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Photolabelling of a mitochondrially encoded subunit of NADH dehydrogenase with [^{3}H]dihydrorotenone

Fergus G.P. Earley, Salil D. Patel, C. Ian Ragan and G. Attardi*

Department of Biochemistry, University of Southampton, Southampton, SO9 3TU, England and **Division of Biology, California Institute of Technology, Pasadena, CA 91125, USA*

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Mitochondrial NADH dehydrogenase from bovine heart was photolabelled with the inhibitor [^{3}H]dihydrorotenone. A constituent of the hydrophobic domain of the enzyme of M_r 33000 was the major site of labelling. The identity of this protein with the mitochondrially encoded ND-1 gene product was established by immunoblotting and immunoprecipitation with an antiserum raised to the expected C-terminal sequence of the human ND-1 gene product.

NADH dehydrogenase; Mitochondrial genome; Rotenone; ND-1 gene product

1. INTRODUCTION

Seven of the reading frames of the mammalian mitochondrial genome have been shown recently to encode subunits of the respiratory chain NADH dehydrogenase [1,2]. The putative amino acid sequences of these proteins are available for several species, not only mammalian (e.g. [3]), but interspecies comparisons have not suggested any obvious role for these proteins in NADH dehydrogenase function despite regions of sequence which are strongly conserved [3]. For example, none of these ND gene products contain sufficient numbers of conserved cysteines to be iron-sulphur proteins [3] while the FMN-containing subunit of the enzyme is almost certainly nuclear-encoded [3]. However, the sequences are mostly hydrophobic and suggest the presence of several transmembrane α-helical regions. It is highly likely therefore that some of these subunits are in direct contact with the bilayer lipid and might therefore serve to bind hydrophobic ligands which partition into the hydrocarbon region of the membrane. Possible ligands are the substrate, ubiquinone-10, and inhibitors such as rotenone and piericidin which are believed to act very close to the site of ubiquinone-10 reduction [4].

In previous work we had attempted to identify the NADH dehydrogenase subunit involved in rotenone binding by photoaffinity labelling. A photoactivatable rotenone analogue, arylazidoamorphigenin, specifically labelled a protein of M_r 33000 in purified beef heart NADH dehydrogenase (complex I) [5]. The low specific radioactivity of the arylazidoamorphigenin hampered further studies, but here we show that this protein can be photolabelled directly by [^{3}H]dihydrorotenone to a much higher specific activity. Using an antibody directed against the C-terminal sequence of the human ND-1 product [6] we also show that this rotenone-binding protein is mitochondrially encoded.

Correspondence (present) address: Merck Sharp and Dohme Research Laboratories, Neuroscience Research Centre, Eastwick Road, Harlow CM20 2QR, England

2. MATERIALS AND METHODS

Complex I was prepared as in [7]. Inhibition of

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NADH-ubiquinone-1 reductase activity by rotenone or dihydrorotenone was measured as in [8]. Photolysis and determination of protein-bound radioactivity were carried out as in [8]. Gel electrophoresis was carried out in 12–16% (w/v) polyacrylamide gradient gels using the Laemmli buffer system [9] unless otherwise indicated. M_r standards were from BDH Chemicals (Poole, England). Radioactivity in gels was determined by scintillation counting of slices [5], or by fluorography using Amplify (Amersham International, Bucks, England) as scintillant and preflashed Kodak X-Omat AR film at −70°C. Immunoblotting on nitrocellulose was carried out using the streptavidin-biotin system (Amersham International) for detection. For description of the immunoprecipitation procedure refer to the legend to fig.4.

Dihydrorotenone was synthesized by hydrogenation of rotenone in ethyl acetate using 10% Pd/C as catalyst. [^{3}H]Dihydrorotenone (spec. act. 58 Ci/mmol) was synthesised by Amersham International in the same way. The crude material (3 mCi) was dried down under N_2, dissolved in 100 μl acetonitrile/H_2O (60:40, v/v) and applied to a Waters Resolve reverse-phase C_{18} column, developed with the same solvent and monitored at 295 nm. The major absorbing species had a retention time identical to that of unlabelled dihydrorotenone. This material was collected and diluted with cold dihydrorotenone to give final specific activities as indicated.

3. RESULTS

The possibility that rotenone would photolabel its binding site was suggested by the presence in the structure of an arylketone (rings C and D of fig.1), a group which forms a biradical upon activation by UV light [10] and which has been used as a basis for photolabelling reagents. To obtain a radiolabelled derivative, [^{3}H]dihydrorotenone was produced by reduction of the isopropenyl side chain. Dihydrorotenone inhibited the NADH-ubiquinone-1 reductase activity of complex I with the same potency as rotenone, e.g. 50% inhibition at 0.35 nmol/mg protein [5].

