Photoaffinity labelling of mitochondrial NADH dehydrogenase with ary lazidoamorphigenin, an analogue of rotenone

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A photoaffinity-labelling analogue of the respiratory inhibitor rotenone was synthesized from the naturally occurring rotenoid amorphigenin. The analogue inhibits NADH-ubiquinone oxidoreductase activity at concentrations comparable with those of rotenone. Photolysis of the radiolabelled analogue bound to isolated NADH-ubiquinone oxidoreductase resulted in preferential incorporation of radioactivity into a polypeptide of $M_r$ 33000, particularly at low concentrations of the inhibitor. Preparations of the enzyme differ in a parallel fashion in the content of this polypeptide, the degree of photolabelling by the analogue and their sensitivity to rotenone, providing further evidence that the 33000-$M_r$ protein forms part of the rotenone-binding site.

Mitochondrial oxidation of NADH is specifically inhibited by two naturally occurring compounds, rotenone (Lindahl & Öberg, 1961) and piericidin (Hall et al., 1966). These compounds inhibit at concentrations stoichiometric with the enzyme and are characterized by a high affinity for their binding site(s) (Singer & Gutman, 1971). Both types of inhibitor are believed to act at or very close to the site of reduction of ubiquinone, the acceptor of NADH dehydrogenase, since the flavin and iron–sulphur groups of the enzyme are fully reduced by NADH in the presence of these compounds (Gutman & Singer, 1970). It is believed that inhibitors of redox reactions involving quinones (e.g. antimycin, myxothiazol etc.) do so by displacing the fully reduced or oxidized quinol from its binding site on the enzyme (Crofts & Wraight, 1983). It is very likely that rotenone and piericidin exert their inhibitory effects in the same way, and direct competition between ubiquinone and piericidin has been demonstrated (Gutman et al., 1971). Thus the identification of the binding site for rotenone or piericidin may also identify the binding site for ubiquinone on NADH dehydrogenase. Unfortunately, despite the high affinity of rotenone and, particularly, piericidin, for mammalian NADH dehydrogenase, the binding of these compounds is not covalent. We have therefore synthesized an analogue of rotenone, ary lazido-

amorphigenin, designed to modify the binding site covalently by photoaffinity labelling. The present paper describes the synthesis and properties of this compound and the results obtained from photoaffinity labelling of NADH-ubiquinone oxidoreductase (EC 1.6.99.3) from ox heart mitochondria.

Materials and methods

Enzyme preparations

NADH-ubiquinone reductase (Complex I in the nomenclature of Hatefi et al., 1962) was prepared by the method of Hatefi & Rieske (1967). Resolution of the enzyme by NaClO$_4$ was performed as described by Smith & Ragan (1980).

Isolation of amorphigenin

The method was based on the small-scale isolation procedure of Crombie et al. (1973). Seeds of *Amorpha fruticosa* (Thomson and Morgan, Ipswich, U.K.) were washed, soaked in NaOCl solution (10–14% free Cl$_2$) for 10 min, rinsed with water and heated in distilled water at 60°C for 10 min. The drained seeds were left for 10 days in the dark on constantly moist filter paper in covered plastic trays. The resulting seedlings (5–10 cm tall) were frozen at −20°C.

The seedlings (150 g wet wt.) were homogenized in a blender with 250 ml of ethanol and 150 ml of water. The homogenate was poured into 500 ml of

