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PHOTOAFFINITY LABELLING OF MITOCHONDRIAL NADH:UBIQUINONE REDUCTASE WITH PETHIDINE ANALOGUES

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Abstract—1. Chemically reactive derivatives of pethidine analogues—novel potent inhibitors of the mitochondrial NADH:ubiquinone reductase (complex I)—were synthesized.

2. Dose–response curves of these components revealed that the photoactivatable aryl azido derivative has retained most of the inhibitory activity displayed by the parent substance. After introduction of a radioactive iodine isotope into the molecule, it was used as a probe for the localization of the inhibitor binding polypeptides within complex I.

3. Photolysis of the radiolabelled derivative bound to isolated complex I both from *Neurospora crassa* and beef heart resulted in a covalent incorporation of the inhibitor into 6–7 individual subunits of the enzyme. Essentially the same labelling patterns were obtained, when whole mitchondrial membranes were incubated with the reactive derivative.

4. Applying a double isotope labelling technique, the inhibitor-binding polypeptides in *N. crassa* were identified as mitochondrially synthesized constituents of complex I (ND gene products). In the beef heart enzyme the ND-1 product was detected to be among the polypeptides reacting with the inhibitor.

5. Competition experiments employing either NADH or decylbenzoquinone (DB), together with the pethidine analogue, showed that both enzyme substrates interfere specifically with the inhibitor binding to complex I.

Mitochondrial oxidation of NADH* implicates the transfer of electrons from the pyridine nucleotide to the acceptor ubiquinone. This formally simple process seems to involve a highly complex mechanism including a number of intermediate steps. All of them are associated with the puzzling structure of the respiratory chain enzyme NADH:ubiquinone reductase, also known as complex I (for review see Ref. 1).

While many studies deal either with structural or functional aspects, our knowledge of their actual relationships is still rather poor. One obvious approach towards this goal is the identification of the particular sites where substrate or inhibitor molecules interact with the catalytic unit.

Rotenone is a strong and relatively specific inhibitor of NADH:ubiquinone dehydrogenase [2], probably acting close to the site of ubiquinone reduction [3]. Using derivatives of this drug, Early and coworkers [4, 5] have shown that these components bind to a hydrophobic component of the protein complex isolated from beef heart. This 33 kDa binding protein is most likely identical with the mitochondrially encoded ND-1 gene product [5], as judged by immunological criteria.

We have recently reported that several pethidine analogues are powerful inhibitors of complex I, nearly equipotent to rotenone, and we suggested a similar mode of action for both classes of compounds [6]. The advantages of the novel inhibitors (e.g. amphipathic nature of the molecules, high specificity, superior derivatization potential—for discussion see Ref. 6) prompted us to employ them as probes for the identification of their binding site(s) within the oligomeric protein of *Neurospora crassa* and beef heart. The incorporation experiments show that about six individual subunits of complex I interact with the inhibitor, nearly all of them representing mitochondrially encoded gene products. The binding process is specifically influenced by the presence of the enzyme substrates, ubiquinone and NADH.

* Abbreviations: [^{125}I]APBnp, 1-(4-azido-3-[^{125}I]iodophenyl)-2,3-dimethyl-2-butenyl-norpethidine, or 1-[(4-azido-3-[^{125}I]iodophenyl)-2,3-dimethyl-2-butenyl]-4-phenyl-4-piperidinecarboxylic acid ethyl ester; DB, 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone; Q_0, 2,3-dimethoxy-5-methyl-1,4-benzoquinone. Enzyme: NADH:ubiquinone reductase (EC 1.6.99.5).

MATERIALS AND METHODS

Chemistry

Melting points were determined in open capillary tubes and are uncorrected. ^{1}H-NMR spectra were recorded on a Varian XL-200 instrument. The various splitting patterns were designated as follows: s, singlet; br s, broad singlet; d, doublet; t, triplet; q, quartet or quintuplet; m, multiplet. Mass spectra (Varian MAT CH-5), i.r. spectra (Perkin–Elmer 283 B), UV spectra (Perkin–Elmer 550 S) were obtained by the indicated equipment. For thin-layer chromatography (TLC) (analytical and preparative) precoated silica gel 60 plates (Merck) were used and developed with methanol, if not otherwise stated. Flash column chromatography was conducted with aluminium oxide (Fluka, acid type 504 C).

1-(4-Nitrophenyl-2,3-dimethyl-2-butenyl)-4-phenyl-4-piperidine-carboxylic acid ethyl ester (III—

roman numbers refer to the synthesis scheme given in Fig. 1) was produced as already described [6].

1-(4-*Aminophenyl*-2,3-*dimethyl*-2-*butenyl*)-4-*phenyl*-4-*piperidine-carboxylic acid ethyl ester* (*IV*). Four hundred milligrams of III (hydrochloride) were dissolved in 60 ml of ethanol, 92 mg of hydrazine hydrate were added and the mixture was warmed to 60–70° and then treated subsequently with small amounts of Raney Ni, as reported by Balcom and Furst [7]. After 40 min of stirring the solution was briefly heated to boiling and filtered. The solvent was evaporated, the residual material taken up in CH_2Cl_2 and chromatographed on 150 g of Al_2O_3. The column was first eluted with CH_2Cl_2 and finally with CH_2Cl_2/acetone, 1:1 (vol/vol). The latter eluate was collected and the solvent evaporated. The material was dissolved in ether, bubbled with hydrogen chloride and the creamy white precipitate was dried. Yields were 85–90%; mp.167°; TLC R_f = 0.61 (0.74 for the nitroderivative); positive reaction for aromatic amines; UV λ_{max} (0.1 N HCl) 209 nm ($\varepsilon = 25.4\ mM^{-1} \times cm^{-1}$); i.r. (KBr) 3400 cm^{-1} (R-NH_2); ^{1}H-NMR 200 MHz ($CDCl_3$), d 1.2 (t, 3H, CO_2—CH_2—CH_3), 1.6 (s, 3H, CH_3—C=C(Ar)—CH_3), 1.9 (s, 3H, CH_3—C=C(Ar)—CH_3), 2.3 (m, 2H, pip.), 2.6 (m, 2H, pip.), 3.0 (m, 2H, pip.), 3.15 (s, 2H, C=C—CH_2—Ar), 3.3 (s, 2H, C=C—CH_2—pip.), 3.5 (br m, 2H, —NH_2), 4.1 (q, 2H, CO_2—CH_2—CH_3), 6.6 (m, 2H, Ar), 6.9 (m, 2H, Ar), 7.3 (m, 5H, Ph); MS, m/e 406, 246, 173, 158.

