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PHOTOLABELLING OF DOPAMINE-β-HYDROXYLASE BY BLEOMYCIN

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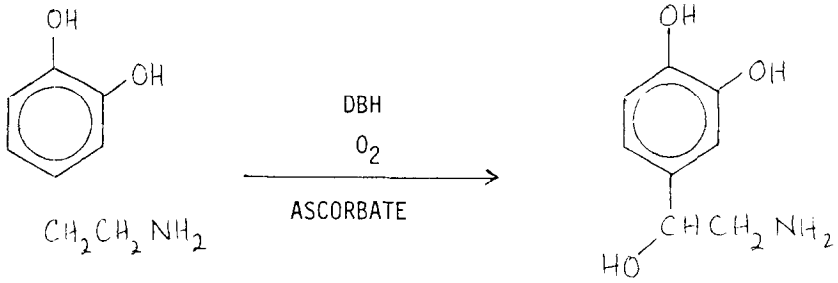
Summary: Irradiation of a mixture of dopamine-β-hydroxylase and bleomycin with light of wavelengths >300 nm. leads to irreversible inhibition which is not prevented in the presence of tyramine. Prephotolysis of bleomycin followed by incubation (in the dark) with dopamine-β-hydroxylase did not lead to irreversible inhibition. Thus bleomycin is a photolabel of this enzyme.

Dopamine-β-Hydroxylase, DBH (EC 1.14.17.1), catalyses the conversion of dopamine to norepinephrine (equation (1)). This enzyme is of interest in view of its position in the final step of the



DOPAMINE NOREPINEPHRINE

biosynthesis of the hormone/neuro-transmitter, norepinephrine and removal of dopamine (1). Interest in it also comes from its release into cerebrospinal fluid and serum during nervous activity (2), its localisation in specific chromaffin granules in the adrenal

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Abbreviations: DBH, dopamine β-hydroxylase; BLM, bleomycin.

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gland and in synaptic vesicles in the noradrenergic nervous system (3,4).

A number of reversible inhibitors of DBH have been described (5) as well as a few covalent inhibitors (1,5). A recent report (6) of powerful, reversible inhibition of DBH by bleomycin (50% inhibition at ~ 8×10^{-8}M) prompted us to investigate the possibility of using bleomycin (BLM) as a photolabel of DBH in view of the recent discovery of the extreme photosensitivity of BLM (7). We report below some preliminary findings in this area.

Materials and Methods

Blenoxane (bleomycin sulphate Lot. No. BOX 06) obtained from Bristol Laboratories, was dissolved in sodium acetate buffer (0.2M, pH 5.0). DBH (from bovine adrenals) was from Sigma Chemical Company, (specific activity, 2.6 Units/mg). A dual-wavelength spectrophotometric assay was employed (8,9). The incubations were carried out at pH 5.0, 37°C for 45 minutes and the assay solution contained 100 µL of buffer (2M. sodium acetate), 150 µL of N-ethylmaleimide (0.2M), 25 µL of catalase (25 mg/mL), 25 µL of pargyline hydrochloride (40 mM), 50 µL of ascorbic acid (0.2M), 50 µL of tyramine hydrochloride (0.4M) and 50 µL of sodium fumarate (0.2M).

Spectrophotometric measurements were made on a Carlo-Erba Spectracomp. 601 or Pye-Unicam SP8-100 UV-Visible spectrophotometer. Photolyses were conducted using an Applied Photophysics quantum yield photoreactor with a medium pressure mercury lamp. The light from the source was focussed on a quartz cell, thermostatted by means of a Dewar flask with a quartz window. Light of wavelengths >300 nm was selected by means of a tin chloride chemical filter solution (10). DBH (1.43 units) and BLM (9.62×10^{-5}M) were photoirradiated together for one minute at 4°C in a quartz tube. Protein was reisolated by gel filtration (Sephadex G-50, 27 x 1.3 cm). Eluted material was collected by means of a Volumetric Minipuls 2 Gilson pump and fraction collector, arranged such that protein eluted reproducibly from run to run. Fractions were analysed in terms of absorbances at 280 nm and dopamine-β-hydroxylase activity. Enzyme similarly photoirradiated in the absence of BLM was used as a control.