Abbreviation used: SDS, sodium dodecyl sulphate.
boiling ethanol, refluxed for 10 min and filtered. The residue was extracted twice with 200 ml of hot ethanol and filtered, and the extracts were combined. After evaporation of the solution to near-dryness, 250 ml of water was added and the mixture was extracted three times with 50 ml of diethyl ether. The ether extracts were dried over anhydrous Na₂SO₄ and the ether was removed by evaporation. The residue was dissolved in 3 ml of chloroform and applied to a column (24 cm long x 2.5 cm internal diam.) of silica gel (Kieselgel 60, 70–230 mesh; from Merck, Darmstadt, W. Germany) equilibrated with chloroform. The column was developed with benzene/chloroform (1:1, v/v), and 50 ml fractions were collected after the elution firstly of a yellow fraction and secondly of a greeny-brown fraction. The progress of the elution was followed by t.l.c. on silica-gel sheets containing a bound fluorophor (Schleicher und Schüll, Dassel, W. Germany). The solvent was chloroform/propan-2-ol (19:1, v/v). Spots were detected by short-wavelength u.v. light or staining with I₂ vapour. Rotenoids were detected by their blue colour after spraying with HI reagent (Delfel, 1966). Two rotenoids were found, the major one being eluted as a single spot in fractions 3–8 (RF 0.47) and co-migrating with authentic amorphigenin. These fractions were pooled and evaporated to dryness, and the residue was crystallized from benzene as white needles. The yield was 130 mg, and n.m.r. and i.r. spectra of the product were identical with published spectra for amorphigenin (Claisse et al., 1964).

Synthesis of arylazido-β-alanine

4-Fluoro-3-nitrophenyl azide was synthesized as described by Fleet et al. (1972) from 4-fluoro-3-nitroaniline. Arylazido-β-alanine [3-amino-N-(4-azido-3-nitrophényl)propanoic acid] was synthesized by the method of Guilory & Jeng (1977) from β-alanine (1.07 g) and 4-fluoro-3-nitrophényl azide (1.8 g). The yield was 1.33 g (54%) and the compound appeared pure by t.l.c. on silica with water-saturated butan-1-ol and ethyl acetate/light petroleum (b.p. 40–60°C)/acetic acid (25:25:1, by vol.) as solvents.

Synthesis of arylazido-β-[3H]alanine

β-[3H]Alanine (32 Ci/mmol) was obtained from Amersham International, Amersham, Bucks., U.K. Arylazido-β-[3H]alanine was synthesized from β-[3H]alanine (5 mCi), β-alanine (22.3 mg) and 4-fluoro-3-nitrophényl azide (145 mg) as described above. The yield was 18 mg (29%) and the specific radioactivity was 20 Ci/mol.

Synthesis of aryliazidoamorphigenin

Arylazido-β-alanine (0.2 mmol) and carbonyldimidazole (0.22 mmol) were dissolved in 4 ml of dry tetrahydrofuran and allowed to react for 1 h at room temperature under dry N₂. Amorphigenin (0.2 mmol) was added, followed by imidazolylsodium (0.2 mmol in 0.3 ml of tetrahydrofuran) made by dissolving Na in tetrahydrofuran containing excess imidazole. After 15 min at room temperature the reaction was stopped with 4 ml of ice-cold 0.5 M HCl. The reaction mixture was extracted with chloroform, and the extracts were washed with NaHCO₃ solution, water and then dried over anhydrous Na₂SO₄. T.l.c. on silica gel, with benzene/methanol (19:1, v/v) as solvent, showed very little free amorphigenin (RF 0.21). Of the major reaction products (RF 0.55 and RF 0.62), that with RF 0.55 predominated and was isolated by preparative t.l.c. on silica gel (Kiesel-gel 60PF₂₅₄ from Merck) with benzene/methanol (19:1, v/v) as solvent. Crystallization from chloroform/methanol gave aryliazidoamorphigenin (0.05 mmol, 25% yield).

The structure was confirmed by 1H n.m.r. (100.1 MHz). All resonances attributed to amorphigenin (Claisse et al., 1964) were observed unchanged, except for those of the hydroxy proton, which disappeared, and the 8'-methylene protons (see Scheme 1), shifted downfield to τ 5.26, as expected, after esterification. Other resonances, assigned by comparison with the spectrum of arylazido-β-alanine, were as follows: τ 1.91 (broad singlet, N–H); τ 2.14 (doublet, J = 2 Hz, e-H); between τ 2.7 and 3.22 (multiplets, γ-H and δ-H); τ 6.36 (quartet, two β-H) and τ 7.29 (triplet, two α-H).

Arylazido[3H]amorphigenin was synthesized from 0.072 mmol of arylazido-β-[3H]alanine by the same method.

