

Fluorescence labelling of NADPH-cytochrome *P*-450 reductase with the monobromomethyl derivative of syn-9,10-dioxabimane

Frank VOGEL and Ludwig LUMPER

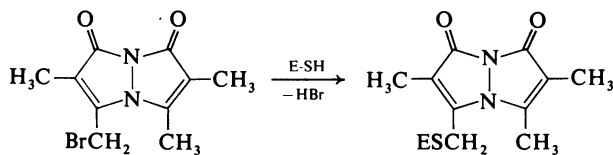
Biochemisches Institut der Justus Liebig-Universität, Fachbereich Humanmedizin, Friedrichstrasse 24,
6300 Giessen, Federal Republic of Germany

(Received 17 March 1983/Accepted 9 June 1983)

The kinetics of thiol-group alkylation in NADPH-cytochrome *P*-450 reductase during its inactivation by monobromobimane has been studied using the fluorimetric determination of *S*-bimane-L-cysteine by high-performance liquid chromatography. Loss of activity during the reaction of NADPH-cytochrome *P*-450 reductase with monobromobimane is caused by the alkylation of one single critical cysteine residue, which can be protected against thiol-specific reagents by NADP(H). The chemical stability of the bimane group allows the digestion of bimane-labelled NADPH-cytochrome *P*-450 reductase by CNBr. The critical cysteine residue could be located in a CNBr-cleaved peptide purified to homogeneity with M_r 10 500 \pm 1000 and valine as *N*-terminus.

The fluorogenic monobromomethyl derivative of syn-9,10-dioxabimane (monobromobimane) synthesized by Kosower *et al.* (1978, 1981) promises to be a useful tool for the covalent labelling of cysteine residues (Scheme 1) before carrying out sequence studies. It is superior to other fluorescent thiol-specific reagents with respect to group specificity as well as to the chemical stability and photochemical properties of its reaction product with L-cysteine. NADPH-cytochrome *P*-450 reductase contains one critical cysteine residue located at or near the NADPH-binding site (Lazar *et al.*, 1977; Yoshinaga *et al.*, 1982). To study the location of the critical cysteine residue within the peptide chain of the NADPH-cytochrome *P*-450 reductase the use of a specific thiol-group label of great sensitivity and high stability under the conditions of chemical cleavage of peptide bonds proved to be necessary. In the present paper we report the modification of the NADPH-cytochrome *P*-450 reductase by the fluorogenic monobromobimane as a convenient alternative to

Abbreviations used: BNPS-skatole, 3-bromo-3-methyl-2-(nitrophenylsulphenyl)-1*H*-indole; h.p.l.c., high-performance liquid chromatography; monobromobimane, 4-bromomethyl-3,6,7-trimethyl-1,5-diazabicyclo[3.3.0]octa-3,6-diene-2,8-dione; bimane group, 4-methylene-3,6,7-trimethylbimane (see Kosower *et al.*, 1981); NaDodSO₄, sodium dodecyl sulphate; NADPH-cytochrome *P*-450 reductase, trypsin-solubilized NADPH-cytochrome *P*-450 reductase (EC 1.6.2.4); Nbs₂, 5,5'-dithiobis(2-nitrobenzoate); Nbs, 2-nitro-5-thiophenyl; TFA, trifluoroacetic acid.



Scheme 1. Reaction of monobromobimane with thiol groups of proteins

Abbreviation used: E-SH, thiol group of enzyme.

radiolabelling of cysteine residues and the distribution of the bimane-group on the CNBr peptides obtained from bimane-inactivated NADPH-cytochrome *P*-450 reductase.

Materials and methods

Most of the materials and methods used were as previously described (Lazar *et al.*, 1977; Lumper *et al.*, 1980). Pronase E was from E. Merck; aminopeptidase (cytosol) and the synthetic hemisulphate of leupeptin was obtained from Sigma. Monobromobimane was sold from Calbiochem under the trade name Thiolyte MB.