1-(4-*Azidophenyl*-2,3-*dimethyl*-2-*butenyl*)-4-*phenyl*-4-*piperidine-carboxylic acid ethyl ester* (*VIb*). Seventy-five milligrams of IV were dissolved in 8 ml 1 N HCl/dioxan, 1:1 (vol/vol). The solution was chilled to 0°, 14 mg of $NaNO_2$ (in 0.5 ml H_2O) were added over 30 min and the mixture was incubated for another 30 min. All following operations were performed under subdued light. Fourteen milligrams of NaN_3 (in 0.5 ml H_2O) were slowly added (15 min) to the reaction assay; it was stirred for 30 min at 0° and then for an additional 30 min at room temperature. The resulting mixture was made alkaline by a concentrated solution of $NaHCO_3$ and extracted several times with ether. The combined organic layers were filtered, dried over Na_2CO_3, and finally bubbled briefly with hydrogen chloride. The precipitated hydrochloride was crystallized from ethanol/ether. Yield about 70%; mp. 173°; TLC R_f = 0.72; UV λ_{max} (0.1 N HCl) 207 nm ($\varepsilon = 25.8\ mM^{-1} \times cm^{-1}$), 252 nm ($\varepsilon = 13.4\ mM^{-1} \times cm^{-1}$); i.r. (KBr) 2110 cm^{-1} (R—N_3); ^{1}H-NMR 200 MHz ($CDCl_3$), d 1.2 (t, 3H, CO_2—CH_2—CH_3), 1.65 (s, 3H, CH_3—C=C(Ar)—CH_3), 1.8 (s, 3H, CH_3—C=C(Ar)—CH_3), 2.0 (m, 2H, pip.), 2.6 (m, 2H, pip.), 2.8 (m, 2H, pip.), 2.95 (s, 2H, C=C—CH_2—Ar), 3.4 (s, 2H, C=C—CH_2—pip.), 4.1 (q, 2H, CO_2—CH_2—CH_3), 6.9 (m, 2H, Ar), 7.1 (m, 2H, Ar), 7.3 (m, 5H, Ph); MS, m/e 432 (M^+), 404, 246.

1-(4-*Isothiocyanatophenyl*-2,3-*dimethyl*-2-*butenyl*)-4-*phenyl*-4-*piperidine-carboxylic acid ethyl ester* (*VII*). Fifty milligrams of IV were dissolved in 25 ml $CHCl_3$ and vigorously stirred with 25 ml of a saturated $NaHCO_3$-solution. To the biphasic system 500 µl of redestilled thiophosgene were added. Two further portions, 500 µl and 3 ml respectively, of thiophosgene were given to the mixture under continuous stirring at one hour intervals. Ninety minutes after the last addition, the organic layer was removed, combined with two $CHCl_3$ extracts of the aqueous layer and dried over Na_2CO_3. The solvent was evaporated and the residual material was chromatographed on a preparative silica gel TCL plate (solvent methanol). The band migrating with a R_f = 0.76 was cut out, eluted with $CHCl_3$ and dried. Yield was about 30% of an oily, pinky base. It reacted readily with various compounds possessing primary amino groups. i.r. (KBr) 2100 (vs) and 2180 (s) cm^{-1} (R—NCS); ^{1}H-NMR data were similar to those already described for the derivatives IV and VIb.

1-[(4-*Azido*-3-[^{125}I]*iodophenyl*)-2,3-*dimethyl*-2-*butenyl*]-4-*phenyl*-4-*piperidine-carboxylic acid ethyl ester* (*VIa* = [^{125}I]*APBnp*). Eight micrograms of IV were taken up in 8 µl of dimethyl sulfoxide. At room temperature 24 µl of sodium acetate buffer (1 M, pH 5.6) and 1 mCi of carrier-free Na[^{125}I] (2300 Ci/mmol) were added to the solution, followed by 7 µl of chloramine T (1 mg/ml in H_2O). After an incubation period of 3 min the reaction was halted with 5 µl of $NaHSO_3$ (1 mg/ml in H_2O) and the mixture was made basic by the addition of 5 µl 1 N NaOH. The solution was immediately extracted three times with 0.4 ml portions of ethyl acetate. The organic layers were pooled and dried under a stream of nitrogen. An aliquot of the resulting V was analysed on TLC. Exposure of the plate to a film revealed the presence of one major radioactive spot of R_f = 0.64. The above preparation of V was taken up in 200 µl of dioxan/H_2O, 1:1 (vol/vol), mixed with 30 µl of 1 N HCl and chilled in an ice bath. Strictly at 0°, the solution was first treated with 30 µl of $NaNO_2$ (6.9 mg/ml in H_2O), and after an incubation period of 15 min, with 30 µl of NaN_3 (6.5 mg/ml in H_2O). Twenty minutes later, 35 µl of 1 N NaOH were added and the mixture was extracted into ethyl acetate (three times, 0.5 ml). The combined organic layers were briefly dried over Na_2CO_3 and the solvent was evaporated under a stream of nitrogen. Resulting VIa was dissolved in 200 µl of absolute methanol and stored at −20°. Thin-layer chromatography analysis resolved a single radioactive compound of R_f = 0.70. Usually, about 20% of the applied [^{125}I] was incorporated into the derivative. The specific radioactivity of [^{125}I]APBnp obtained by this procedure was in the range of 20 Ci/mmol.

Ubiquinone derivatives. 2,3-Dimethoxy-5-methyl-6-decyl-1,4-benzoquinone (DB) was synthesized according to Wan *et al.* [8]. 2,3-Dimethoxy-5-methyl-1,4-benzoquinone (Q_0) was purchased from Sigma.

Preparations of complex I

N. crassa. The isolation was carried out essentially as reported by Ise *et al.* [9] with minor modifications [6].

Beef heart. As an alternative to the relatively time consuming "ammonium sulfate precipitation technique" described by Hatefi [10], the following, more rapid, isolation procedure was developed. All steps were carried out at 4°. Two-point-three grams of mitochondria were diluted with TSH buffer (0.66 M

sucrose, 50 mM Tris–HCl, 1 mM histidine, pH 8.0) to a protein concentration of 20 mg/ml. Desoxycholate (0.3 mg/mg protein) and KCl (72 mg/ml) were added to the suspension, which was stirred for 10 min and then centrifuged for 60 min at 100,000 *g*. The pellet was suspended in 40 ml Tris buffer (50 mM; pH 7.5) and Na_2SO_4 (final conc. 0.1 M), and Triton X-100 (final conc. 3%) were added. The mixture was stirred for 10 min and centrifuged for 20 min at 50,000 *g*. The supernatant was loaded onto a hydroxylapatite column (Bio-gel HTP, Bio-Rad) (3 × 20 cm) equilibrated with TST buffer (20 mM Tris–HCl, 100 mM Na_2SO_4, 0.3% Triton X-100, pH 7.5). The column was eluted subsequently with TST buffer alone and then with TST buffer containing 25 mM, 60 mM and 120 mM sodium phosphate, respectively. The eluates obtained with phosphate concentrations between 60 and 120 mM were treated with an amount of Amberlite XAD-2 (20–50 mesh, prewashed with methanol and water) sufficient to remove 85% of the detergent. After an incubation period the resin was removed by filtration through a cheesecloth and the solution was dialysed (6 hr) against water. The pellet (400 mg) recovered from a centrifugation step (60 min, 100,000 *g*) was suspended in 30 ml Tris–HCl, pH 7.5. Triton was added to a final concentration of 3% and the resulting lysate was applied on a DEAE–Sepharose column (6 × 5 cm) equilibrated with TT buffer (20 mM Tris–HCl, 0.3% Triton X-100, pH 7.5). The elution was performed with the following step gradient: TT buffer alone, and buffer containing 100 mM, 200 mM, and 300 mM NaCl, respectively. The protein developed with 200 mM NaCl was incubated with XAD resin as described above, and the filtered solution was centrifuged for 60 min at 100,000 *g*. The precipitate (250 mg of complex I) was taken up in 20 mM Tris buffer (pH 7.5) and frozen. The enzymatic activities and the gel electrophoretic patterns observed with these preparations compared well to those obtained by the "classical Hatefi procedure" [10].

Photoaffinity labelling

All experiments were carried out at 4°.

