polypeptides in most other species, but these generally have rather different M_r values, as summarized in Table 1. These polypeptides are clearly visible in the immunoprecipitates shown in Fig. 5, and the human counterpart of the 13000- M_r protein can be seen in the blots shown in Fig. 6.

Table 1. Apparent M_r of iron-sulphur protein subunits. Subunits crossreacting with antisera to the bovine heart proteins of M_r 30000 and 13000 were identified by immunoblotting as in Fig. 7. Differences in M_r values were measured either from the immunoblots or from stained gels of immunoprecipitates (Fig. 5). Abbreviation: N.D., homologue not detected.

Species	$10^{-3} \times$ Apparent difference in M_r	
	Of 30000- M_r homologue	Of 13000- M_r homologue
Rabbit	-1.3	+0.2
Chicken	-2.5	+1.0
Pig	0	+1.0
Rat	-2.0	+0.2
Human	-2.0	+0.3
Quail	-2.5	N.D.
Mouse	-2.5	N.D.

Discussion

The large number of polypeptides found in isolated bovine heart NADH dehydrogenase (Complex I) has inevitably raised doubts about the purity of the preparation, despite the consistency of the subunit composition. In previous work we used anti-holoenzyme and antiserum to the iron-protein fragment to immunoprecipitate the bovine heart enzyme from solubilized mitochondria and submitochondrial particles and had concluded that all the subunits present are indeed part of the Complex (Heron *et al.*, 1979; Smith & Ragan, 1980). In the present work, we have further substantiated this conclusion.

Immunoprecipitates generated by antisera that react with very few polypeptides have been analysed on highly resolving SDS/polyacrylamide gels and appear to be effectively identical with isolated Complex I.

Direct immunoprecipitation of the enzyme from other mammalian species has revealed a similarly complicated polypeptide profile. The antisera used react in some instances with a very limited number of subunits, providing further evidence that all the polypeptides found are constituents of the enzyme.

The polypeptide profiles for all mammalian species tested are very similar in the high- M_r region, and homology of several of these proteins was directly demonstrated by immunoblotting with subunit-specific antisera or anti-holoenzyme sera of limited polypeptide specificity. This is reminiscent of the behaviour of cytochrome *c* oxidase (Kadenbach & Merle, 1981) and can be explained in the same way. The larger subunits of NADH dehydrogenase contain most, if not all, of the redox groups of the enzyme, and strong

Fig. 7. Immunoblotting of submitochondrial particles from different species with subunit-specific antisera. Conditions were as in Fig. 6 legend except that a mixture of antisera to the 75000- M_r , 49000- M_r , 30000- M_r and 13000- M_r polypeptides (each at 1:100 dilution) was used. Antigen-antibody-complex formation was detected by using ^{125}I -labelled protein A and autoradiography. Track 1, bovine heart preparation; track 2, rabbit liver preparation; track 3, chicken liver preparation; track 4, pig liver preparation; track 5, rat liver preparation; track 6, human heart preparation; track 7, quail liver preparation; track 8, mouse liver preparation. Each track contained 50 μg of protein.

conservation of sequence would therefore be expected. Indeed, the immunological cross-reactivity of some of the subunit-specific antisera raised against iron-sulphur proteins extends to plants (Cottingham *et al.*, 1984), insects (F. G. P. Earley & C. I. Ragan, unpublished work) and *P. denitrificans* (C. L. George, S. J. Ferguson, M. W. J. Cleeter & C. I. Ragan, unpublished work). Most of the smaller subunits of NADH dehydrogenase do not carry redox groups and we have suggested that their role is purely structural (Ragan, 1980). Greater variability between species might therefore be expected, and indeed cross-reaction with antiserum to the 13000- M_r polypeptide is very weak or undetectable in non-mammalian species. However, the differences in the smaller polypeptides of the mammalian enzymes might be more apparent than real. The demonstration that six of the so-called unidentified reading frames of the human mitochondrial genome, including the very small URF 3 and URF 4L, code for NADH dehydrogenase subunits (Chomyn *et al.*, 1985) indicates that further homologies undoubtedly exist and that these encompass not only mammalian species but insects, filamentous fungi and protozoa (see, e.g., Grivell, 1983).

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