Purification of the NADPH-cytochrome *P*-450 reductase

Microsomes from pig liver were prepared by the method of Schenkman and Cinti (1978). The NADPH-cytochrome *P*-450 reductase was purified

as described previously (Lumper *et al.*, 1980) introducing the following modifications. (a) The reductase adsorbed to 2',5'-ADP-Sepharose 4B (*N*⁶-6-aminohexyladenosine 2'-phosphate 5'-phosphate coupled to Sepharose 4B) column (1.4 cm × 20 cm) was washed with 500 ml of 0.5 M-KCl/0.05 M-sodium phosphate buffer, pH 7.5, and eluted with 40 ml of 1 mM-2'-AMP/1 mM-Ti triplex III/0.05 M-sodium phosphate buffer, pH 7.5. (b) 2'-AMP was removed by gel filtration of the column eluate through a Sephadex G-25 column (2.5 cm × 100 cm) equilibrated with 0.05 M-sodium phosphate buffer, pH 7.5. Finally the enzyme solution was concentrated on an Amicon PM-10 Diaflo membrane to 4 mg of protein/ml. After this step leupeptin was added to the enzyme [reductase/leupeptin (50:1, w/w)].

Modification of the (Nbs)₂-reductase with monobromobimane

(Nbs)₂-reductase (15 μM) synthesized as described by Lazar *et al.* (1977) was incubated with monobromobimane (0.3–1.5 mM; 0.01–0.05 mol of enzyme/mol of reagent) in 0.1 M-NH₄HCO₃ buffer, pH 8.2, at 4°C. Removal of excess reagents at the end of the reaction was performed by filtration through a Sephadex G-25 column (2.5 cm × 100 cm) equilibrated with 0.1 M-NH₄HCO₃ buffer, pH 8.2, at 4°C.

CNBr cleavage of the NADPH-cytochrome P-450 reductase

(Un)modified reductase (30–70 nmol) was *S*-carboxymethylated by the procedure described by Kuhn *et al.* (1974). The reaction mixture was acidified with 100% acetic acid and excess reagents were removed by filtration through Sephadex G-50 (1.9 cm × 26 cm) in 30% acetic acid before freeze-drying. A solution of *S*-carboxymethylated reductase (2 mg/ml; 30–70 nmol) made in 70% (v/v) formic acid was supplemented with a 250-fold molar excess of CNBr over methionine, left in the dark for 24 h at 30°C under N₂ and finally after dilution with water dried in a Speed-Vac concentrator (Savant).

Gel chromatography of the CNBr peptides

The freeze-dried CNBr peptides were redissolved in 1 ml of 10% (v/v) acetic acid and separated on a Sephadex G-75 column (1.7 cm × 130 cm) in 10% acetic acid.

Determination of S-bimane-L-cysteine in NADPH-cytochrome P-450 reductase

Bimane-labelled reductase (0.5–1.5 nmol) was incubated for 6 h at 37°C in 0.2 ml of 0.1 M-NH₄HCO₃ buffer, pH 8.2, containing aminopeptidase (cytosol) (0.13 μg/μg of reductase). After addition of Pronase E (1 μg/μg of reductase) the

digest was incubated for a further period of 16–20 h under identical conditions. The samples were dried in a Speed-Vac concentrator (Savant). The residual material was dissolved in aq. 0.1% (v/v) TFA (50 μl). Of each sample 40 μl was injected on to a du Pont Zorbax CN column (0.46 cm × 25 cm) in aq. 0.1% (v/v) TFA, pH 2.0, equipped with a Perkin-Elmer Chromatograph Series 3, an RF-530 Fluorescence Spectromonitor and a C-R1A recording integrator (Shimadzu). *S*-Bimane-L-cysteine was eluted with a linear gradient of propan-1-ol (1–30%; 40 min; flow rate 1 ml/min). The fluorescence of the column eluate was monitored. The excitation wavelength was set at 395 nm and the emission wavelength at 475 nm. Calibration runs were performed with standard solutions of *S*-bimane-L-cysteine prepared daily under exclusion of light as described by Fahey *et al.* (1981) with the modification that no thiol-agarose was added.

Electrophoretic studies

Samples (5–20 μg of protein or peptide) were incubated in 1% (w/v) NaDodSO₄/1% β-mercaptoethanol for 3 min at 100°C and then subjected to NaDodSO₄/20% (w/v) polyacrylamide-gel electrophoresis, pH 6.8 (Swank & Munkres, 1971). Urea was omitted from the buffer. The gels were stained for protein with Coomassie Brilliant Blue. Standard *M_r* markers were bovine serum albumin, trypsinogen, cytochrome *c*, myoglobin, insulin B-chain, bacitracin [fixed with glutaraldehyde in 1% (v/v) alkaline borate buffer before staining (Oakley *et al.*, 1980)] and the CNBr peptides of myoglobin.

General methods

A Biotronic model 6001 instrument equipped with a Hewlett-Packard integrator terminal 3388 A was used for amino acid analysis after sample hydrolysis in 6.1 M-HCl *in vacuo* at 105°C for 24 h. U.v. spectra were made on a Beckman 25 recording spectrophotometer. Fluorimetric measurements were performed with a Perkin-Elmer spectrofluorimeter (model MPF 2A).