Isolated complex I. Fifty micrograms of isolated complex I were finely dispersed in 0.5 ml of phosphate buffer (0.1 M, pH 8.0) by a brief sonication step. When indicated, the suspension was preincubated for 15 min with NADH, NAD, NADPH, NADP, 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone (DB), 2,3-dimethoxy-5-methyl-1,4-benzoquinone, rotenone, and various pethidine analogues, respectively. Then, [^{125}I]APBnp (about 0.1–0.2 nmol; 20 mCi/μmol), dissolved in 1–2 μl of methanol, was added and incubated for a further 15 min. The mixture was transferred to a semi-micro quartz cuvette and illuminated for 3 min with a UV-lamp (model XX-15, Ultra-Violet Products Inc., San Gabriel, CA.) at a distance of 1 cm. Finally, cholate and desoxycholate were admixed to give final concentrations of 0.25%, each. The protein was precipitated by addition of 0.35 ml of a saturated solution of ammonium sulfate and the resulting pellet was subjected to SDS–gel electrophoresis.

Mitochondrial membranes. One milligram of mitochondrial membrane was incubated (15 min) in 1 ml of phosphate buffer (0.1 M, pH 8.0) together with 0.2–0.3 nmol [^{125}I]APBnp and then photolysed for 8 min as detailed above. The reacted mixture was centrifuged (20 min, 50,000 *g*) and the pellet obtained was solubilized by the addition of either Triton X-100 (1% in phosphate buffer) or 100 μl SDS (1% in phosphate buffer). The lysates were treated with antisera to the whole complex I and to the ND-1 (URF-1) gene product in separate experiments.

Immunological techniques. Immunoprecipitations from Triton lysates [11] and from solutions containing SDS [12] have been described elsewhere. Immunodecorations were performed with a gold-labelled second antibody (Auro Probe BL plus GAR) and a silver enhancement system (IntenSE II) supplied by Janssen (Life Science Products, Belgium).

Other methods. Sodium-dodecyl-sulphate–gel electrophoresis, gel slicing, nitrocellulose blotting, autoradiography, *in vivo* labelling techniques of *N. crassa* with radioactive amino acids, preparation of mitochondria, and enzyme assays have been reported [12–14, 11, 15–17, 6]. Mitochondrial membranes were prepared as described previously [17]. IC_{50} values (inhibitor concentrations resulting in a 50% loss of activity) were extrapolated from a series of enzyme determinations varying the concentrations of the inhibitor only.

RESULTS

Synthesis of chemically reactive inhibitor compounds

Figure 1 displays the scheme used for the design of chemically reactive derivatives of a particular pethidine analogue (III, compound No. 49); this drug has been shown to be a highly potent inhibitor of the NADH : ubiquinone reductase activity in mitochondria [6]. The key intermediate for the synthesis of the derivatives, aryl amine IV, was readily produced by the reduction of the corresponding nitrophenyl compound under selective conditions.

In our first approach an isothiocyanate ligand was chosen as alkylating moiety, because of its known high reactivity with primary and secondary amino groups, compared to its slow reaction with hydroxyl groups. For this purpose, the amine IV was treated with thiophosgene in a biphasic chloroform–bicarbonate system to give the desired derivative VII. In a second set of experiments, an aryl azide was exploited as the relevant functional group, which generates an extremely reactive nitrene group upon photolysis. The radioactively labelled photoaffinity ligand [^{125}I]APBnp (VIa) was prepared from the intermediate IV in two synthetic steps. The aryl amine was first radioiodinated using chloramine-T and carrier-free Na[^{125}I], and then quantitatively converted to the corresponding azide compound by diazotization and subsequent reaction with sodium azide. The product was analysed by TLC, visualized by autoradiography and shown to comigrate with fully characterized non-radioactive compound VIb when chromatographed in different solvent systems.

Attachment of the azido (or eventually a diazo) moiety at several alternative loci of the inhibitor molecule (e.g. at the 4-phenyl and the 4′-ethyl ester

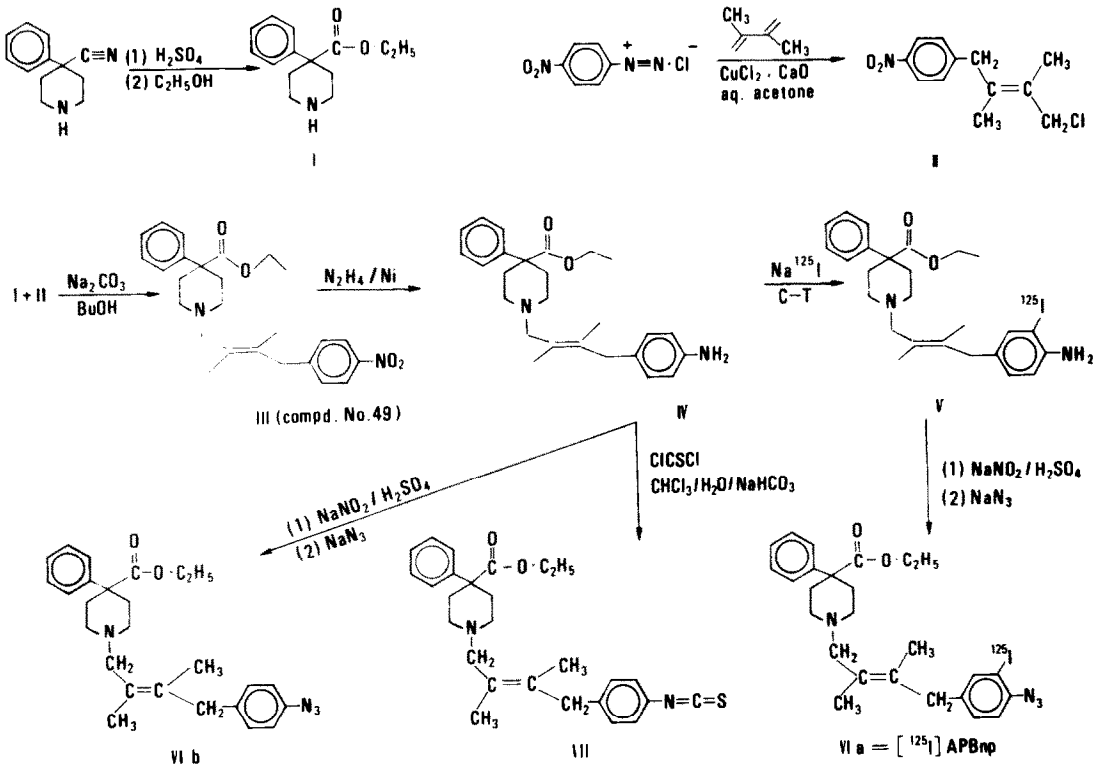

Fig. 1. Syntheses of chemically reactive pethidine analogues.

substituents of the piperidine ring, respectively), or a further elongation of the anilino group of compound IV by a rather bulky 3-nitro-phenylazide ligand, resulted in derivatives of low biological activity and/or insufficient chemical stability upon storage. These syntheses were therefore abandoned (S. Werner, unpublished results).

Properties of the affinity compounds

In order to assess the biological activity of the chemically reactive derivatives synthesized, their inhibitory effect on NADH:ubiquinone reductase was determined in a standard enzymatic assay using beef heart mitochondrial membranes. After addition of the compounds in the dark to the complete enzyme assays a rapid onset of the inhibitory action, with a lag period of a few seconds, took place. Preincubation studies of the enzyme with the pethidine analogues and substrates showed that the inhibition was not reversible. Also, washing steps with buffer of mitochondrial membranes, pretreated with the chemically reactive derivatives, did not influence the extent of inhibition. Figure 2 shows the dose–response curves of the analogues together with those of the parent and intermediate compounds. While the relevant IC_{50} values for the nitro- and amino derivatives are 1 ± 0.3 and $2 \pm 0.4\ \mu M$, respectively, a significantly higher value ($5 \pm 0.7\ \mu M$) was obtained for the photoactivatable azido compound. Despite this loss of activity, the last analogue triggered a well defined enzymatic inhibition. This was in contrast to the results obtained with the isothiocyanate derivative. Its inhibitory potency dropped by about one order of magnitude ($IC_{50} = 10 \pm 1.5\ \mu M$), compared to the parent compound. Moreover, maximal inhibition levels hardly exceeded the 60% range, and higher concentrations of the drug seemed even to activate the enzyme system, depending also on the actual incubation conditions.

