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The Fluorescent Labeling of Mitochondrial Monoamine Oxidase

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SUMMARY

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Purified mitochondrial monoamine oxidase is specifically and irreversibly labeled by 5′-(*N*-dansyl)cadaveryl-*p*-carboxymethylpargyline, a fluorescent analogue of the drug pargyline. The dansyl moiety is bound in a polar environment and has rotational degrees of freedom independent of the macromolecule. Since monoamine oxidase is found only in the outer mitochondrial membrane, probes of this type can be used to monitor chemical events occurring there.

INTRODUCTION

Flavin-linked monoamine oxidase is an integral protein in the outer mitochondrial membrane (1). The role of this enzyme is the oxidative deamination of monoamine neurotransmitters, such as serotonin and dopamine (2). Because of the physiological importance of this enzyme, a large number of inhibitors have been developed for it. Among these are a series of highly specific irreversible inhibitors which require catalytic turnover by monoamine oxidase before the enzyme itself is inactivated. The key point is that the product of this enzymatic turnover is a highly reactive molecule. The reactive product, once generated, engages in a chemical reaction with an active site moiety, resulting in the irreversible inactivation of the enzyme (3). Included in this group of inhibitors are the β,γ-acetylenic amines, one of the most potent of which is the antidepressant drug pargyline (*N*-benzyl-*N*-methylpropargylamine) (4). It would be of interest to incorporate a physical probe into the pargyline skeleton and use the great selectivity of this inhibitor to incorporate the probe specifically into the monoamine oxidase. Since monoamine oxidase is located in the outer mitochondrial membrane, these probes would be directed specifically to this site. Depending on the kind of information required, different probes could be used. For example, fluorescent probes could be used as specific histological stains for mitochondria, and the wavelength of maximal absorption and polarization value of the probe would in addition give information about the lipid environment of the monoamine oxidase. This report demonstrates the feasibility of this method of directing probes. Specifically, it is shown that 5′-(*N*-dansyl)cadaveryl-*p*-carboxymethylpargyline (*1*) is a specific irreversible inhibitor of flavin-linked monoamine oxidase, and the binding of this probe to the purified enzyme is characterized.

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MATERIALS AND METHODS

Sources. Pargyline hydrochloride was

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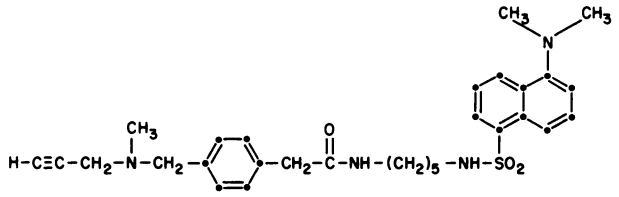
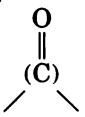


a product of Regis Chemical Company; *N*-methylpropargylamine and *p*-methylphenylacetic acid, of Aldrich Chemical Company; and dansylcadaverine, of Sigma. All products were either distilled or recrystallized before use. Silica gel thin-layer plates were products of EM Laboratories. Other chemicals were reagent grade and obtained from commercial sources.

Synthesis of DCP.[2] Dansylcadaverine (335 mg, 1 mmole) dissolved in 10 ml of dry chloroform was added over 30 min to 1 mmole (247 mg) of *p*-bromomethylethylphenylacetyl chloride [prepared by oxalyl chloride treatment of *p*-bromomethylphenylacetic acid (5)] in 10 ml of dry tetrahydrofuran containing 200 mg of K_2CO_3. The reaction was run at −10° in dry glassware under a stream of dry N_2 with stirring. After the addition was complete, the reaction mixture was allowed to warm to room temperature and remain there for an additional 30 min. The mixture was then taken to dryness on a rotary evaporator, 10 ml of water were added, and the mixture was extracted with chloroform. The chloroform layer was dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness under vacuum at 35°. The oily product was immediately treated with 10 ml of cold (0°), distilled *N*-methylpropargylamine. The solution was allowed to warm to room temperature and remain there for 3 hr, then evaporated to dryness on a rotary evaporator at water pressure. The yellow oily product was dissolved in chloroform, washed several times with 0.1 M K_2CO_3, dried over anhydrous Na_2SO_4, and taken to dryness, yielding 425 mg of the crude product as a heavy yellow oil. On thin-layer plates (EM coated silica gel plates without fluorescent indicators), a single migrating fluorescent compound was observed in a 5% (v/v) methanol-chloroform tank. The DCP was purified by preparative thin-layer chromatography on these plates. The product was isolated as a yellow-green powder. The infrared spectrum ($CHCl_3$) of DCP showed absorption at 3.1 μm (H—C≡C—), 3.4-3.6 μm (alkyl and aromatic (−H), and 6.1 μm