Results and discussion

Spectroscopic characteristics of the bimane-labelled NADPH-cytochrome P-450 reductase

The u.v.-visible spectrum of the unmodified NADPH-cytochrome P-450 reductase in the oxidized state has maxima at 276, 384 and 456 nm with a shoulder at 485 nm (Iyanagi *et al.*, 1974). Modification of the NADPH-cytochrome P-450 reductase by monobromobimane results in an absorbance increase between 310 and 430 nm with a maximum at 385 nm. The u.v.-band of the bimane group in the NADPH-cytochrome P-450 reductase is displaced about 10 nm to shorter wavelengths

compared with the absorption spectrum of monobromobimane dissolved in 0.1 M-NH₄HCO₃ buffer, pH 8.2.

The fluorescence emission spectrum of bimane-labelled NADPH-cytochrome *P*-450 reductase excited at 395 nm shows a broad and asymmetric band between 440 and 600 nm with a maximum at 475 nm. The fluorescence emission spectrum of the unmodified flavoprotein, however, is under an identical set of experimental conditions located between 480 and 560 nm. The ratio of the integrated areas of the fluorescence emission bands for the bimane-induced fluorescence of the modified enzyme and the intrinsic fluorescence of the unmodified reductase between 440 and 660 nm is approx. 17:1.

Determination of *S*-bimane-L-cysteine in the bimane-labelled NADPH-cytochrome *P*-450 reductase

H.p.l.c. with cyanopropylsilyl columns using fluorescence detection allows the identification and the quantification of *S*-bimane-L-cysteine present in aminopeptidase (cytosol)/Pronase E digests of bimane-labelled NADPH-cytochrome *P*-450 reductase in the range 1–1000 pmol (see the Materials and methods section). Hydrolysis of the modified enzyme by proteinases is necessary, since *S*-bimane-L-cysteine is destroyed during acid hydrolysis in 6.1 M-HCl at 105°C. A quantitative yield of *S*-bimane-L-cysteine is obtained by sequential incubation of the modified reductase with aminopeptidase (cytosol) and Pronase E for a total time period of 20 h using a high proteinase/reductase ratio (above 1:1, w/w; see the Materials and methods section). In the h.p.l.c. chromatogram of digests obtained from bimane-labelled NADPH-cytochrome *P*-450 reductase is *S*-bimane-L-cysteine clearly separated from the flavoco-enzymes (FAD and FMN) and an unknown peak comprising 5–7% of the total fluorescence (Fig. 1). *S*-Bimane-labelled peptides cannot be detected. The determination of the fluorescent reaction products arising during the modification of the NADPH-cytochrome *P*-450 reductase by monobromobimane documents the selective modification of cysteine residues. Additional evidence for the thiol-selectivity of monobromobimane is obtained by monitoring of the h.p.l.c. eluates at 385 nm. Under the conditions used non-thiol-containing proteins (e.g. chymotrypsin) and amino acids such as L-lysine, L-serine, L-histidine or L-tyrosine do not form detectable amounts of bimane-derivatives. Our results do not support the supposition of Fahey *et al.* (1981) that nucleophilic side chains in proteins other than thiol groups are attacked by monobromobimane at pH 8.2. During the reaction with NADPH-cytochrome *P*-450 reductase fluorescent bimane derivatives of substances present in buffers commonly

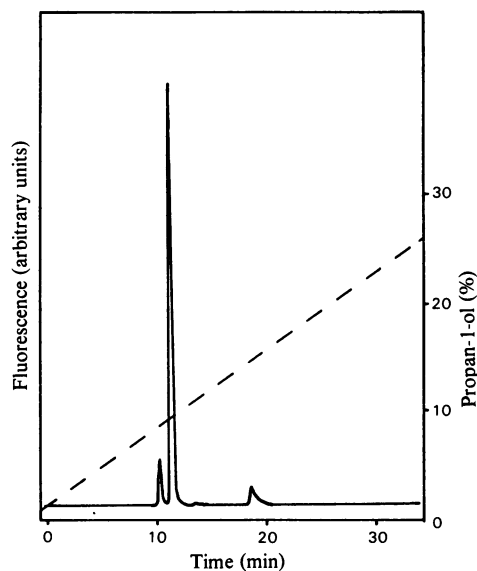


Fig. 1. H.p.l.c. chromatogram of the enzymic hydrolysate of bimane-labelled NADPH-cytochrome *P*-450 reductase