Since the azido compound turned out to be the most promising affinity tool, it was further characterized by its photolytic behaviour. Figure 3 exhibits the typical change in the UV-absorption spectrum upon illumination. The peak disappearing gradually in the 250 nm range is consistent with the wavelength of maximal absorption, reported for the aryl azide moiety [18, 19]. On the other hand, the absorption of the parent amino compound is rather low in this region and no particular photosensitivity was observed with the latter derivative during a similar exposure to UV-light (result not shown).

Covalent photoincorporation of the inhibitor into complex I

Reaction with isolated complex I. First, we tried to locate the polypeptide subunit(s) involved in the binding of the inhibitor to the oligomeric protein complex. The ^{125}I-labelled photoreactive azido derivative ([^{125}I]APBnp) was incubated with enzyme preparations isolated from *N. crassa* and beef heart, respectively. After photoactivation the samples were washed and subjected to SDS–gel electrophoresis. Figure 4 shows the fluorographic resolutions. Applying the *N. crassa* preparation (panel A), several polypeptide subunits become covalently labelled

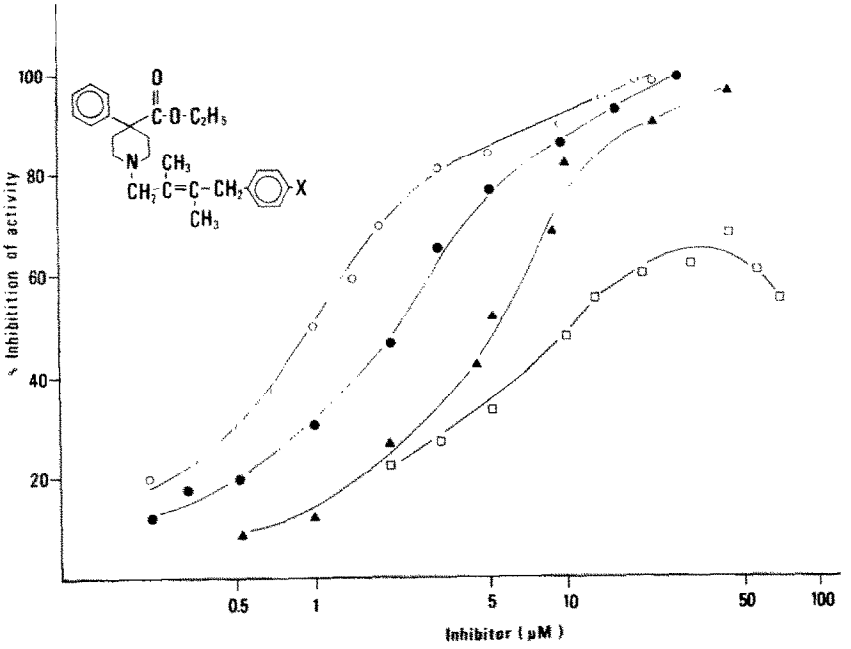

Fig. 2. Semi-logarithmic dose–response curves for the inhibition of NADH:ubiquinone reductase activity by various pethidine analogues. Ligand X of the structure formula represents NO_2 (○), NH_2 (●), N_3 (▲), and NCS (□), respectively. The derivatives were added in the dark to the complete enzyme assay without preincubation. The standard assay contained decyl-benzoquinone (DB) and 100 μg of beef heart mitochondrial membranes (specific activity: 0.36 U/mg protein).

with the pethidine analogue. The radioactive bands correspond to discrete components of complex I, which is made up of at least 25 individual polypeptides, judged by the resolved Coomassie staining pattern. The predominantly labelled polypeptide species have apparent molecular weights of 47, 38, 29, 22, 17 and 12 kDa, respectively. The radioactive material migrating with the dye front of the gel represents inhibitor not covalently linked to protein. The assays carried out under varying incubation con-

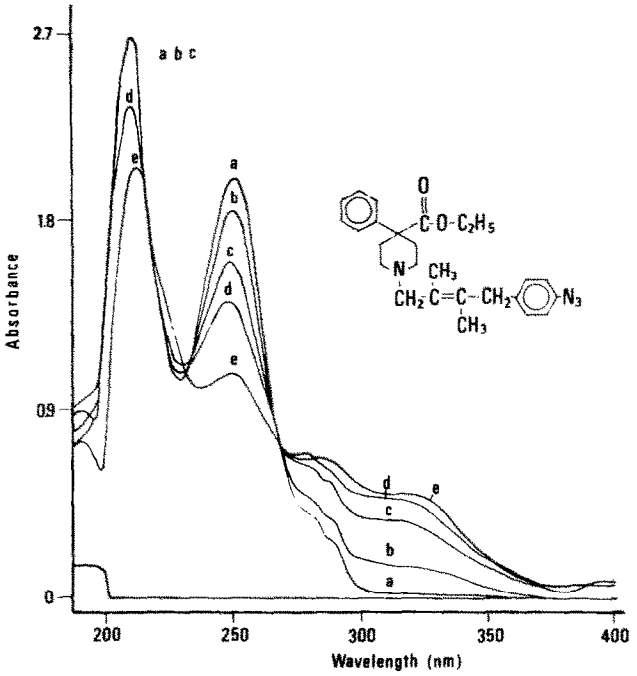

Fig. 3. Photodecomposition of the azido derivative. The compound (about 20 μM in methanol) was photolyzed in quartz cuvettes. UV-spectra were recorded after various periods of irradiation; control (a), 1 min (b), 3 min (c), 5 min (d), 10 min (e).