Binding of inhibitors to Complex I

Complex I was diluted to 2–4 mg of protein/ml with 0.67 M sucrose/50 mM Tris/HCl buffer, pH 8.0, at 4°C. The inhibitors rotenone or amorphigenin were dissolved in ethanol and added to the Complex I to give a final ethanol concentration of 5% (v/v). Samples were incubated at 4°C for at least 5 h before the assay.

Arylazidoamorphigenin was dissolved in dimethyl sulphoxide or tetrahydrofuran, since it is sparingly soluble in ethanol or methanol, and added to the Complex I to give a final organic solvent concentration of at most 2% (v/v). Samples were pre-incubated in the dark for up to 20 h or as specified in the text before assay or photolysis.

Photolabelling of Complex I

Continuously stirred samples in 1 cm-light-path plastic semi-micro cuvettes were exposed to a 125 W high-pressure mercury-vapour lamp (Thorn Electrical, Southampton, U.K.) at a distance of
30 cm for six 30 s bursts interspersed with 30 s dark periods or as indicated in the text.

**Determination of protein-bound radioactivity**

Portions containing 0.2 mg of Complex I protein were mixed with 0.5 ml of ice-cold 10% (w/v) trichloroacetic acid and left on ice for 15 min. Precipitated protein was collected by suction on glass-fibre filters (Whatman GF/C) pre-wetted with 5% (w/v) trichloroacetic acid. The filters were washed successively with 5 ml of 5% trichloroacetic acid, acetone, ethanol/diethyl ether (1:1, v/v) and diethyl ether. When they were dry, they were wetted with 75 µl of 1% (w/v) SDS and incubated in scintillation vials with 0.5 ml of NCS solubilizer (BDH Chemicals, Poole, Dorset, U.K.) for 5 h at 50°C. When they had cooled, 10 ml of a mixture containing 6 g of 2,5-diphenyloxazole and 75 mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene per litre of toluene was added and radioactivity was determined by scintillation counting. Counting efficiency was 27%.

**Gel electrophoresis**

SDS/polyacrylamide-gel electrophoresis was carried out in 11 cm cylindrical gels containing 12.5% (w/v) acrylamide and 0.34% (w/v) bisacrylamide as otherwise described by Weber & Osborn (1969). Alternatively, samples were run on 12 cm-long x 1.5 mm-thick slab gels containing a concave gradient of acrylamide between 13% (w/v) and 16% (w/v) (Earley & Ragan, 1981). Gels were stained and destained by the method of Weber & Osborn (1969). Mf values were determined from the cylindrical-gel electrophoretograms by comparison with standard markers (BDH Chemicals).

**Determination of radioactivity in gels**

For each determination, five identical cylindrical gels were used, each loaded with 150 µg of labelled Complex I protein. Sections (1 mm) of destained gels were cut with a gel slicer (Mickle Engineering Co., Gomshall, Surrey, U.K.), and corresponding slices were pooled and incubated in scintillation vials with 0.8 ml of NCS solubilizer at 50°C overnight. When they had cooled, 10 ml of toluene-based scintillant was added to each and radioactivity determined by scintillation counting.

**Assays**

NADH-ubiquinone oxidoreductase and NADH-K3Fe(CN)6 oxidoreductase activities were measured as described by Ragan (1976). NADH-juglone (5-hydroxy-1,4-naphthoquinone) oxidoreductase and NADH-menaquinone oxidoreductase activities were measured as described by Ragan & Bloxham (1977). Protein was measured by the method of Lowry et al. (1951), with bovine serum albumin (fraction V; Sigma Chemical Co.) as a standard. Flavin in Complex I solutions was released by boiling and assayed spectrophotometrically (Ragan et al., 1982b). Molarities of Complex I solutions were calculated assuming 1 mol of flavin (FMN)/mol of enzyme.

**Immunoprecipitation of Complex I**

Anti-(Complex I) serum was provided by M. W. J. Cleeter, of this department. Complex I was solubilized in Triton X-100 and immunoprecipitated as described by Heron et al. (1979).