. The NMR spectrum ($CDCl_3$) of DCP showed a quartet (5) (benz−H) at 8.4δ, a multiplet (6) (naph−H) at 7.3δ, a multiplet (1) (NH−amido) at 5.6δ, a singlet (2) (benz−CH_2—CO—) at 3.6δ, a singlet (2) (benz−CH_2−N) at 3.5δ, a doublet (2) (C=C−CH_2−) at 3.3δ, a multiplet (4) (NH_2−CH_2−) at 2.9δ, a singlet (6) [$(NCH_3)_2$] at 2.9δ, a singlet (3) (N−CH_3) at 2.3°, a triplet (1) (H—C≡C) at 2.2δ, and a multiplet (6) [−$(CH_2)_3$−] at 1.2δ. The ultraviolet and fluorescence spectra of DCP are given under RESULTS.

$C_{30}H_{38}SO_3N_4$

Calculated:	C 67.41, H 7.12, N 10.44, S 5.99
Found:	C 66.72, H 7.11, N 11.02, S 5.85

Enzyme preparation. Purified, soluble pig liver monoamine oxidase was a generous gift of Professor Lars Oreland (6). This 2-butanone-extracted enzyme was 25% pure (2500 units/mg of protein) according to a benzylamine assay (7), which was used routinely and amounted to following the formation of benzaldehyde spectrophotometrically as a function of time. The spectrophotometric assays were conducted on a Gilford model 240 spectrophotometer. Protein concentrations were determined by the method of Lowry *et al.* (8).

[2] The abbreviation used is: DCP, 5′-(*N*-dansyl)cadaveryl-*p*-carboxymethylpargyline, where dansyl denotes 5-dimethylaminonaphthalene-1-sulfonyl, cadaverine denotes 1,5-diaminopentane, and pargyline is *N*-benzyl-*N*-methylpropargylamine.

Fluorescence measurements. All fluorescence measurements were conducted on an Aminco model SPF 1000 CS double-beam spectrofluorometer with a 500-W lamp and a Hamamatsu R818S photomultiplier. The slit widths for the excitation and emission beams were 5 nm. Neither the excitation spectra nor the emission spectra reported were corrected. Quartz fluorescence cells (Precision Scientific) had either a 2-mm excitation, 10-mm emission or a 10-mm excitation, 10-mm emission path length. Polarization measurements were conducted in mechanically stirred thermostated cuvettes. The excitation light for the polarization experiments was defined by a monochromator at 345 nm. The polarizer was set to transmit vertically polarized light. The calculated polarization was $(I_{vv} - I_{vh})/(I_{vv} + I_{vh})$. I_{vv} and I_{vh} have been defined by Weber (9). The temperature studies were conducted with a Lauda model K2/R constant temperature bath and thermostated cell holders.

Absorption spectra. Absorption spectra were determined on a Cary model 118 spectrophotometer, using quartz cells (Precision Scientific) of 10-mm path length.

RESULTS

Ultraviolet and fluorescence spectra of DCP. The ultraviolet spectrum of a 150 μM solution of DCP in 95% ethanol is shown in Fig. 1. The corresponding fluorescence excitation and emission spectra of the probe at a concentration of 2.5 μM in 95% ethanol are shown in Fig. 2. The fluorescence spectrum of the dansyl moiety is known to be solvent-sensitive. λ_{max} valves for DCP