The effect of BLM (5.00×10^{-7}M) on DBH in the presence of tyramine (0.1M) was also investigated by following a similar procedure. The possibility of inhibition of DBH by the products of BLM photolysis was also considered. BLM (9.62×10^{-5}M) was photolysed (1 minute, 4°C) in a quartz tube and then incubated with DBH in the dark for 40 minutes. During this period aliquots of the mixture were withdrawn at intervals and assayed for enzyme activity.

Results

In agreement with a published report (6), bleomycin inhibited bovine adrenal gland dopamine-β-hydroxylase at pH 5.0, 37°C without photoirradiation, showing 50% inhibition at 10^{-7}M BLM (substrate 0.02M).

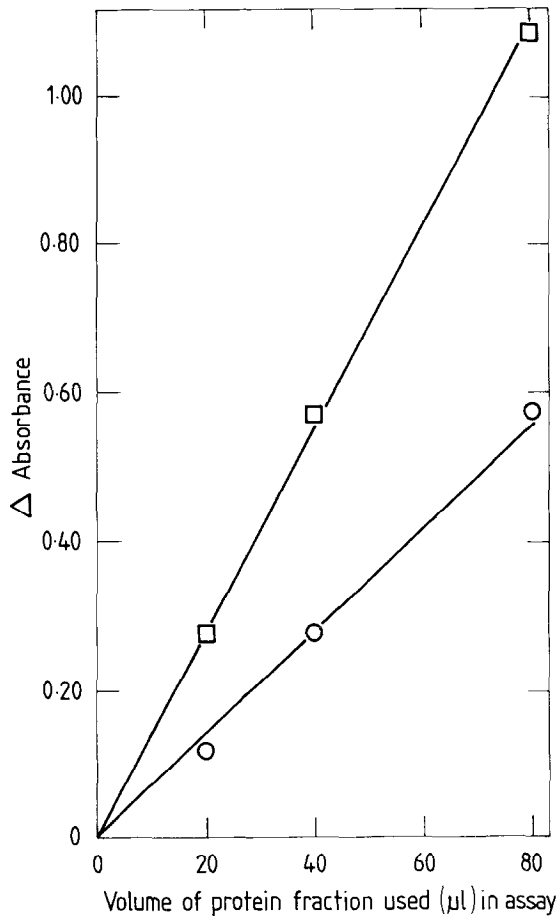

Figure 1 Assay results for one sample of DBH after photoirradiation as described in the text followed by G-50 gel filtration reisolation of protein (- - -, control enzyme; - - -, enzyme plus bleomycin). The points are experimental, lines are by least squares regression analysis through the origin, the relative slopes giving the degree of inactivation (see text).

An unphotoirradiated mixture of DBH and BLM was found to be completely resolved using the G-50 gel filtration procedure described and the activity of the enzyme was fully retained after gel filtration. Enzyme (2.6 units/mg) was found to be fully active after irradiation for 1 minute (4°C, pH 5.0) with light of wavelengths >300 nm, at a distance of 15 cm. from the source.

Photolysis of BLM (9.62×10^{-5}M) in the presence of DBH (1.43 units) at 4°C led to irreversible inhibition (Figure 1). Photoirradiation of

BLM with DBH and photoirradiation of the enzyme alone (as a control) was carried out separately (for 1 minute, at $4^{o}C$ and at a distance of 15 cm from the source). These reaction mixtures after G-50 gel-filtration over a calibrated column, gave protein fractions with similar values of absorbances at 280 nm, 0.061 and 0.063, respectively. After assaying each for DBH activity at three enzyme concentrations 46.5 ± 3.0% inhibition was observed (Figure 1), based on duplicate runs.