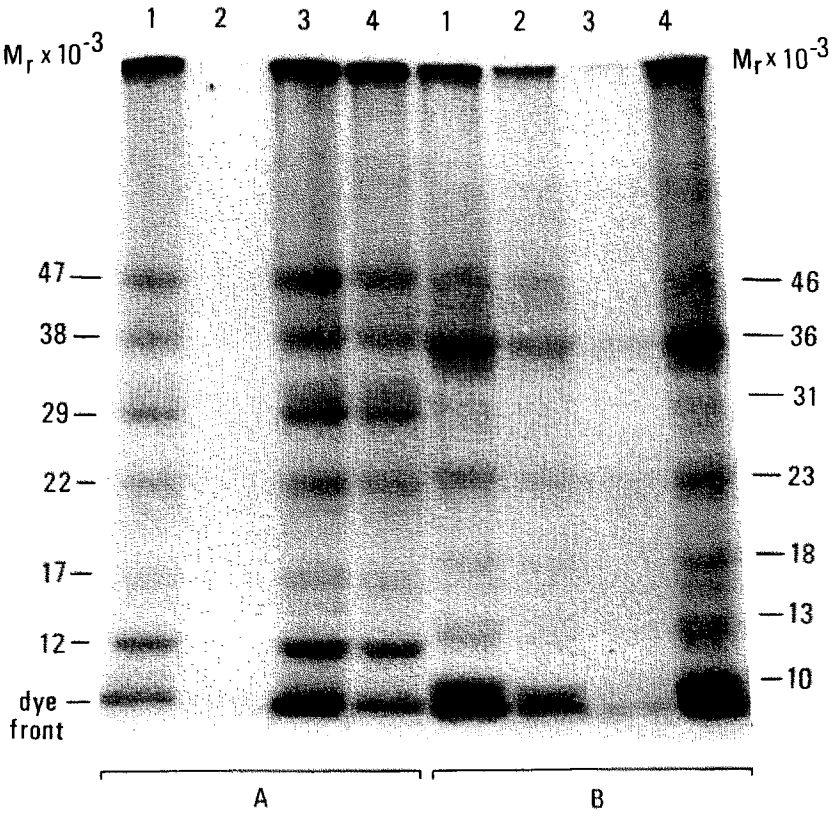

Fig. 4. Covalent photoincorporation of the inhibitor into isolated complex I. Fifty micrograms of complex I were incubated with the photoreactive inhibitor ([^{125}I]APBnp) and processed as described in Materials and Methods. Then all samples were subjected to SDS–gel electrophoresis and the gels were subsequently fluorographed. (A) *N. crassa* isolated complex I: (1) standard procedure (see Materials and Methods); (2) sample was not illuminated; (3) complex I and inhibitor were incubated in the presence of 0.03% Triton X-100; (4) sample was incubated in the presence of 0.2% cholate/0.2% desoxycholate. (B) Beef heart isolated complex I: (1) standard procedure (see Materials and Methods); (2) complex I was washed twice by two subsequent ammonium sulfate precipitations; (3) sample was not illuminated; (4) complex I and inhibitor were incubated in the presence of 0.03% Triton X-100.

ditions produced the same radioactivity patterns, differing only slightly in the relative intensities of the individual bands. Panel B of Fig. 4 shows the binding of the radioligand to isolated beef heart complex I in which, compared to the *N. crassa* preparation, a quite similar behaviour was observed. In this case, most of the incorporated radioactivity was associated with polypeptides exhibiting apparent molecular weights of 46, 36, 31, 23, 18, 13 and about 10 kDa, respectively.

Reaction with mitochondrial membranes. In a second set of experiments an *in situ* labelling of complex I within its "physiological environment" was attempted. For this purpose whole mitochondrial membranes were incubated with the photoreactive inhibitor and, after illumination, complex I was isolated by immunoprecipitation. The protein was analysed again by SDS–gel electrophoresis (Fig. 5). Five to six major radioactive bands are observed in *N. crassa* (panel A), all of which match the components already described in the reaction with the isolated enzyme. However one component, the 17 kDa product, seems to be labelled very faintly in the membrane experiments. Remarkably, most of the incorporated radioactive ligand is now concentrated in the 47 kDa polypeptide. A somewhat analogous result was obtained with beef heart mitochondrial membranes (panel B). The inhibitor binds predominantly to six polypeptides exhibiting an identical migration behaviour to that reported in the assay with isolated complex. However, one major difference was noted: in this case, the 36 kDa component no longer incorporated any radioactivity.

Identification of the inhibitor binding subunits

Preliminary electrophoretic analyses with preparations of *N. crassa* complex I, which contained either a selective label for mitochondrial products (cycloheximide-resistant incorporation of radioactive amino acids) or the covalently bound inhibitor, revealed that similar labelling patterns appeared in the separate lanes upon fluorography (results not shown). This surprising comigration of the relevant bands prompted us to design a double labelling experiment which allows an unambiguous correlation of the mitochondrially synthesized polypeptides with the inhibitor binding constituents of complex I (Fig. 6). Mitochondrial membranes were

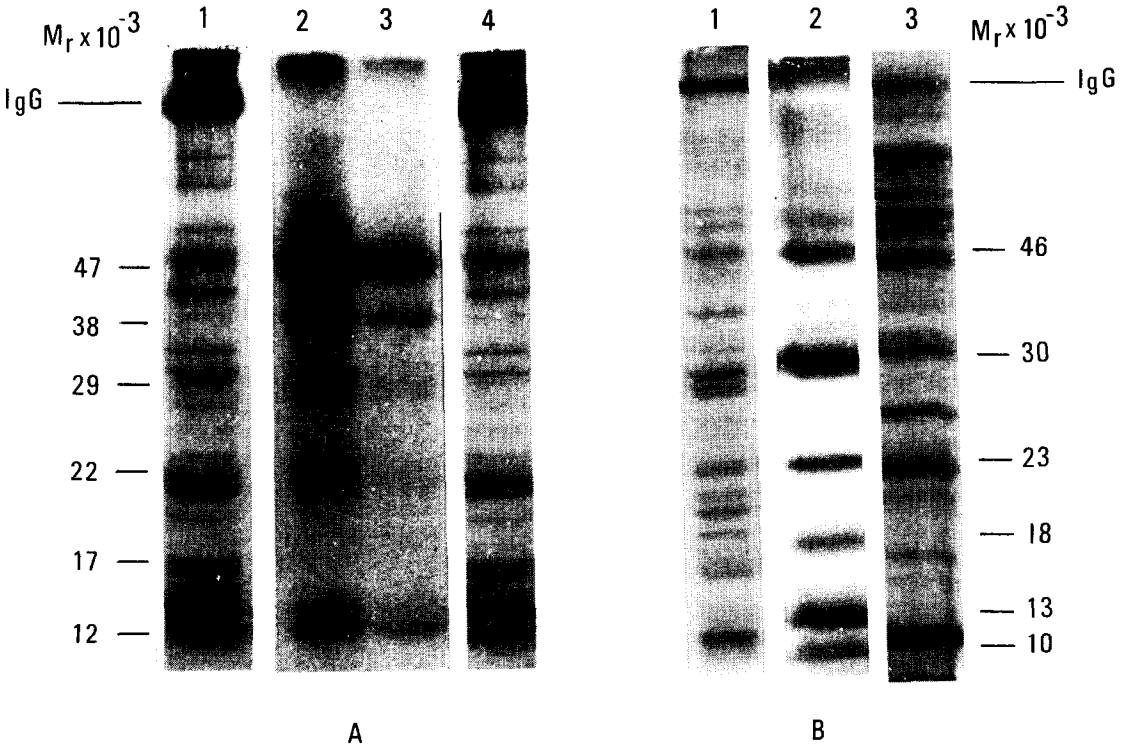

Fig. 5. Covalent photoincorporation of the inhibitor into complex I within mitochondrial membranes. One milligram of mitochondrial membrane was incubated with [^{125}I]APBnp and photolyzed. The mixtures were solubilized with Triton X-100 and complex I was immunoprecipitated with an appropriate antibody to the whole enzyme. The isolated material was subjected to SDS–gel electrophoresis and the gels were first stained with Coomassie Brilliant Blue and then fluorographed. (A) *N. crassa* precipitates of complex I: (1) and (4) Coomassie staining; (2) fluorograph (standard procedure, see Materials and Methods); (3) fluorograph (membranes were illuminated for 3 min only). (B) Beef heart precipitates of complex I: (1) and (3) Coomassie staining; (2) fluorograph (standard procedure, see Materials and Methods).

prepared from *N. crassa* cells labelled *in vivo* with [^{3}H]leucine in the presence of cycloheximide. These membranes were incubated with [^{125}I]APBnp and photolyzed. The complex I was immunoprecipitated from the assay and subjected to SDS–gel electrophoresis (Fig. 6A). Six to seven tritium labelled mitochondrial components with apparent molecular weights of 47, 38, 33, 29, 17 and probably 12 kDa (very poor incorporation into the latter product) can be discriminated. (For reasons of clarity we want to point out that the amounts of ^{3}H-radioactivity present in the individual peaks do not reflect protein mass, but the actual pool sizes of the precursor polypeptides). The ^{125}I-label of the inhibitor clearly coincides with all of the mitochondrially made constituents of complex I, except the 33 kDa species, which contains no pethidine analogue. Most of the inhibitor covalently bound by this *in situ* experiment was recovered with the 47 kDa subunit of *N. crassa*. Moreover, the involvement of the ND-1 (URF-1) polypeptide is particularly demonstrated by the analysis given in Fig. 6B. The 29 kDa subunit was separately precipitated from the same assay by a specific antibody, after dissociation of the membrane preparation with SDS.