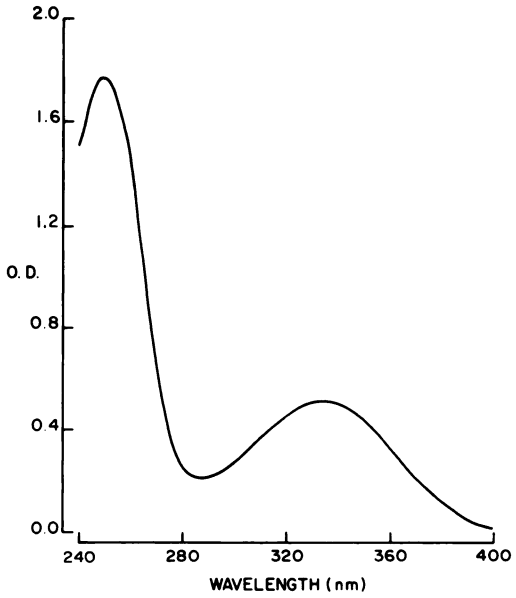


FIG. 1. *Ultraviolet spectrum of DCP*
The ultraviolet spectrum of a 150 μM solution of DCP in 95% ethanol was determined at 23°. The reference cell contained 95% ethanol.

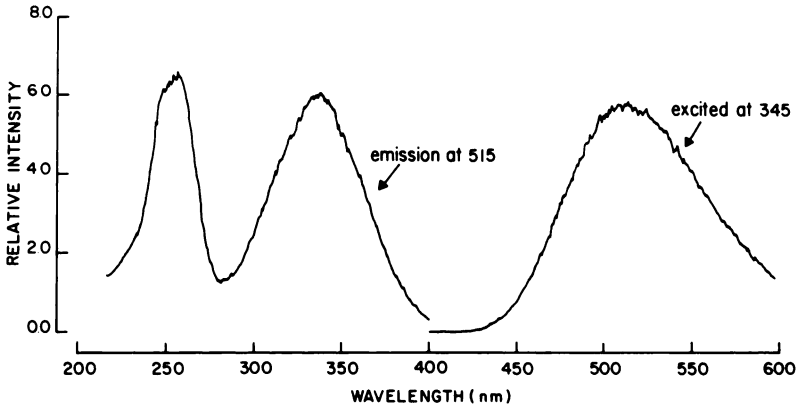


FIG. 2. *Excitation and emission spectra of DC P*
The excitation and emission spectra of a 2.5 μM solution of DCP in 95% ethanol were determined at 23°. The reference cell contained 95% ethanol, and 10 × 10 mm quartz fluorescence cells were used. The gain was set at 1, with 5-nm slit widths for excitation and emission. The emission spectrum was run with a 345 nm excitation wavelength, and the excitation spectrum was run monitoring the 515 nm wavelength emission.

TABLE 1

Emission and absorption maxima of DCP as a function of solvent

Solutions of DCP, at 2 μM for emission spectra or at 150 μM for absorption measurements, were made up in the solvents listed, and absorption and emission maxima were determined, the latter at an excitation wavelength of 345 nm. The temperature was maintained at 23°.

Solvent	λ_{max}	
	Emission	Absorption
	nm	*nm*
Dioxane	489	324
Chloroform	498	320
95% ethanol	515	325
0.005 M potassium phosphate, pH 7.5	555	330

emission in various solvents are given in Table 1. Excitation was at 345 nm in all cases. In highly nonpolar solvents such as hexane, DCP tends to aggregate, yielding uninterpretable spectra. λ_{max} values of absorption spectra in various solvents are also given in Table 1.

Irreversible inhibition of monoamine oxidase by DCP. Purified, soluble monoamine oxidase is rapidly and irreversibly inactivated by DCP (Fig. 3) at concentrations of 1 and 3 μM. The apparent biphasic structure of the inactivation curve may be either due to isoenzymes of monoamine oxidase or, possibly, to the oligomeric nature of the purified enzyme in solution. Monoamine oxidase is, of course, a membrane-bound protein. It is liberated from the membrane by delipidation with 2-butanone. Detergent is not used, so that the enzyme tends toward higher oligomers, with the molecular weights of these complexes ranging from 2×10^5 to 10^6 (6). It should be noted that the same biphasic inhibition curve is observed when pargyline itself is used as the inhibitor. The activity of the irreversibly inactivated enzyme cannot be regained in the least by continued dialysis.

Ultraviolet spectrum of DCP-inactivated monoamine oxidase. It has been well established that pargyline characteristically changes the flavin spectrum of monoamine oxidase as a consequence of the chemical reaction between the oxidized drug and the reduced flavin (10, 11). If DCP interacts with the enzyme as expected, this change in the flavin spectrum should be exhibited, as was the case. The ultraviolet spectrum of the DCP-labeled enzyme described above is shown in Fig. 4. The same spectral change was observed upon inhibition of the enzyme with pargyline (Fig. 4).