**Results**

**Synthesis of aryldiazoidoamorphigenin**

Amorphigenin differs from rotenone by the presence of a hydroxy group in the substituent on the E ring (Fig. 1). Modifications to this region of the rotenoid structure do not seem to affect inhibitory potency (F. G. P. Earley & C. I. Ragan, unpublished work), and the hydroxy group provides a relatively straightforward means of synthesis of a photoaffinity analogue as shown in Scheme 1. Esterification of a hydroxy group by aryldiazio-β-alanine is the method used to synthesize photoaffinity analogues of NAD+ and NADP+ (e.g. Guillory & Jeng, 1977). With use of the carbonyl-di-imidazole alone, the esterification proceeded slowly and resulted in several reaction products. However, with imidazolysodium present (Staab, 1962) the reaction proceeded rapidly to completion and only two products were formed, the major one of which was aryldiazioamorphigenin.

The u.v.-visible-absorption spectrum of aryldiazioamorphigenin is shown in Fig. 2(a). Subtraction of the spectrum of aryldiazio-β-alanine to eliminate the 450 nm peak resulted in a spectrum (Fig. 2a) that is very similar to that of amorphigenin.
Scheme 1. Synthesis of arylazidoamorphigenin
See the text for further details. Abbreviations: CDI, carbonyldi-imidazole; THF, tetrahydrofuran.

Inhibitory properties of arylazidoamorphigenin in the absence of light

Arylazidoamorphigenin was a potent inhibitor of NADH-ubiquinone reductase activity of Complex I, comparable with rotenone or amorphigenin (see below). The extreme insolubility of the analogue may explain the slow kinetics of inhibition (Fig. 3).

The reduction of \( K_3Fe(CN)_6 \) or juglone by NADH was completely insensitive to arylazidoamorphigenin over the concentration range of Fig. 3, and the reduction of menadione was inhibited to a maximum of only 20%. The analogue therefore resembles rotenone in its effects and differs from another inhibitor of Complex I, diphenyleneiodonium, which inhibits menadione reductase activity (Ragan & Bloxham, 1977).

Photolabelling of Complex I

After incubation of Complex I with arylazido[\(^3\)H]amorphigenin in the dark, photolysis resulted in the incorporation of label into protein. The incorporation was nearly maximal after a 3min period of illumination, which was therefore adopted as standard; no incorporation was found in the absence of light. Between 20 and 40% of the starting radioactivity became protein-bound. Photolysis of the uninhibited enzyme for the standard period of 3min did not lead to any inactivation of the enzyme. Moreover, photolysis of enzyme that had been inhibited to various extents by pre-incubation with arylazidoamorphigenin did not lead to any lesser or greater extent of inhibition.

Incorporation of radioactivity into protein was nearly linearly related to the amount of added inhibitor over the range 0.25–2 mol of arylazidoamorphigenin/mol of Complex I. Since there was very little tendency of incorporation to saturate, we conclude that, in addition to specific binding of the
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Complex I was incubated with arylazidoamorphigenin for 40 min (○), 5 h (▲) or 24 h (▲) in the dark and assayed for NADH–ubiquinone oxidoreductase activity as described in the Materials and methods section. The line at 90% shows the maximum inhibition obtained with rotenone under the same assay conditions.

Table 1. Photolabelling of Complex I

<table>
<thead>
<tr>
<th>Labelling conditions</th>
<th>Radioactivity incorporated into protein (% of that added)</th>
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<tbody>
<tr>
<td>Arylazidoamorphigenin</td>
<td>39.2</td>
</tr>
<tr>
<td>Arylazidoamorphigenin + glutathione</td>
<td>21.3</td>
</tr>
<tr>
<td>Arylazido-β-alanine</td>
<td>12.6</td>
</tr>
<tr>
<td>Arylazido-β-alanine + glutathione</td>
<td>1.4</td>
</tr>
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These results indicate that the major sites of labelling are different for the two compounds and that labelling with the inhibitor analogue occurs predominantly in a hydrophobic environment, inaccessible to glutathione.