The effect of incubating pre-photolysed BLM ($4.81 \times 10^{-5}M$, exposed for 1 minute, 15 cm from the source at $4^{o}C$) with unphotoirradiated enzyme (2.6 units/mg) led to no irreversible loss in activity even after 40 mins. For control enzyme assay velocities (normalised) at 0, 10, 20 and 40 minutes the mean activity was 1.00 ± 0.04 and for DBH incubated with prephotolysed BLM the mean activity of measurements at similar times was 1.04 ± 0.09 (using control velocity normalised to 1.00).

Discussion

Clearly dopamine-β-hydroxylase is strongly inhibited by bleomycin (with 50% inhibition at 80 nM (6)) and 100 nM (this work under dark conditions). This inhibition , readily reversed by dialysis (6) or by simple gel filtration,may represent chelation of the active-site copper atoms of DBH (11). We have found that brief exposure of a BLM-DBH mixture to light (>300 nm) leads to 46.5% inhibition,irreversible after G-50 passage. Under these conditions, the enzyme alone retains full activity on photoirradiation. The inhibition is apparently due to primary photoproduct(s) from BLM or a very strong short-lived photolysis product generated at and trapped by the enzyme's BLM-binding site because the photoproducts of BLM (1 min photoexposure) led to no irreversible inactivation, even on prolonged incubation with DBH. Consequently, BLM is behaving as a photolabel for this enzyme. It is perhaps unwise to call it a photoaffinity label, as bleomycin although a powerful DBH inhibitor, bears no obvious structure resemblance to the natural substrate(s) or cofactors

for this enzyme. It is interesting that the photolabelling of DBH by BLM was not prevented by high concentrations of substrate (tyramine) in the medium.

Although the primary photolytic processes for BLM have not yet been clarified, it is likely that the bithiazole moeity is the photoreactive centre (7) and some degree of analogy may lie in the photoreactions of 2-benzoylamino-Δ^2-thiazoline, which photolyses with C-S bond cleavage (12).

This irreversible labelling of DBH by BLM is probably caused by covalent binding, although an extremely tight-binding inhibition (with a low rate of dissociation of inhibitor from the enzyme-inhibitor complex) cannot be excluded yet. In view of the fact that the attack of BLM on plasmid DNA is affected by the presence or absence of light (13), the present photolabelling of a biological macromolecule (DNH) by BLM on photoexposure suggests BLM as a potential photolabel for other biological macromolecules/systems.

Acknowledgements

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References

1. Villafranca, J.J. (1981) in "Copper Proteins", pp 265-289 Ed. T.G. Spiro, Wiley-Interscience, New York.

2. De Schaepdryver, A.F., Moerman, E.S. amd Smith, A.D. (1969) J. Physiol (London) 204, 102-104.

3. Hortnagl, H., Winkler, H. and Lochs, H. (1972) Biochem. J. 129, 187-195.

4. Levitt, M., Spector, S., Sjoerdsra, A. and Undenfriend, S. (1965) J. Pharmacol. Exp. Therap. 148, 1-6.

5. Creveling, C.R., Daly, J.W., Witkop, B. and Undenfriend, S. (1962) Biochim. Biophys. Acta 64, 125-131.

6. Matsui, H., Kato, T., Yamamoto, C., Takita, T., Takeuchi, T., Umezawa, H. and Nagatsu, T. (1980) J. Antibiotics 33(4), 435-440.
7. Thakrar, N. and Douglas, K.T. (1981) Cancer Letters 13, 265-268.
8. Kato, T., Wakui, Y., Nagatsu, T. and Ahnishi, T. (1978) Biochem. Pharmacol. 27, 829-831.
9. Kato, T., Kuzuya, H. and Nagatsu, T. (1974) Biochem. Med. 10, 320-328.
10. Zimmermann, H.E. (1971) Mol. Photochem. 3, 281-292.
11. Friedman, S. and Kaufman, S. (1965) J. Biol. Chem. 240, 552-555.
12. Corval, A., Gaudi, D., Jardon, P. and Gautron, R. (1982) Photochem. Photobiol. 36, 489-490.
13. Douglas, K.T., Thakrar, N., Minter, S.J., Davies, R.W. and Scazzochio, C. (1982) Cancer Letters, 16, 339-345.