Autocatalytic alkylation of the cytochrome *P*-450 prosthetic haem group by 1-aminobenzotriazole

Isolation of an *NN*-bridged benzyne*–protoporphyrin IX adduct

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Destruction of hepatic cytochrome *P*-450 during catalytic processing of 1-aminobenzotriazole is accompanied by an equal loss of microsomal haem but not by loss of cytochrome *b*₅, or stimulation of lipid peroxidation. An abnormal porphyrin, tentatively identified as an *NN*-bridged benzyne–protoporphyrin IX adduct, appears to be formed by the addition of catalytically generated benzyne to prosthetic haem.

Recent studies in this and other laboratories have shown that olefins (De Matteis, 1978; Ortiz de Montellano & Mico, 1980*a*), acetylenes (White, 1978; Ortiz de Montellano & Kunze, 1980*a*), allenes (Ortiz de Montellano & Kunze, 1980*b*) and 1,2,3-benzothiadiazoles (Ortiz de Montellano & Mathews, 1981) destroy cytochrome *P*-450 enzymes engaged in their metabolism through highly specific 'suicidal' (Ortiz de Montellano & Mico, 1980*b*) interactions. A rational search for structures able to inactivate cytochrome *P*-450 through a catalysis-driven interaction led us to investigate 1-aminobenzotriazole, a heterocycle that yields benzyne upon mild chemical oxidation (Campbell & Rees, 1969). The oxidative fragility of 1-aminobenzotriazole, the well-established ability of cytochrome *P*-450 to oxidize amines, and the exceptional chemical reactivity of benzyne made the investigation of 1-aminobenzotriazole a highly promising venture. The fulfilment of this promise is reported here.

Materials and methods

Chemicals

The synthesis of 1-aminobenzotriazole was accomplished as described by Campbell & Rees (1969). The mixture of *NN*-dimethylprotoporphyrin IX (dimethyl ester) isomers synthesized as described previously (Ortiz de Montellano *et al.*, 1980), was provided by Mr. K. L. Kunze of this laboratory.

* The term 'benzyne' indicates the presence of a triple bond and is used in the text to describe the formal reactive species that adds, by a 1,2-benzo linkage, to haem.

Incubations with hepatic microsomes (microsomal fractions)

Washed hepatic microsomes from phenobarbital-pretreated Sprague–Dawley male rats were prepared as previously described (Ortiz de Montellano *et al.*, 1979). Microsomes were suspended at a 1 mg/ml protein concentration in 0.1 M-sodium/potassium phosphate buffer, pH 7.4, containing KCl (150 mM) and EDTA (1.5 mM). Incubations with various concentrations of 1-aminobenzotriazole (see Table 1 below), initiated by addition of 1 mM-NADPH to the rest of the mixture after a 3 min preincubation period, were performed at 37°C. Portions withdrawn after 10 or 30 min of incubation were used for cytochrome *P*-450, cytochrome *b*₅, haem, and malonaldehyde determinations. Similar control incubations were performed in the absence of either NADPH or 1-aminobenzotriazole. In the appropriate experiments, glutathione (10 mM) or CO was included in the incubation.

Assay procedures

Cytochrome *P*-450 concentrations were measured by difference spectroscopy on an Aminco DW-2a instrument as previously described (Ortiz de Montellano *et al.*, 1979; Estabrook *et al.*, 1972). A molar absorption coefficient of 100 mm⁻¹.cm⁻¹ was used for the absorbance difference between 450 and 520 nm in order to calculate cytochrome *P*-450 concentrations. Cytochrome *b*₅ was assayed as described by Omura & Sato (1964). Microsomal haem was measured by the pyridine haemochromogen method also as described by Omura & Sato (1964), by using a molar absorption coefficient of 34.4 cm⁻¹.mM⁻¹ for the 557–600 nm absorbance

difference (Waterman, 1978). Malonaldehyde concentrations were measured as described by Asakawa & Matsushita (1980). Protein concentrations were determined by the Lowry procedure (Lowry *et al.*, 1951), human serum albumin being used as the standard. A difference binding spectrum for 1-aminobenzotriazole was obtained by adding a 10 mM concentration of this agent to one of two spectroscopically balanced cuvettes containing microsomes (1 mg/ml) in 0.1 M-sodium/potassium phosphate buffer, pH 7.4, (Estabrook *et al.*, 1972; Ortiz de Montellano & Mico, 1980a).