The location of the binding site(s) was further investigated by resolution of the inhibited enzyme with the chaotropic agent NaClO₄ (Galante &
Hatefi, 1979; Heron et al., 1979). This leads to solubilization of 30% of the protein, including the FMN and most of the constituent iron–sulphur centres (Ragan et al., 1982a,b). The solubilized material may be separated into two fractions, namely the flavoprotein fraction and the iron–protein fraction (Hatefi & Stempel, 1969; Ragan et al., 1982a,b). The insoluble residue contains the more hydrophobic proteins and phospholipids and all the proteins that are in contact with the apolar region of the membrane (Earley & Ragan, 1980, 1981). When Complex I that had been treated in the dark with 1 mol of arylazidomorphigenin/mol of enzyme was resolved with NaClO₄, 74% of the radioactivity was recovered bound to protein. Of this, 83% was associated with the insoluble residue and only 9% and 8% was associated with the flavo-protein and iron–protein fractions respectively. This small labelling of the soluble material may have resulted either from non-specific binding of the inhibitor to the Complex I or from non-specific transfer of inhibitor during the resolution process.

Gel electrophoresis of labelled Complex I

Fig. 4 shows the distribution of radioactivity between Complex I polypeptides after photolabelling with arylazidomorphigenin (1 nmol/mg of protein). Nearly all the radioactivity was associated with a major polypeptide, of Mr, 33000. This protein is known to fractionate into the insoluble residue from NaClO₄ resolution (Heron et al., 1979). However, the recovery of radioactivity in this protein was only 4% of that added to the enzyme or 16% of that which was protein-bound after photolysis. The small amounts of radioactivity associated with other proteins cannot account for the discrepancy, and it is clear that the protein-bound radioactivity is unstable during electrophoresis. Staining and destaining of the gel had no effect on the incorporation of radioactivity into the 33000-Mr protein, but the label was partially lost when the protein was dissolved in SDS and 2-mercaptoethanol. In one experiment, protein-bound radioactivity decreased by 30% after only 1 h at room temperature in 1% (w/v) SDS/1% (v/v) 2-mercaptoethanol. The radioactive profile was little affected by dissociating the sample at 20°C instead of 100°C, or by using N-ethylmaleimide in place of 2-mercaptoethanol.

Fig. 5 shows the incorporation achieved with higher concentrations of the photolabel (2 nmol and 10 nmol/mg of protein). Although labelling of the 33000-Mr polypeptide did not increase proportionally (i.e. it becomes saturated), labelling of other proteins can be seen, particularly at the highest concentration of analogue, where there is little specificity to the incorporation. Fig. 5 also shows that rotenone, in a 10-fold molar excess over arylazidomorphigenin, led to a 50% decrease in the extent of labelling of the 33000-Mr protein. Incorporation into the 33000-Mr protein depended strongly on the particular preparation of Complex I used, and varied from 0.01 to 0.04 nmol/mg of enzyme when labelling was carried out with 1 nmol of analogue/mg of protein. This might have been due to slight conformational differences between preparations, resulting in a different affinity, geometry or specificity of binding of the photolabel. However, these variations also correlated with differences in the polypeptide profiles on gels and in the dependence of inhibition on rotenone concentration, as described in the next section.

Variability in the amount of 33000-Mr protein

Fig. 6 shows two different preparations of Complex I analysed on gradient slab gels. The stained profiles differ only in the relative intensity of one polypeptide (marked with the arrow), which is the 33000-Mr protein previously identified on Weber & Osborn (1969) gels (Fig. 4). Some
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Fig. 5. Effect on labelling of arylazidoamorphigenin concentration and rotenone
Complex I or Complex I pre-incubated with rotenone was labelled with arylazido[3H]amorphigenin and analysed by SDS/polyacrylamide-gel electrophoresis as described in the Materials and methods section. The Figure shows labelling with 2 nmol of arylazidoamorphigenin/mg of protein in the presence (a) or in the absence (b) of rotenone (20 nmol/mg of protein) and labelling with 10 nmol of arylazidoamorphigenin in the absence of rotenone (c). Profiles (b) and (c) are displaced upwards by 50 d.p.m. and 100 d.p.m. respectively for convenience of presentation.

diminution of the intensity of this band can be brought about by dissociating the Complex I in SDS at 100°C rather than at 20°C. This causes aggregated protein to appear at the electrophoretic origin (Fig. 6), but did not lead to a decrease in the incorporation of arylazidoamorphigenin into the 33000-Mr polypeptide. Thus there are probably two different proteins that migrate to this position on gels, one of which can be aggregated by boiling, and the other of which is the site of labelling by the analogue. Even after 100°C treatment, though, the relative stain intensity of the band differs markedly in the two preparations.