UV irradiation of [^{3}H]dihydrorotenone in the presence of complex I gave a time-dependent incorporation of label into protein which, under our conditions, began to saturate after 20 min of il-

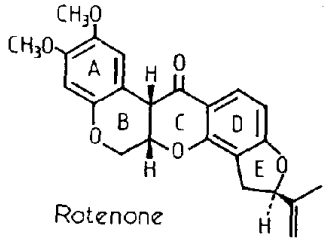

Fig.1. Structure of rotenone.

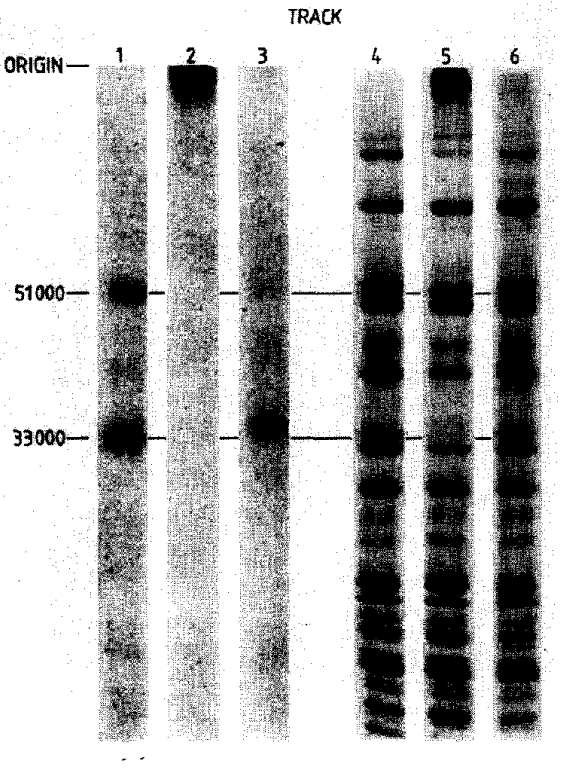

Fig.2. Photolabelling of complex I with [^{3}H]dihydrorotenone. Complex I (3 mg/ml in 0.67 M sucrose/50 mM Tris-HCl, pH 8.0) was incubated with [^{3}H]dihydrorotenone (1 nmol/mg protein, spec. act. 3 Ci/mmol) for 15 min on ice and then photolysed for 30 min either under argon (tracks 1, 2, 4, 5) or in air (tracks 3, 6). Samples were dissociated in SDS either at 20°C (tracks 1, 3, 4, 6) or 100°C (tracks 2, 5), prior to electrophoresis (150 μg protein/track) and fluorography. Tracks 1–3, fluorograph; 4–6, Coomassie blue-stained gel.

lumination. Photolysis times were routinely varied between 30 and 45 min. Analysis by SDS gel electrophoresis and fluorography of complex I labelled with 1 mol inhibitor/mol enzyme revealed substantial labelling of the 33 kDa polypeptide (fig.2, tracks 3,6) which had been shown in previous work to be preferentially photolabelled by arylazidoamorphigenin [5]. In addition to the 33 kDa subunit, some larger (51 and 42 kDa) and smaller subunits were also labelled, but to a much lesser extent. When the complex I sample was dissociated in SDS at 100°C (fig.2, track 2), extensive aggregation of the labelled material took place and nearly all radioactivity was found at the origin. Dissociation of complex I at 100°C also resulted in the loss of the 33 kDa protein (track 5) presumably as a result of aggregation. When photolabelling was carried out under anaerobic conditions, more extensive labelling of the protein of 51 kDa was obtained (track 1). It seems likely

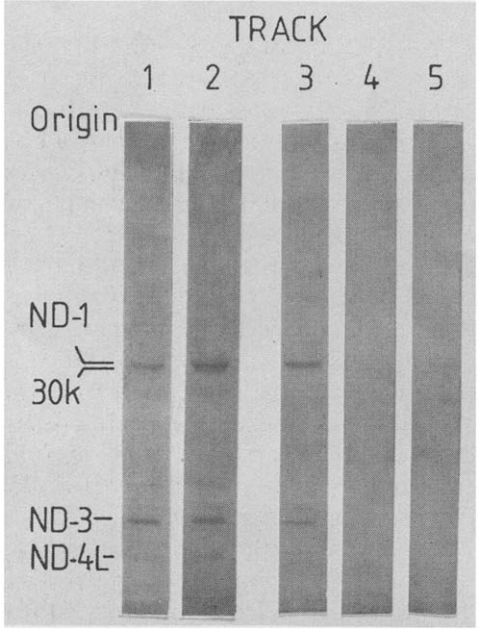

Fig.3. Immunoblotting of complex I and subfractions. Proteins were separated by SDS gel electrophoresis and transferred to nitrocellulose. Immunoblotting was with a mixture of antibodies to the ND-1, 3 and 4L products (each diluted 100-fold) or, additionally, in track 2, with antiserum to the 30 kDa protein of complex I (1:100). Complex I was resolved with $NaClO_4$ (e.g. [11]) and separated into flavoprotein, iron protein and hydrophobic protein fractions. Tracks: 1,2, complex I (100 μg protein); 3, hydrophobic protein (70 μg protein); 4, iron protein (30 μg protein); 5, flavoprotein (10 μg protein).