This latter strategy was also applied to the beef heart enzyme. The electrophoretic analysis (Fig. 7) shows that the ND-1 product, immunoprecipitated from beef heart mitochondria preincubated with radioactive inhibitor, contains covalently bound ligand (lane 1). The isolated polypeptide exhibits the same migration behavior as the beef heart ND-1 product visualized in (unlabelled) mitochondria by an immunodecoration technique (lane 2). The position of the ND-1 polypeptide of *N. crassa*, which migrates more slowly than its mammalian counterpart, represents an internal calibration marker (lane 3).

Specific interaction of NADH and ubiquinone with the binding of the inhibitor

In order to characterize in more detail the binding of pethidine derivatives to the individual subunits of the enzyme, isolated complex I (either from *N. crassa*, or from beef heart) was preincubated with a variety of compounds including nucleotides, ubiquinone derivatives and unlabelled inhibitors, respectively. Then, the mixtures were treated with [^{125}I]APBnp, photolyzed, and the protein was subjected to gel electrophoresis. Figure 8 shows the fluorographs. About 5000-fold molar excess of NADH over the inhibitor prevented completely the covalent binding of the radioligand to complex I. Surprisingly, the protective action of the pyridine

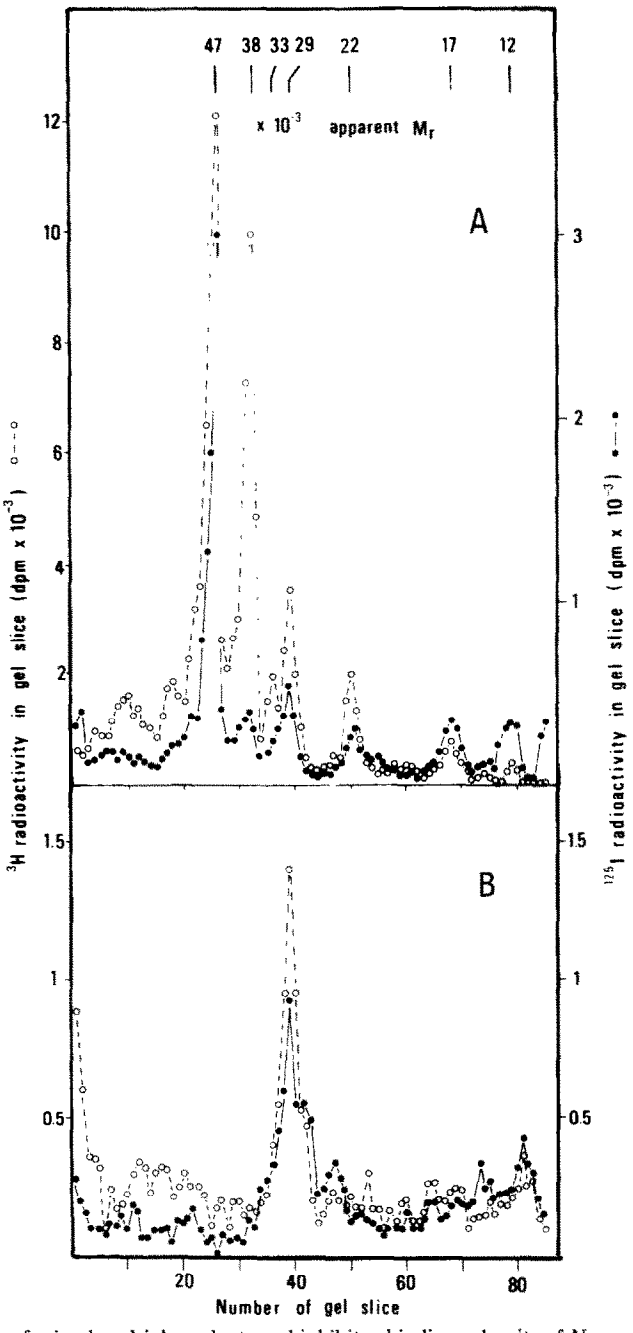

Fig. 6. Correlation of mitochondrial products and inhibitor binding subunits of *N. crassa* complex I. *N. crassa* cells were labelled with [^{3}H]leucine in the presence of cycloheximide. Mitochondrial membranes were prepared, incubated with [^{125}I]APBnp and photolyzed. One portion of the suspension was solubilized with Triton X-100 and complex I was immunoprecipitated with an antibody to the whole enzyme (panel A). The residual portion was dissociated with SDS and the ND-1 product was isolated using a subunit-specific antibody (panel B). The samples were subjected to SDS–gel electrophoresis, and the sliced gel was counted for ^{3}H- and ^{125}I-isotopes.

nucleotide was observed with all subunit species, which were radioactively labelled in the control experiment. Lower concentrations of NADH in the preincubation assay resulted in a less pronounced labelling with no obvious preference for any of the individual subunits in question (see also Fig. 9, lane 3). In contrast to NADH, the presence of other nucleotides, such as NAD, NADPH and NADP did not affect the incorporation of the pethidine analogue into subunits of complex I. On the other hand, the second substrate for the enzyme, ubiquinone, also prevented the binding of the inhibitor. However, this was only true for the more "physiological" decyl-benzoquinone derivative of ubiquinone (DB), but not for 2,3-dimethoxy-5-methyl-1,4-benzoquinone (Q_o). Fully effective concentrations

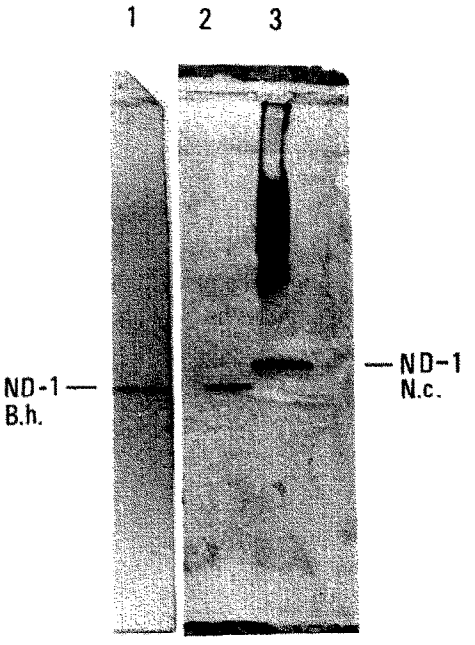

Fig. 7. Gel electrophoretic analysis of the ND-1 product from beef heart mitochondria. One-point-five milligrams of beef heart mitochondrial membranes were incubated with [^{125}I]APBnp and photolyzed. The material was dissociated with SDS and the ND-1 product was isolated by means of a subunit-specific antibody. The precipitate was applied to lane 1 of the gel. Lanes 2 and 3 contained whole mitochondrial membranes (80 μg each, solubilized with SDS) prepared from beef heart and *N. crassa*, respectively. After electrophoretic resolution, the protein in the gel was blotted onto a single sheet of nitrocellulose. Then the paper was cut between lanes 1 and 2. The first lane on the nitrocellulose was autoradiographed, the second and third were immunodecorated with anti-ND-1 serum and visualized with a gold-labelled second antibody.

for decyl-benzoquinone were in the range of 0.06–0.1 mM. Addition of lower concentrations to the preincubation assay resulted in similar effects as already described for NADH. Finally, treatment of complex I with excess of either unlabelled pethidine analogues or rotenone did not allow a covalent attachment of the inhibitor.