Amount injected was equivalent to 80 µg of bimane-labelled NADPH-cytochrome *P*-450 reductase (3.0 mol of *S*-bimane-L-cysteine/mol). All other conditions were as given in the Materials and methods section. The peak corresponding to *S*-bimane-L-cysteine was eluted at 12.1 min.

used (e.g. sodium phosphate, NH₄HCO₃, ammonium acetate) are formed. The rates of fluorescence increase are in the range 0.2–2 µM · h⁻¹ for 0.1 M buffers at 25°C. Labelling of the NADPH-cytochrome *P*-450 reductase by monobromobimane can therefore not be observed by continuous monitoring using fluorescence spectroscopy. Gel filtration on Sephadex G-50 is required to remove the interfering side products before the determination of *S*-bimane-L-cysteine.

ϵ_{380} of the bimane group attached to the NADPH-cytochrome *P*-450 reductase was calculated, correlating the corrected absorbance increase $A_{380} = A_{380}^{\text{reductase}}$ (the absorbance of the bimane-labelled reductase at 380 nm) – A_{380}^{flavin} (the absorbance induced by the flavin chromophores in the bimane-labelled reductase) with the content (mol) of *S*-bimane-L-cysteine/mol of modified reductase estimated by h.p.l.c. A_{380}^{flavin} was calculated from the absorbance of the bimane-labelled NADPH-cytochrome *P*-450 reductase at 455 nm using the factor 1.15 ± 0.02 , which is identical with the ratio A_{455}/A_{380} obtained for the unmodified NADPH-cytochrome *P*-450 reductase. On the basis of this calculation ϵ_{380} of the *S*-bimane group was de-

terminated to be $6000 \pm 200 \text{ M}^{-1} \cdot \text{cm}^{-1}$. This value is in good agreement with that reported for monobromobimane dissolved in acetonitrile (Kosower *et al.*, 1979).

Stoichiometry and time course of modification of the NADPH-cytochrome P-450 reductase by monobromobimane

Modification of the NADPH-cytochrome P-450 reductase with monobromobimane has been studied to obtain specific labelling of its critical cysteine residue with a fluorophore. Controlled modification of this residue has been attained already with Nbs_2 and *N*-ethylmaleimide (Lumper *et al.*, 1980). Therefore the modification reaction between monobromobimane and the NADPH-cytochrome P-450 reductase has been studied at pH 8.2 under conditions successfully used previously (Table 1). Consistent with the results reported for Nbs_2 (1 mM), 3 ± 0.2 thiol groups/mol of reductase are accessible to monobromobimane at 4°C (and pH 8.2). As shown in Table 1, complete alkylation of the cysteine residues accessible to 1.5 mM-monobromobimane is achieved within 24 h (4°C). Data presented in Table 1 suggest that Nbs_2 is more reactive than monobromobimane. The sum of the thiol groups/mol of reductase alkylated by monobromobimane and that modified by 1 mM- Nbs_2 after pre-incubation of the NADPH-cytochrome P-450 reductase with monobromobimane remained the same at 3 ± 0.27 during the course of the reaction (Table 1). This result indicates the attack of identical thiol groups by both thiol-specific reagents. At 25°C the number of cysteine residues/mol of reductase accessible to 1.5 mM-monobromobimane increases up to 4 (Table 1). A 85–95% loss of the original catalytic activity is caused by the modification of more than 2.5 thiol groups/mol of reductase by monobromobimane. From preliminary calculations of the flavin content from A_{455} of the modified reductase it can be concluded that flavin loss is not a controlling factor in the inactivation process, since considerable dissociation of flavins does not occur at 4°C.

The kinetics of the inactivation by monobromobimane is not linearly dependent on the reagent concentration in the range 18–250 μM at 0.7 μM -NADPH-cytochrome P-450 reductase in 0.1 M- NH_4HCO_3 , pH 8.2 (4 and 25°C). The apparent first-order rate constant (k_{app}) for the inactivation of the NADPH-cytochrome P-450 reductase obtained from the slope of the $\ln(E/E_0)$ -versus-time plot, where E is the activity at any time and E_0 is the activity at zero time, shows a hyperbolic dependence on monobromobimane. This result can be discussed in terms of the mechanism:

$$E + \text{inhibitor} \xrightleftharpoons[k_{-1}]{k_{+1}} (E \times \text{inhibitor}) \xrightarrow{k_{+2}} E - \text{inhibitor}$$