Fluorescence spectrum of DCP-inactivated enzyme. The DCP-inactivated enzyme was thoroughly dialyzed to remove

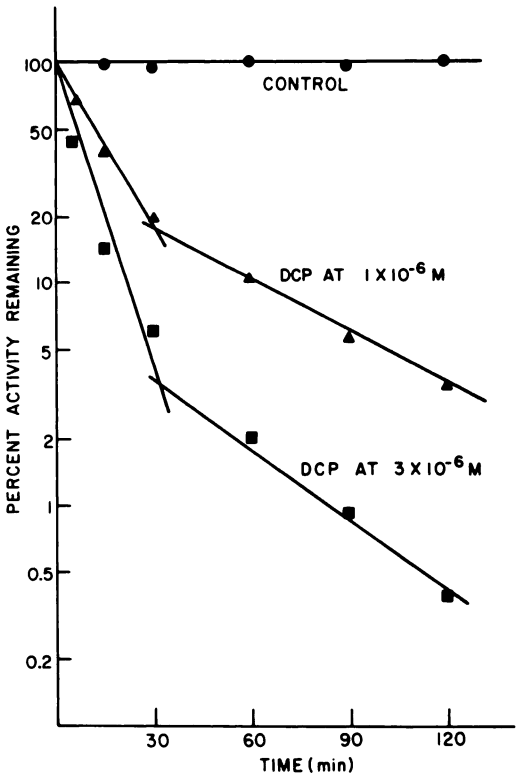


FIG. 3. *Irreversible inhibition of monoamine oxidase by DCP*

Enzyme (0.56 mg/ml) in 0.1 ml of 0.005 M potassium phosphate buffer, pH 7.5, was incubated with 1 μM DCP at 37°. At the times indicated, 0.01-ml aliquots were removed and immediately added to 3 ml of 3.3 mM benzylamine in the phosphate buffer. The rate of oxidation of benzylamine to benzaldehyde was followed spectrophotometrically. Enzyme which proved to be inactive by this assay also proved to be inactive for the oxidation of serotonin and dopamine. The inactivated enzyme was not in the least reactivated by continuous dialysis for 2 days against 10 changes of the phosphate buffer.

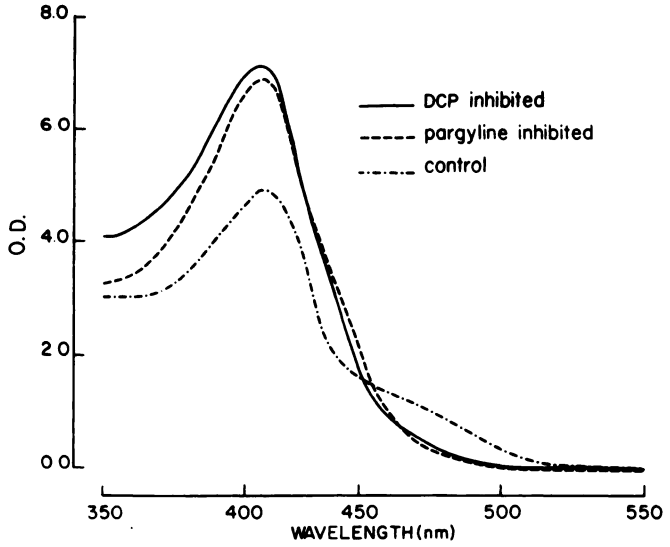


FIG. 4. *Ultraviolet spectrum of DCP-inactivated monoamine oxidase*

Three samples of the enzyme (1 mg/ml) were prepared in 1 ml of 0.005 M phosphate buffer, pH 7.5. The first sample was treated with 100 μM pargyline for 40 min at 37°. The second sample was treated with 10 μM DCP at 37° for 40 min, after which the enzyme proved to be inactive. The third sample was untreated. All three samples were placed in dialysis sacs and dialyzed independently against 1 liter of the phosphate buffer. The dialysis was extended over 2 days at 4°, with four buffer changes. After equalizing the protein concentrations of these three samples, their ultraviolet spectra were determined. The final protein concentration in each cuvette was 0.8 mg/ml.