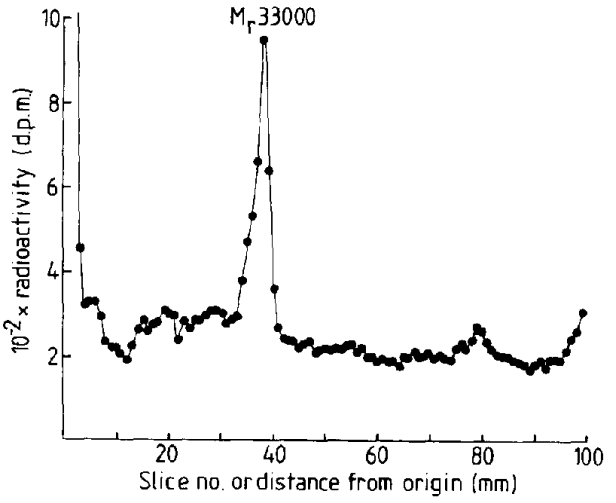

Fig.4. Immunoprecipitation of labelled ND-1 product. Complex I (2 mg protein/ml) was incubated with [^{3}H]dihydrorotenone (0.4 nmol/mg, 58 Ci/mmol) for 10 min followed by photolysis for 30 min under argon at 0°C. The following method was adapted from that of Anderson and Blobel [12]. The protein (100 μg) was solubilized by addition of 5 μl of 25% (w/v) SDS and 200 μl of a mixture containing 1.25% (w/v) Triton X-100, 190 mM NaCl, 60 mM Tris-HCl, pH 7.6, 6 mM EDTA. Normal serum (10 μl) was added and incubated for 4 h at 4°C. Fixed *S. aureus* (100 μl of a 10% suspension) was added and mixed for 1 h at room temperature. After centrifugation, the supernatant was treated with 50 μl anti-ND-1 product antiserum for 16 h at 4°C and then absorbed with 500 μl of 10% *S. aureus* for 1 h at room temperature. The *S. aureus* was washed three times with 1 ml of a mixture containing 145 mM NaCl, 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.1% (w/v) Triton X-100, 0.02% (w/v) SDS by centrifugation and resuspension. After a further wash in detergent-free buffer, the cells were suspended in 50 μl of 0.5 M Tris-HCl, pH 6.8, 50 μl 10% (w/v) SDS and 50 μl of 10% (v/v) 2-mercaptoethanol and incubated at 37°C for 40 min. The supernatant fraction was separated by electrophoresis on a 12.5% polyacrylamide cylindrical gel (11 cm × 5 mm i.d.) using the buffer system described in [13]. After staining and destaining, the gel was sliced into 1 mm slices for scintillation counting. The Weber and Osborne [13] buffer system provides excellent separation of the 33 and 30 kDa polypeptides [11]; a parallel gel pattern of complex I was used as a molecular mass marker.

that labelling of this protein was suppressed by oxygen which is a radical scavenger. From similar experiments in which the gel was sliced and counted for radioactivity, it was established that the recovery of radioactivity on the gel was only 5–10% of that added to the enzyme. The low degree of incorporation of the label was a problem encountered in the related work with arylazidoamorphigenin [5].

The 33 kDa protein is a constituent of the hydrophobic domain of complex I which is associated with the membrane lipid [3,11]. This property is shared with the ND-1 gene product. As shown in fig.3, immunoblotting with antiserum to the C-terminal undecapeptide of the human ND-1 product specifically identifies the ND-1 product as a protein of 33 kDa (track 1), distinct from the closely migrating subunit of 30 kDa (track 2) and located exclusively in the hydrophobic domain of the enzyme (tracks 3–5). Moreover, the ND-1 product is aggregated following dissociation of complex I in SDS at 100°C (not shown).

Definite proof of the identity of the rotenone-labelled protein with the ND-1 gene product was obtained from the experiment of fig.4. Complex I, photolabelled to a high specific activity with [^{3}H]dihydrorotenone, was dissociated in SDS and Triton and the ND-1 product immunoprecipitated by addition of antiserum and fixed *S. aureus*. Analysis by SDS gel electrophoresis revealed a major radiolabelled species of 33 kDa, and a minor species of much lower molecular mass which might be a proteolytic breakdown product.