In a further series of otherwise identical experiments the sequence of substrate additions was reversed: first complex I preparations were incubated with [^{125}I]ABPnp and later NADH or decyl-benzoquinone was admixed at the given concentrations. Figure 9 presents examples, carried out with the *N. crassa* enzyme. The electrophoretic analysis reveals that under these conditions labelled inhibitor became associated, to a considerable extent, with the particular polypeptide subunits. A partial protective effect was observed only with decyl-benzoquinone.

In a final approach, we wanted to rule out the possibility that especially NADH or the decyl-benzoquinone derivative might react (chemically) with the inhibitor, or even precipitate the radioactive pethidine analogue. Since the latter was obviously not the case, as judged by appropriate centrifugation steps, the incubation mixtures (before and after illumination) were chromatographed on silica gel plates. No significant difference in the migration behaviour or in the intensity of the resolved radioactive spots was observed when compared to those obtained with authentic samples of [^{125}I]APBnp, treated in an identical way.

DISCUSSION

Among the chemically reactive derivatives of a particular pethidine analogue, which inhibit the activity of NADH:ubiquinone reductase in a highly specific manner [6], the azido compound appears to satisfy most of the requirements of a photoaffinity labelling reagent. (1) The derivative still retains an amphiphatic nature so that the molecule dissolves in aqueous solutions at the relevant inhibitory concentrations. This allows an effective washing of the applied biological material. (2) The probe binds both to isolated complex I and to mitochondrial membranes in a concentration-dependent manner. Nevertheless, the inhibitory potency of the derivative is negatively affected by the introduction of the azido moiety into the inhibitor molecule. Although this moderate loss of "specificity" may be tolerated in most experimental approaches, we are currently trying to improve the structural design of the affinity probe on the basis of the computer-generated model described previously [6].

The main aim of this paper is to identify the polypeptide subunit(s) of complex I, which bind the inhibitor of the reductase activity. Two approaches have been reported in the literature, applying aryl-azido-morphigenin [4], an analogue of rotenone, and, very recently, dihydrorotenone [5]. Although these compounds exhibit some unfavourable properties (extreme hydrophobic character, relatively low specific radioactivity, and/or poor coupling efficiencies upon photoactivation—see discussion in the cited papers), a predominant labelling of a 33 kDa polypeptide was observed when these derivatives were incubated with isolated complex I from beef heart [4, 5]. When investigating the same organism, both with respect to the isolated enzyme and whole mitochondrial membranes, the pethidine analogue binds to several subunits of complex I, among them a 30 kDa polypeptide.

Considering the well documented fact that the determination of apparent molecular weights depends on the particular gel systems applied, we assume that the 33 and 30 kDa bands represent the same polypeptide. This view is supported by a detailed comparison of the Coomassie staining patterns to the actual positions of this radioactive band.

Recently, Earley *et al.* [5] reported that the 33 kDa polypeptide of beef heart complex I, which had been photolabelled with [^{3}H]dihydrorotenone, most probably represents the ND-1 product. We tried to answer this question for our inhibitor-binding subunit in the 30 kDa region by means of an antiserum directed against a synthetic peptide which corresponds to a highly conserved segment of the ND-1 polypeptide [12]. The electrophoretic comigration of the precipitated ND-1 product associated with ^{125}I-labelled inhibitor and of the immunodecorated polypeptide from mitochondria, leads to the same

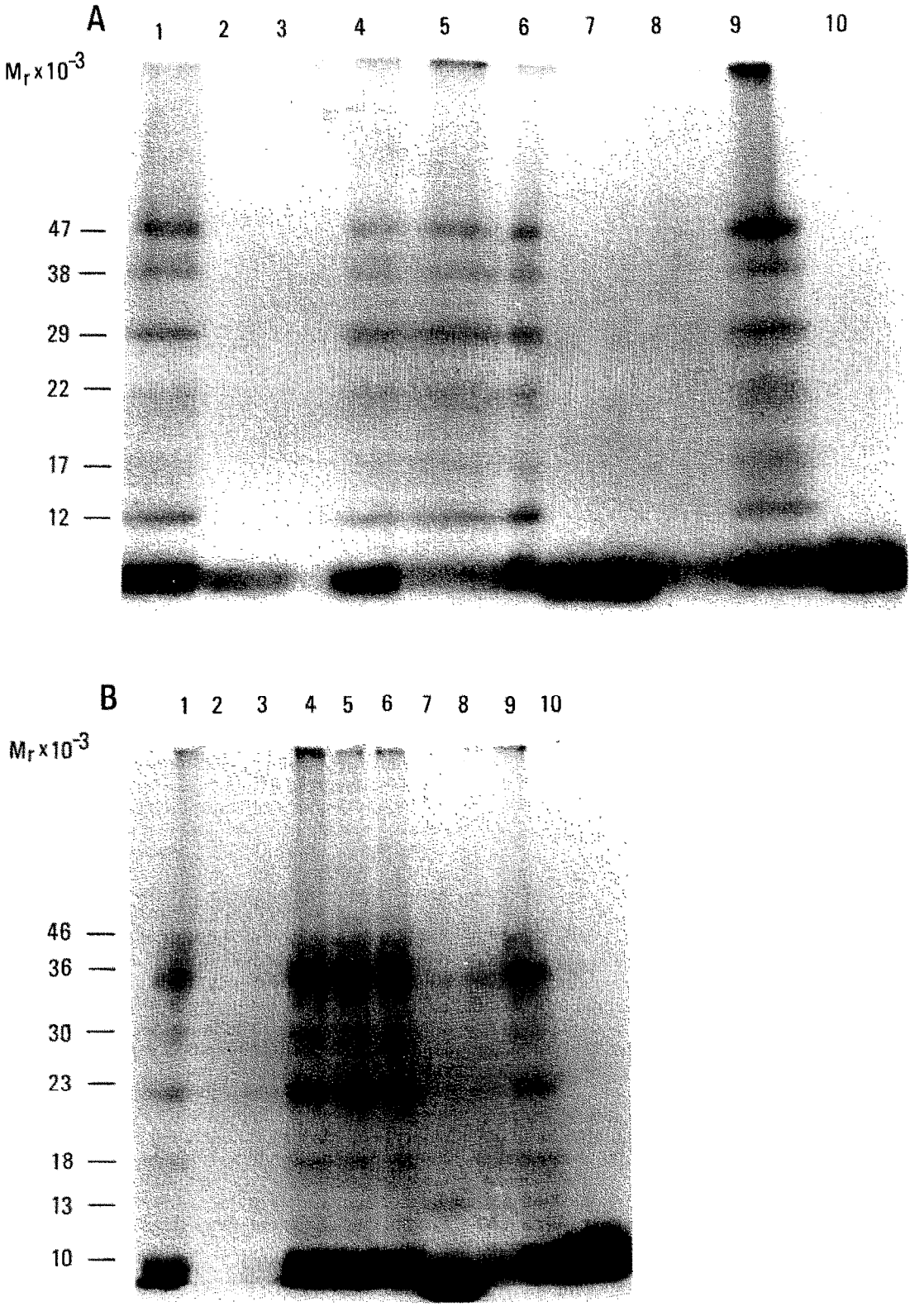

Fig. 8. The effect on the inhibitor labelling of complex I by preincubation of the enzyme with various compounds. Fifty micrograms of isolated complex I were preincubated for 15 min with one of the compounds listed below. Then, [^{125}I]APBnp was added (conc. about 0.4 μM). The mixture was photolyzed and washed as outlined in Materials and Methods. Finally, the material was subjected to SDS–gel electrophoresis and the gels were fluorographed. (A) *N. crassa* complex I: (1) no preincubation (control); (2) as in (1), but no illumination; (3) 2 mM NADH; (4) 2 mM NAD; (5) 2 mM NADPH; (6) 2 mM NADP; (7) 400 μM compound No. 49, the unlabelled nitro derivative (III) of the inhibitor; (8) 200 μM rotenone; (9) 2 mM Q_0; (10) 0.1 mM decyl-benzoquinone (DB). (B) Beef heart complex I: Loading of the gel slots was analogous to the *N. crassa* experiment described above.