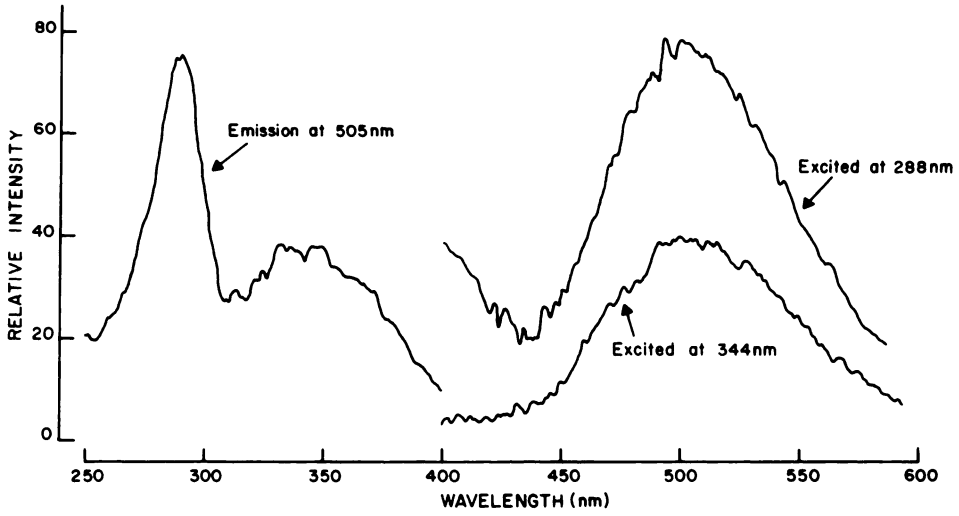


FIG. 5. *Fluorescence spectrum of DCP-inactivated monoamine oxidase*

The excitation and emission spectra of the same enzyme preparation as used in Fig. 4 were determined independently. Both the pargyline-treated and the blank showed negligible fluorescence. The fluorescence spectrum of the DCP-inactivated enzyme (0.8 mg/ml) is shown run against the pargyline-treated enzyme in the reference cuvette at 25°. In a separate experiment it was found that enzyme treated with pargyline and then incubated with DCP did not show any dansyl fluorescence. The spectrum was run in 10 × 10 mm quartz cuvettes at excitation and emission slit widths of 5 nm.

all noncovalently bound inhibitor. Its excitation and emission spectra were recorded (Fig. 5). Enzyme treated with pargyline, then subjected to DCP under standard labeling conditions, did not show any incorporation of dansyl fluorescence. In fact, this sample was used as a reference in the double-beam fluorescence spectrometer. The fluorescence spectrum of the DCP-inactivated enzyme yielded two observations. First, the excitation spectrum (emission at 505 nm) is more complicated than that of the probe by itself. Excitation at 288 nm is not characteristic of the probe but is characteristic of tryptophan (12). This suggests that singlet energy transfer must occur between an excited tryptophan residue and the dansyl moiety. Furthermore, it is obvious from the spectrum that the quantum yield for this transfer is greater than that for direct excitation (344 nm). This is not unusual (12). This result means that tryptophan residues must occur within approximately 50 Å of the bound dansyl probe (13). The second observation relates to the polarity of the environment of the probe. As shown earlier, the emission spectrum of DCP is solvent-dependent. The λ_{max} of the emission of the enzyme-bound probe is 505 nm. This suggests that the environment of the probe is approximately as polar as ethanol.

Polarization studies on DCP-inactivated enzyme. In order to determine whether the probe was bound tightly to the enzyme or was relatively free to rotate, the polarization of the enzyme-bound probe was measured as a function of T/η (14). Plots of $1/p$ vs. T/η are shown in Fig. 6, in which both temperature and viscosity were independently varied. The viscosities were increased by the addition of sucrose to the