4. DISCUSSION

The product of the mitochondrial gene ND-1 (previously URF-1) was first identified by immunoprecipitation from labelled HeLa cell mitochondria as a protein of apparent M_r 24000 on SDS-urea gels as estimated relative to water-soluble marker proteins [6]. The subsequent assignment of this and other mitochondrial gene products to NADH dehydrogenase [1,2] has enabled us to identify this protein directly in the purified bovine heart enzyme by immunoblotting. The undecapeptide used as antigen differs by only three amino acids from the equivalent bovine sequence [14,15]. The M_r of 33000, determined using the Laemmli buffer system, is very close to the value of 35675 predicted from the amino acid sequence [15].

Our previous conclusion from using arylazidoamorphigenin that the 33 kDa subunit was the site of rotenone binding was qualified by the low recovery of label on the SDS gel [5]. While the same problem applies to the use of dihydrorotenone, the labelling of the same subunit by two photolabels whose structure, site of photoactivation and photochemistry are quite different, provides much more compelling evidence in favour of our original proposal. Dihydrorotenone seems rather less specific than arylazidoamorphigenin; however, the appearance of label in other polypeptides need not be ascribed only to nonspecific binding but rather it may reflect the participation of other proteins in forming the rotenone-binding site.

The identification of seven mitochondrial gene products as subunits of NADH dehydrogenase has been hitherto rather indirect. The parallel immunoprecipitation of these proteins by antiserum to complex I [1] or the 49 kDa iron-sulphur protein subunit [2] and the demonstration by immunoblotting of three of these proteins (the ND-1, 3 and 4L products) in isolated complex I could be ascribed to their fortuitous association with the enzyme. However, the immunoprecipitation by a monospecific antiserum of a protein labelled with a dehydrogenase inhibitor provides direct evidence that the ND-1 gene product is a dehydrogenase subunit and, moreover, one which may be intimately involved in the enzyme's function. It may be significant, in this connection, that ND_1 is the most conserved evolutionarily among the mitochondrially encoded subunits of NADH dehydrogenase [16], and that its gene has been found in all mitochondrial genomes so far analysed from organisms which exhibit site 1 energy coupling [17].

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REFERENCES

[1] Chomyn, A., Mariottini, P., Cleeter, M.W.J., Ragan, C.I., Matsuno-Yagi, A., Hatefi, Y., Doolittle, R.F. and Attardi, G. (1985) Nature 314, 592–597.

[2] Chomyn, A., Cleeter, M.W.J., Ragan, C.I., Riley, M., Doolittle, R.F. and Attardi, G. (1986) Science 234, 614–618.

[3] Ragan, C.I. (1987) Curr. Top. Bioenerg., in press.

[4] Gutman, M., Coles, C.J., Singer, T.P. and Casida, J.E. (1971) Biochemistry 10, 2036–2043.

[5] Earley, F.G.P. and Ragan, C.I. (1984) Biochem. J. 224, 525–534.

[6] Chomyn, A., Mariottini, P., Gonzalez-Cadavid, N., Attardi, G., Strong, D.D., Trovato, D., Riley, M. and Doolittle, R.F. (1983) Proc. Natl. Acad. Sci. USA 80, 5535–5539.

[7] Hatefi, Y. and Rieske, J.S. (1967) Methods Enzymol. 10, 235–239.

[8] Earley, F.G.P. and Ragan, C.I. (1980) Biochem. J. 191, 429–436.

[9] Laemmli, U.K. (1970) Nature 227, 680–685.

[10] Hammond, G.S., Baker, W.P. and Moore, W.M. (1961) J. Am. Chem. Soc. 83, 2795–2799.

[11] Smith, S. and Ragan, C.I. (1980) Biochem. J. 185, 315–367.

[12] Anderson, D.J. and Blobel, G. (1983) Methods Enzymol. 96, 111–120.

[13] Weber, K. and Osborne, M. (1969) J. Biol. Chem. 244, 4406–4412.

[14] Anderson, S., Bankier, A.T., Barrell, B.G., De Bruijn, M.H.L., Coulson, A.R., Drouin, J., Eperon, I.C., Nierlich, D.P., Roe, B.A., Sanger, F., Schreier, P.H., Smith, A.J.H., Staden, R. and Young, I.G. (1981) Nature 290, 457–465.

[15] Anderson, S., De Bruijn, M.H.L., Coulson, A.R., Eperon, I.C., Sanger, F. and Young, I.G. (1982) J. Mol. Biol. 156, 683–717.

[16] Attardi, G. (1985) Int. Rev. Cytol. 93, 93–145.

[17] Attardi, G., Chomyn, A., Doolittle, R.E., Mariottini, P. and Ragan, C.I. (1986) Cold Spring Harbor Symp. Quant. Biol. L1, 103–114.