Hepatic-pigment isolation and characterization

The procedure for extraction of hepatic pigments has been described (Ortiz de Montellano & Mico, 1980a). In brief, phenobarbital-pretreated (80 mg/kg, 4 days) Sprague-Dawley male rats were injected intraperitoneally with 1-aminobenzotriazole (100 mg/kg) dissolved in 1 ml of water. The rats were decapitated 4 h later and their livers, perfused *in situ* with ice-cold iso-osmotic 0.9% NaCl solution, were homogenized and added to H₂SO₄/methanol (1:19, v/v). After the homogenates had been left overnight at 4°C, equal volumes of water and methylene chloride were added and the organic layer was washed thrice with water. The residue obtained after drying the solution and removing the solvent was taken up in zinc acetate-saturated chloroform, and was chromatographed on a preparative silica-gel plate with chloroform/acetone (3:1, v/v). Two green (red fluorescing) bands were thus isolated, the minor one with an R_F value of 0.9 and the major one with an R_F value of 0.1. The major fraction, extracted with methanol, was subjected to high-pressure liquid chromatography on a 4.6 mm × 250 mm Whatman Partisil 10-PAC column eluted with a 15 min 0–100% exponential gradient of methanol in methylene chloride (detector set at 417 nm). The green (red-fluorescing) porphyrin emerged as a single peak. Electronic absorption spectra were recorded on a Cary-Varian 118 spectrophotometer. Field-desorption mass spectra were obtained on a Kratos/

AEI MS 902 instrument as previously reported (Ortiz de Montellano *et al.*, 1980).

Results

Destruction of cytochrome P-450

The cytochrome P-450 content of hepatic microsomes from phenobarbital-pretreated rats decreases precipitously on incubation with 1-aminobenzotriazole in the presence of NADPH, although no enzyme is lost if either NADPH or 1-aminobenzotriazole is withheld from the incubation (Table 1). Incubation of microsomes with 10 mM-1-aminobenzotriazole resulted in an 81% loss of cytochrome P-450 in the first 10 min and a further 5% loss in the subsequent 20 min. The bulk of the enzyme is thus lost in the first few minutes, an observation confirmed by even shorter (1–2 min) incubations (results not shown). Lower concentrations of 1-aminobenzotriazole also rapidly destroyed the enzyme (Table 1).

The loss of cytochrome P-450 *in vitro*, which was inhibited by CO (Table 1), was matched by an approximately equimolar loss of microsomal haem (Table 2). Cytochrome *b₅* concentrations, however, were unaffected (Table 2). No protection was afforded to cytochrome P-450 by inclusion of 10 mM-glutathione in the incubation mixture (Table 1), a result that argues against the involvement of a diffusible electrophilic species. This conclusion was strengthened by the finding that malonaldehyde concentration, and consequently lipid peroxidation, were not enhanced by 1-aminobenzotriazole. The malonaldehyde concentration thus was the same in microsomes before ($1.6 \pm 0.2 \mu\text{M}$) and after ($1.4 \pm 0.1 \mu\text{M}$) incubation for 30 min with 10 mM-1-aminobenzotriazole. Similar incubation with carbon tetrachloride raised the malonaldehyde concentration to $49.3 \pm 2.1 \mu\text{M}$, as expected. A typical type-II difference spectrum (Estabrook *et al.*, 1972), with a maximum at 426 nm and a minimum at 397 nm, was observed on addition of 1-aminobenzotriazole (10 mM) to hepatic microsomes (results not shown). Clear evidence for binding of this agent at the active site of cytochrome P-450 is thus provided.