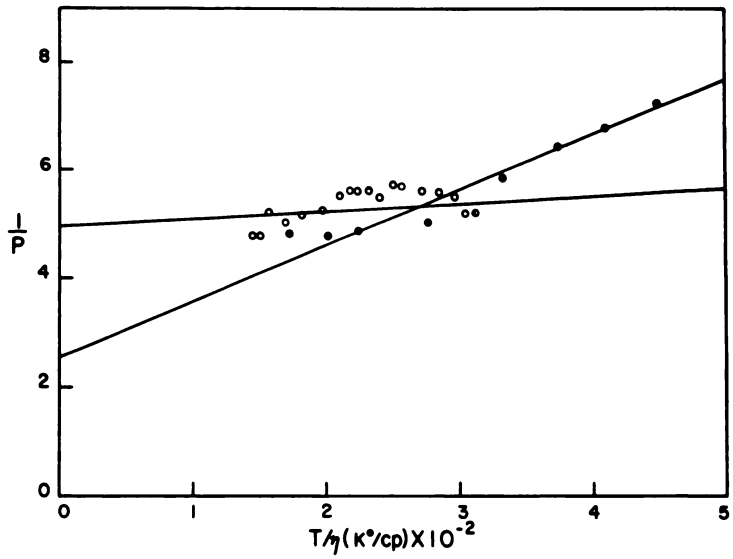


FIG. 6. *Polarization studies on DCP-inactivated enzyme*

DCP-labeled monoamine oxidase at a concentration of 0.5 mg/ml in 0.005 M phosphate buffer, pH 7.5, was used in these experiments. The depolarization of vertically polarized light was calculated according to $p = (I_{vv} - I_{vh})/(I_{vv} + I_{vh})$. The depolarization was measured both by varying the temperature (●) between 6° and 38° and by altering the viscosity (○) of the buffer by adding successively increasing amounts of 50% sucrose. The viscosities of these solutions were determined independently. The studies of depolarization vs. viscosity were carried out isothermally at 22°. All studies were carried out in 10 × 10 mm quartz cells with stirring. The excitation wavelength was 345 nm, and the emission wavelength was 505 nm. Both the emission and excitation bandwidths were 20 nm. No depolarization was observable when the excitation wavelength was 288 nm. In addition, the polarization value for DCP itself in the phosphate buffer at 22° was 0.04. The polarization values measured for the DCP-inactivated enzyme were also shown not to be concentration-dependent.

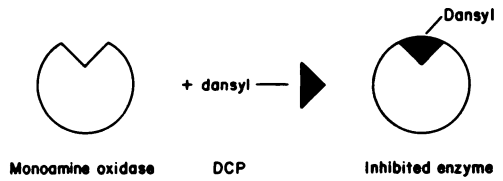
cuvette containing the labeled enzyme. At lower values of T/η no slope was observed. At higher temperature, however, a slope could be measured with an extrapolated p_0 of 0.4. This latter value is probably due to localized rotation of the dansyl moiety independently of the protein as a whole. The flat part of the curve indicates that the dansyl probe cannot be used to measure the rotational relaxation time of a protein which has as high a molecular weight as monoamine oxidase. Under the conditions of these experiments, the enzyme was excluded by a Sephadex G-200 column, indicating that molecular aggregates of at least 200,000 mol wt were present. Very high concentration of cholic acid will eventually break down the complexes into 60,000 mol wt monomers (6).

DISCUSSION

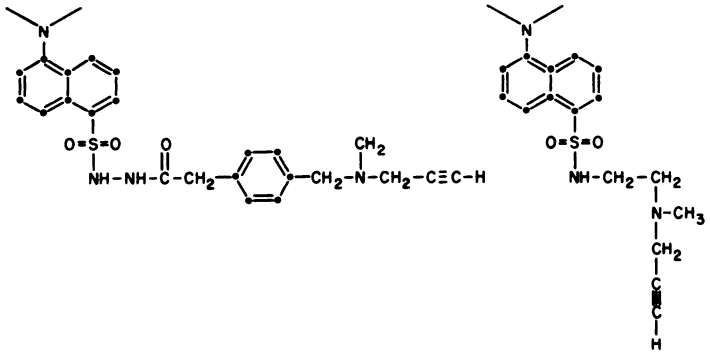
The covalent introduction of fluorescent and spin-label probes into biological macromolecules has led to an understanding of many of the structural parameters underlying their biological function. One problem in the use of covalent probes of this type has been their relative lack of specificity (15). In general, these compounds are made by attaching a chemically reactive functional group, for example, bromoketone, to the probe being used. Since the chemically reactive functional groups are generally reactive to nucleophiles, they do not show specificity when introduced into biological systems. Consequently heterogeneous labeling occurs, and the average of many signals is observed. This severely limits the usefulness of the information obtained. On the other hand, irreversible enzyme inhibitors that require catalytic activation by the target enzymes are highly specific (3). Inhibitors of this type would obviously offer many advantages over the conventional affinity-labeling reagents for the specific introduction of an environmentally sensitive probe into an enzyme. In the example shown here, a dansyl probe is incorporated via a spacer moiety into pargyline. The resultant compound, DCP, is still a highly potent irreversible inhibitor of flavin-linked monoamine oxidase.