Further evidence for this interpretation comes from Fig. 7. The two Complex I preparations were incubated in Triton X-100 with antiserum to Complex I, and the immunoprecipitates were analysed by SDS/polyacrylamide-gel electrophoresis. As previously reported, the profiles are very similar to that of untreated Complex I except for the absence of a polypeptide of Mr 42000

Fig. 6. Polypeptide composition of two preparations of Complex I
The complex I preparations were analysed by SDS/polyacrylamide-gradient-gel electrophoresis as described in the Materials and methods section. Tracks 1 and 2, preparation A (as used in the experiment of Fig. 4); tracks 3 and 4, preparation B; tracks 1 and 3, samples dissociated at 20°C; tracks 2 and 4, samples dissociated at 100°C.

Fig. 7. Immunoprecipitation of two Complex I preparations
The two preparations, A and B, were precipitated from solution in Triton X-100 by anti-(Complex I) serum as described in the Materials and methods section. The precipitates were analysed by SDS/polyacrylamide-gradient-gel electrophoresis. Identification of tracks was exactly as in the legend to Fig. 6.
Fig. 8. *Rotenone inhibition of two Complex I preparations*  
The two preparations A (○) and B (△) were incubated with rotenone and assayed for NADH-ubiquinone reductase activity as described in the Materials and Methods section. Activity is expressed as the percentage of that which is inhabitable by $30\text{nmol}$ of rotenone/mg of protein (25 mol/mol of enzyme).

(Smith & Ragan, 1980). Treatment at 100°C no longer caused aggregated material to appear at the origin, and the intensity of the 33000-$M_r$ band was unaffected. We therefore conclude that the material that moves from this position to the origin on heating is an impurity. However, the relative stain intensity of the remaining band was still very different in the two preparations. Since anti-(Complex I) serum does not contain any antibodies to this protein (M. W. J. Cleeter & C. I. Ragan, unpublished work), we conclude that it is a true constituent of Complex I. In support of this, immunoprecipitates obtained from solubilized submitochondrial particles from ox heart, rat heart, rat liver and rabbit liver all contain a polypeptide running on gels at this position (M. W. J. Cleeter & C. I. Ragan, unpublished work).

The preparation with the higher concentration of the 33000-$M_r$ protein could be labelled in this polypeptide by arylazidoamphetamine to a much greater extent than the other (2–3-fold). Moreover, the two preparations also differed in their sensitivity to rotenone, as shown in Fig. 8. The Complex I with the higher amount of the 33000-$M_r$ protein was considerably less inhibited at intermediate concentrations of rotenone than was the other preparation. The initial activities of the two preparations and the maximum extents of inhibition were, however, very similar. The unusual shapes of these titration curves, in which 35–60% inhibition is achieved by as little as 0.2 mol of rotenone/mol of Complex I, has been noted before (Ragan & Heron, 1978), and has also been encountered in rotenone inhibition of an NADH-induced semiquinone e.s.r. signal (Suzuki & King, 1983).

**Discussion**

We have described the synthesis of a photo-activatable analogue of rotenone that causes substantial inhibition of NADH-ubiquinone oxido-reductase at concentrations less than 1 mol of inhibitor/mol of enzyme, as do rotenone and the starting material for the synthesis, amorphigenin. At low concentrations, therefore, the binding of the analogue must be highly specific. Photolysis of the enzyme-bound analogue did not alter the inhibition achieved in the dark, showing that the reaction products were in themselves inhibitory and did not migrate from the specific binding site. Nevertheless, only 20–40% of the inhibitor remained covalently attached to the enzyme, suggesting that the photogenerated nitrene was less likely to become cross-linked to the protein than, for example, to react with phospholipid or with another part of the inhibitor molecule or to react with protein without cross-linking. We favour the latter possibilities, since very little incorporation into the phospholipid fraction was found (results not shown). With increasing concentrations of the inhibitor, incorporation into protein following photolysis did not show saturation, presumably as a result of reaction at sites other than that of inhibition. This is undoubtedly a consequence of the extremely hydrophobic nature of the molecule. Its insolubility in water will ensure that the inhibitor will inevitably associate non-specifically with hydrophobic regions of the enzyme or phospholipids. However, at low concentration binding to the inhibitory site is clearly preferred.