Table 1. Destruction of hepatic microsomal cytochrome P-450 by 1-aminobenzotriazole

Hepatic microsomes from phenobarbital-pretreated rats were incubated with 1-aminobenzotriazole for the indicated time and their cytochrome P-450 content was determined as described in the Materials and methods section. Values ($\pm$ S.D.) given below are averages for three experiments.

[1-Aminobenzotriazole] (mM)	Incubation conditions	Time (min)	Loss of cytochrome P-450 (%)	
			10	30
10.0	Normal	...	81 $\pm$ 1	86 $\pm$ 1
1.0	Normal		70 $\pm$ 1	77 $\pm$ 0
0.1	Normal		47 $\pm$ 1	59 $\pm$ 1
10.0	–NADPH		0	0
10.0	+CO		—	64 $\pm$ 1
10.0	+Glutathione (10 mM)		—	83 $\pm$ 2

Table 2. Cytochrome *P*-450, haem, and cytochrome *b*₅ losses in hepatic microsomes incubated with 1-aminobenzotriazole
Liver microsomes were incubated with 1-aminobenzotriazole (10 mM) and assays were performed as described in the Materials and methods section. Cited values ($\pm$ S.D.) are averages for three determinations.

Component	Microsomal concentration (nmol/mg of protein)		Loss (nmol/mg of protein)
	Before incubation	After 30 min incubation	
Cytochrome <i>P</i> -450	1.97 $\pm$ 0.01	0.44 $\pm$ 0.02	1.53
Haem	3.07 $\pm$ 0.01	1.37 $\pm$ 0.09	1.70
Cytochrome <i>b</i> ₅	0.72 $\pm$ 0.01	0.71 $\pm$ 0.01	—

Isolation and characterization of the abnormal hepatic porphyrin

Two green (red-fluorescing) porphyrin fractions have been isolated from the livers of phenobarbital-induced Sprague-Dawley rats injected with 1-aminobenzotriazole (100 mg/kg). These porphyrins, the carboxy groups of which were methylated during the isolation procedure, were separated by t.l.c. The minor high-*R*_F fraction has not yet been characterized. The major low-*R*_F fraction, after further purification by high-pressure liquid chromatography, has been shown to have the electronic absorption spectrum depicted in Fig. 1(a). The same spectrum is observed if zinc acetate is added to the sample or if the sample is first treated with H₂SO₄/methanol (1:19, v/v) to remove complexed metals (Ortiz de Montellano *et al.*, 1979). The chromatographic behaviour of the porphyrin is also not altered by these treatments. The electronic absorption spectrum of a mixture of synthetic *NN*-dimethylprotoporphyrin IX (dimethyl ester) isomers is also given in Fig. 1. A startling resemblance between the spectrum of the synthetic porphyrin and that of the 1-aminobenzotriazole-derived porphyrin is apparent. The spectrum and chromatographic behaviour of the synthetic *NN*-dimethylated porphyrin isomers are also not altered by addition of zinc acetate, as expected from the known reluctance of *NN*-dialkylated porphyrins to form complexes with metals (Johnson *et al.*, 1975). A clean field-desorption mass spectrum was obtained for the 1-aminobenzotriazole-derived porphyrin (result not shown). The spectrum exhibited a monoprotonated molecular ion at *m/e* 665 and essentially no other peaks. This molecular ion corresponds to that expected for a structure incorporating the elements of the dimethyl ester of protoporphyrin IX (mol.wt. 590) and benzyne (mol.wt. 76) if two protons are lost from the porphyrin on covalent attachment of benzyne.