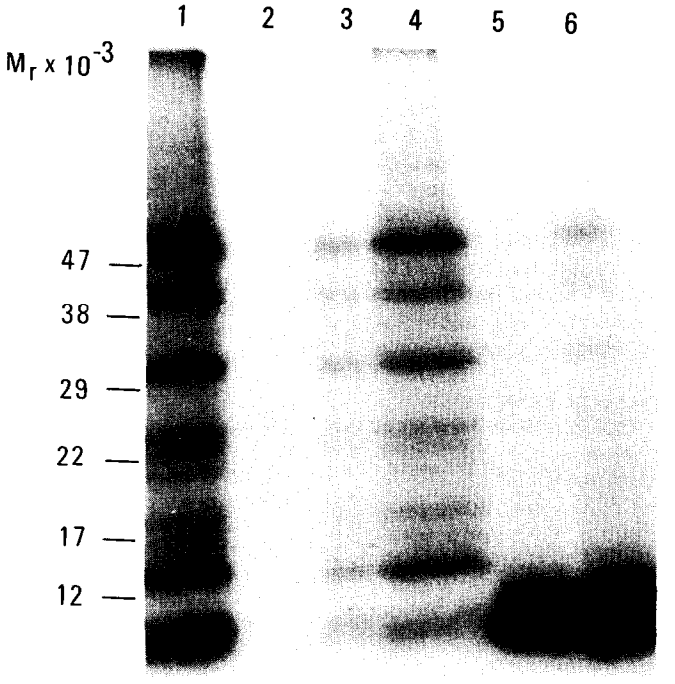

Fig. 9. Competition of NADH and decyl-ubiquinone with the inhibitor binding to complex I. Fifty micrograms of *N. crassa* complex I were incubated with NADH or decyl-ubiquinone, either prior to, or after, addition of [^{125}I]APBnp (0.4 μM): (1) treatment with inhibitor only (control); (2) as in (1), but no illumination; (3) and (5) preincubation with 0.4 mM NADH and 0.1 mM decyl-ubiquinone, respectively, then addition of the inhibitor; (4) and (6) preincubation with the inhibitor, followed by the addition of 0.4 mM NADH and 0.1 mM decyl-ubiquinone, respectively. The mixtures were processed as described in the legend of Fig. 8.

conclusion (Fig. 7). In *N. crassa* the demonstration of identity is most convincing, since in this organism it is possible to employ membranes, which include selectively labelled mitochondrial products (see double isotope labelling experiment depicted in Fig. 6).

Despite the previously mentioned similarities in the binding of rotenone and pethidine analogues to complex I, the main finding in our system is a clear affinity of a number of different subunits to the inhibitor, both in *N. crassa* and in beef heart. Instead of one predominantly labelled polypeptide [4, 5] we observe a marked incorporation of the radioactive derivative into at least 6 individual subunits. In order to rule out possible artefacts when using isolated complex I (e.g. partial denaturation of the preparations), all experiments have been repeated with whole mitochondrial membranes.

Numbers and apparent molecular weights of the labelled subunits remain largely the same. (Only one exception has been noted: the heavily labelled 36 kDa polypeptide present in isolated beef heart complex I hardly incorporates inhibitor when whole membranes are used). However, a distinct change in the relative intensities of the covalent incorporation of inhibitor into the individual subunits is observed. In *N. crassa*, for example, the 47 kDa polypeptide takes up most of the pethidine analogue under *in situ* conditions. This quantitively different distribution of inhibitor molecules can certainly be explained by the altered accessibility of the pethidine analogue to the individual constituents of the oligomeric unit upon its integration into the inner mitochondrial membrane.

The organism *N. crassa* offers an opportunity to identify these inhibitor binding subunits. This is due to the convenient labelling of the mitochrondrial products with radioactive amino acids in the presence of cycloheximide. We found that the fungal complex I contains 7 subunits of mitochondrial origin with apparent molecular weights of 47, 38, 33, 29, 22, 17 and 12 kDa. Here we disagree somewhat with the report of Ise *et al.* [9]. For instance, we do not recover significant label with a 70 kDa component, however, we find labelling of two other species (33 and 12 kDa). Moreover, the ND-1 product is not the 38 kDa subunit, but the 29 kDa polypeptide, as revealed by the immunological reactions. (A more detailed discussion on this topic will be published separately—Videira and Werner, in preparation).

The results of the double labelling experiment depicted in Fig. 6 allow a fair correlation of mitochondrially-synthesized polypeptides with components photolabelled by [^{125}I]APBnp. All six inhibitor binding subunits match mitochondrial products in *N. crassa* complex I. Only the 33 kDa polypeptide seems not to incorporate the pethidine analogue. The question of whether the inhibitor binding proteins of beef heart complex I also represent mitochondrially synthesized components can presently only be answered positively for the ND-1

product (see above). Nevertheless, in *N. crassa* it is quite clear that the ND (URF) gene products are involved in the interaction of the inhibitor with complex I. This result could point to structurally related binding domains within the different polypeptides, suggesting further similar functional roles of these mitochondrial constituents. Indeed, a considerable sequence homology among several ND proteins has been established, indicating the evolution of their genes from a single ancestral precursor by a series of duplications [20]. The high affinity of the pethidine analogues to these polypeptides may be exploited as a useful tool to approach both structural and functional aspects of the electron transfer mechanism.

The idea that several ND gene products could be involved in the electron transfer from NADH to ubiquinone is also supported by the competition experiments between the inhibitor and the enzyme substrates. The protection of the inhibitor binding sites at the individual subunits is remarkably specific both for the reduced pyridine nucleotide and the "more physiological" decyl-benzoquinone. Whereas relatively high concentrations of NADH (about 2 mM) are required to prevent completely the labelling of complex I with the inhibitor, ubiquinone derivatives seem to be considerably more effective (about 0.06 mM). The fact that prebound radioactive inhibitor can be displaced (at least partially) by decylbenzoquinone, but not by NADH (see Fig. 9), stresses the firm binding of the inhibitor to complex I, and is also an indication of a similarity in the interaction of the enzyme with coenzyme Q and the inhibitor. (A detailed kinetic study will be presented elsewhere).

Although, the pethidine analogues may well mimic parts of the structural pecularities of both substrates, the binding sites of ubiquinone and NADH need not compete directly in this process. The major binding site of NADH, for instance, was shown to be on a 51 kDa subunit of beef heart complex I [21], a component which obviously is not labelled by the inhibitor. Furthermore the enzyme kinetics data, revealing a non-competitive type of inhibition for pethidine analogues, both with respect to ubiquinone and NADH [6], suggests an indirect mechanism. Possibly, it involves conformational changes of the whole catalytic unit. The determination of the amino acid sequences participating in the binding of inhibitor and substrates (especially ubiquinone) could highlight some interesting features of this complex structure–function relationship.

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