Structure-activity studies showed that the *para* position of pargyline is open to substitution and that the bulky dansyl group can be attached to this position when an appropriate spacer group is used. *Para*-substituted pargyline derivatives, such as DCP, are still quite potent irreversible inhibitors of flavin-linked monoamine oxidase, effectively inhibiting the enzyme when used in the micromolar range. The dansyl moiety of the DCP-inhibited enzyme finds itself in a relatively polar environment and has rotational degrees of freedom independent of the macromolecule. The former conclusion is based on the λ_{max} of emission of the enzyme-bound probe, and the latter conclusion is based on the depolarization studies. The relatively short lifetime of the dansyl group is such that it could not measure the rotational correlation time of a macromolecule the size of monoamine oxidase in solution. Therefore altering the viscosity of the labeled enzyme solution does not change the observed polarization value. Only at higher temperatures is a slope observable in the plot of $1/p$ vs T/η. At the higher temperatures the local rotation of the dansyl moiety is increased. This motion is independent of the rotation of the macromolecule as a whole. The kind of effect observed here finds analogy in the seminal work of Weber and his colleagues on the dansyl chloride labeling of thyroglobin, a protein of 600,000 mol wt (16). In this case also, alteration of the viscosity of the medium by successive addition of sucrose does not change the observed polarization value. However, at higher temperatures a slope in the $1/p$ vs. T/η plot is observed (16). Weber attributed this latter slope to thermally activated local rotations of the dansyl moiety.

The observations above suggest that the dansyl moiety of DCP binds to the enzyme as shown in Scheme 1. Since the distance

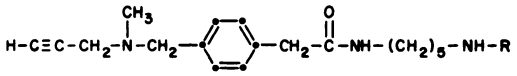


SCHEME 1



between the acetylenic moiety and the dansyl group is considerable, it would be surprising if the probe were found to be bound rigidly at the active site. Hence the dansyl probe really samples the environment of the enzyme and not the active site region. Further evidence for this conclusion is based on the structure-activity relationships found with probes similar to DCP. Analogues *2* and *3* have been found to be ineffective irreversible inhibitors of monoamine oxidase when assayed at 100 μM for 2 hr.[3] Compound *2* is similar to DCP but lacks the 5-carbon extender present in DCP. This molecule is synthesized in the same way as DCP, save for the substitution of dansylhydrazine for dansylcadaverine in the synthesis. The lack of inhibitory activity of this compound toward monoamine oxidase is thought to be a ramification of the fact that dansyl does not fit into the active site region. Compound *3*, which is synthesized by condensing *N*-methylpropargylamine with dansylaziridine, is also thought to be a weak inhibitor, for similar reasons. Furthermore, probes other than dansyl can be used when the 5-carbon spacer is placed between the phenylacetic acid moiety and the probe itself. For example, the tricyclic derivatives made with fluorescein and rhodamine are essentially as potent as DCP as inhibitors of monoamine oxidase.[3] Since these groupings are chemically very different from dansyl, it must be assumed that the probe moiety of the inhibitor does not bind specifically to the active site region but hangs more or less freely from the enzyme.

[3] R. R. Rando, unpublished observations.



The finding that the probe moiety of DCP is not bound at the active site region of monoamine oxidase is very important. As mentioned earlier, flavin-linked monoamine oxidase is an integral protein in the outer mitochondrial membrane. These probes can then be used to sample the immediate environment of the enzyme in the lipid matrix. The DCP inhibitor rapidly and irreversibly inhibits monoamine oxidase in intact mitochondria.[3] This means that the outer mitochondrial membrane can be specifically and irreversibly labeled with a host of biophysical probes. The question of the role of the membrane in modulating the substrate specificities of monoamine oxidase could be answered by using the appropriate probe (17, 18). Furthermore, it would clearly be of interest to insert a pH-sensitive probe into the 5-carbon-spaced pargyline derivative and incorporate it into the outer mitochondrial membrane. The pH at the outer membrane could then be measured. This would be of interest with reference to the question of proton gradients and oxidative phosphorylation. The great advantage of using monoamine oxidase as the receptor for these probes is that its histology is well understood so that one knows exactly where the probe is being attached. Since the probe is exceedingly specific, it can be used at low concentrations and will not interfere with other biological processes.

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