Discussion

The destruction of cytochrome *P*-450 *in vitro* by 1-aminobenzotriazole is distinguished from that mediated by 2-isopropyl-pent-4-enamide, for example (Ortiz de Montellano & Mico, 1980b), by its

Fig. 1. Electronic absorption spectra in chloroform of (a) the abnormal porphyrin obtained biologically with 1-aminobenzotriazole and (b) a synthetic mixture of *NN*-dimethylprotoporphyrin IX (dimethyl ester) isomers

exceptional rapidity, by the fact that at least 90% (rather than 40%) of the enzyme in hepatic microsomes is vulnerable, and by the finding that even the lowest concentration of the heterocycle so far assayed (0.1 mM) is highly effective (Table 1). The extraordinary destructive ability of 1-aminobenzotriazole is reflected in our recent finding that *in vivo*, *in vitro* and with purified enzymes, this agent destroys phenobarbital- and 3-methylcholanthrene-inducible cytochrome *P*-450 isoenzymes with equal facility (P. R. Ortiz de Montellano, B. A. Mico, J. M. Mathews, K. L. Kunze, G. Miwa & A. Y. H. Lu, unpublished work). Catalytic participation of cytochrome *P*-450 in its own destruction is supported by the requirement for NADPH, by the inhibitory effect of CO (Table 1), and by the observation of a characteristic type-II difference spectrum on binding of 1-aminobenzotriazole to unreduced microsomes. Three further observations suggest that the destruction of cytochrome *P*-450 during catalytic interaction with 1-aminobenzotriazole does not result from the formation of an indiscriminately reactive metabolite: (a) cytochrome *b*₅, another haemoprotein, is not affected (Table 2); (b) glutathione does not prevent cytochrome *P*-450 destruction (Table 1), and (c) lipid peroxidation, measured by malonaldehyde formation, is not stimulated.

The above results implicate a destructive mechanism in which the enzyme is inactivated by specific interaction with a catalytically activated form of 1-aminobenzotriazole. The target of this destructive interaction is, at least predominantly, the prosthetic haem moiety of the enzyme. This conclusion derives from the observations that slightly more than 1 mol of microsomal haem is lost per mol of enzyme destroyed (Table 2) and that haem loss is coupled to the formation of an abnormal protoporphyrin IX derivative. The destruction of cytochrome *P*-450 by 2-isopropylpent-4-enamide (De Matteis, 1978; Ortiz de Montellano *et al.*, 1979) and other unsaturated substrates provides strong precedent for such a mechanism, although the abnormal porphyrin formed with 1-aminobenzotriazole is radically different from the *N*-monoalkylated protoporphyrin IX derivatives obtained with olefins (De Matteis & Cantoni, 1979; Ortiz de Montellano *et al.*, 1980a). The virtual identity of the electronic absorption spectrum of the 1-aminobenzotriazole derived porphyrin with that of synthetic *NN*-dimethylprotoporphyrin IX (dimethyl ester) (Fig. 1), the resistance of these two porphyrins to formation of zinc complexes, and their unusually high polarity, leave little doubt that the abnormal porphyrin biologically obtained with the heterocycle is an *NN*-dialkylated protoporphyrin IX derivative. In view of this, the observation of the mass-spectrometric molecular ion (*m/e* 665, monoprotonated) expected of a structure in which a benzene ring is bound to protoporphyrin IX by two covalent bonds permits formulation of the structure shown in Fig. 2 for the porphyrin. The bridging of two vicinal rather than distal porphyrin nitrogen atoms, although not experimentally established, can be inferred from models that show that the distance between two vicinal nitrogen atoms is ideal whereas that between two distal nitrogens is impossibly large. The substituents on the protoporphyrin IX periphery are not shown in the Figure, since no evidence is available as to which pair of nitrogen atoms is specifically alkylated. The results delineate a mechanism for the destructive activity of the heterocycle in which catalytically formed benzyne adds across two nitrogen atoms in the prosthetic haem moiety of the enzyme. The resulting iron-complexed reduced porphyrin, after re-oxidation and loss of iron, yields the isolated bridged porphyrin. Although further evidence is required to define the details of this mechanism, the first use of a benzyne precursor as a suicide substrate has resulted in an extraordinarily effective cytochrome *P*-450-destructive agent and in the isolation of an unprecedented *NN*-aryl-bridged porphyrin.

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Fig. 2. Proposed structure for the abnormal 1-aminobenzotriazole-derived porphyrin

The substituents on the periphery of the porphyrin ring, the same as on protoporphyrin IX, are not explicitly shown, since the two nitrogen atoms that are specifically bridged remain to be defined.

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