The major problem in identifying the particular polypeptide that the analogue photolabels lies in the instability of the covalent attachment in the presence of SDS. After separation of the proteins by SDS/polyacrylamide-gel electrophoresis, most of the label is associated with the 33000-$M_r$ polypeptide. However, this amounted to, at best, 0.04 mol/mol of enzyme. It could be argued, therefore, that labelling of this protein is a minor side reaction and that labelling of the true binding site does not survive exposure to SDS. We cannot exclude this possibility totally, but we have presented other evidence to suggest that the 33000-$M_r$ protein is indeed involved in the binding of rotenoids. Firstly, at low concentrations of the
analogue, this polypeptide is the only one that retains label on SDS/polyacrylamide-gel electrophoresis. At higher concentrations of inhibitor, many other proteins become labelled as well. Therefore many Complex I proteins can retain label after SDS/polyacrylamide-gel electrophoresis, but only the 33000-Mr protein is labelled when the inhibitor is confined to the specific inhibitory site. Secondly, labelling of this polypeptide is decreased by prior treatment of the enzyme with rotenone. Rather surprisingly, a 10-fold molar excess of rotenone resulted in only a 2-fold decrease in the labelling, suggesting that the affinity of the analogue for the binding site on this protein is considerably greater than the affinity for rotenone. This again may result from the greater hydrophobicity of the analogue, which renders its binding effectively irreversible. Rotenone, on the other hand, can be removed from the enzyme by bovine serum albumin, for example (Horgan et al., 1968). Thirdly, we find that the 33000-Mr protein is present in Complex I from rat and rabbit tissue even though the overall polypeptide compositions of the enzyme in ox, rat and rabbit are appreciably different. This suggests a functional rather than a purely structural role for this protein. Fourthly, the variability of the content of this protein in different Complex I preparations correlates, not only with variation in the amount of label that can be incorporated, but also with the sensitivity of the enzyme to inhibition by rotenone.

The shapes of the rotenone inhibition curves of Fig. 8 could, in principle, be due to several causes. The possible dimeric nature of respiratory-chain complexes, including Complex I, has received considerable attention (e.g. Beinert & Albracht, 1982; De Vries et al., 1982), and negative cooperativity between the monomers could explain the rotenone effect. Alternatively there may be two populations of Complex I molecules present that differ substantially in activity, presumably as a result of some alteration during the course of purification. If this alteration is, in fact, the loss of the 33000-Mr polypeptide, this could explain the variation in the rotenone titration curves, if we assume that activity through the deficient complexes is still rotenone-sensitive, but with decreased affinity for the inhibitor. This explanation fails to account for the observation that the initial activity of the two preparations of Fig. 8 was substantially the same, as was that of other preparations whose rotenone titration curves fell between the two examples given. Clearly it is essential to establish whether or not the rotenone inhibition curve is a preparation artifact, and to correlate inhibition with rotenone binding rather than the concentration of rotenone added. In this context, it is known that there is only one class of specific piericidin-binding sites on Complex I (Gutman et al., 1970); nevertheless, the variation in sensitivity to rotenone shown in Fig. 8 is paralleled by a similar variation in sensitivity to piericidin (F. G. P. Earley & C. I. Ragan, unpublished work). The low specific radioactivity of the analogue and its poor incorporation into protein made it impossible to confirm our results with intact mitochondria and submitochondrial particles. Further work should be directed towards the synthesis of alternative photoaffinity-labelling analogues of both rotenone and piericidin to help resolve some of the peculiarities uncovered by the work described here.

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References