discussed by Kitz & Wilson (1962) for the ir-

reversible inhibition of enzymes using the equilibrium assumption. The low value of the second-order rate inactivation constant k of approx. $4 \text{ M}^{-1} \cdot \text{s}^{-1}$ at 25°C provides strong evidence that the equilibrium condition $k \ll k_{+1}$ is valid (Brocklehurst, 1979). The double-reciprocal plot $1/k_{\text{app}}$ against $1/[\text{monobromobimane}]$ yields therefore a straight line with the ordinate intercept equal to $1/k_{+2}$ and the reciprocal value for the true dissociation constant K_1 for the reversible NADPH-cytochrome P-450 reductase–monobromobimane complex as abscissa intercept. From the experimental data, we calculated $K_1 = 25 \mu\text{M}$ and $k_{+2} = 4.7 \times 10^{-3} \text{ min}^{-1}$ at 4°C. A temperature increase to 25°C changes K_1 to 83 μM and k_{+2} to $17.4 \times 10^{-3} \text{ min}^{-1}$.

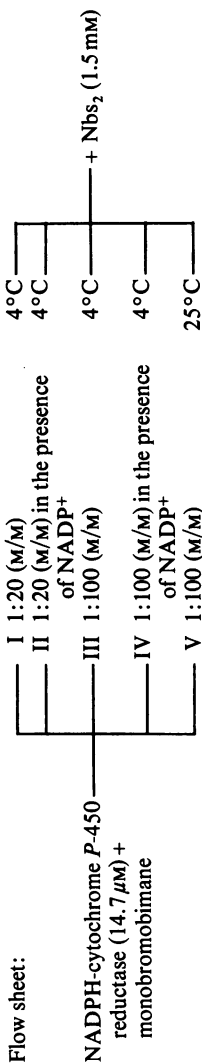
The number of thiol groups/mol of reductase attacked by monobromobimane remains constant when varying the pH from 8.2 to 7.5. The inhibitory effect of monobromobimane is independent of the buffer used. In contrast 0.1 mM-iodoacetate fails to affect the reductase activity (1 μM -reductase) in 0.05 M-phosphate buffer of pH 7.5 and 8.2 by more than 10% within 20 h at 4°C, whereas the activity loss observed by incubation of the enzyme under otherwise identical conditions in 0.05 M-Tris/HCl buffer increases from 30% at pH 7.2 to 70% at pH 8.3. Protection of a cationic site is the likely explanation for the fact that iodoacetate does not destroy the activity of the NADPH-cytochrome P-450 reductase (Nishibayashi-Yamashita & Sato, 1970; Nisimoto & Shibata, 1982). However, the mechanism of inactivation by iodoacetate is not yet clear, since under all conditions studied 1.5–2 mol of carboxymethyl groups/mol of reductase are incorporated into cysteine residues within 20 h (1.5 mM-iodoacetate; 14.7 μM -reductase).

To avoid degradation by endogeneous proteinases leupeptin [total protein/leupeptin, 50:1 (w/w)] was added to the stored protein and all reaction mixtures. Modification sensitizes the NADPH-cytochrome P-450 reductase against the attack of these proteinases as shown by NaDodSO_4 /polyacrylamide-gel electrophoresis (Fig. 2). The markedly enhanced sensitivity of the (Nbs)₃-reductase (3.1 mol of thiol groups/mol of reductase modified by Nbs_2) is obviously caused by a conformational change induced by the introduction of three 1-carboxy-2-nitrophenyl-5-thio groups into the protein. Complete suppression of the proteolytic activity by leupeptin is obtained (Fig. 2). The content of interfering proteinases can be largely reduced by using the modified procedure for the purification of the NADPH-cytochrome P-450 reductase described in the Materials and methods section.

Our data in Table 1 show that the critical thiol group can be protected by 1–2 mM-NADP⁺ against the attack by 0.3 or 1.5 mM-monobromobimane. According to the results of *S*-bimane-L-cysteine

Table 1. Reaction of monobromobimane with NADPH-cytochrome P-450 reductase

The flow sheet shows the protocol of the modification experiments I-V. The reaction was performed in 0.1 M-NH₄HCO₃ (pH 8.2). NADP⁺ was present at 1.5 mM. The number of thiol groups modified by monobromobimane/mol of reductase were determined as mol of S-bimane-L-cysteine by h.p.l.c. (see Materials and methods section). The number of thiol groups accessible to Nbs₂ within 1 h (pH 8.2) is shown. Time given is the reaction period for bimane modification. Flavin was calculated from A₄₃₅/mg of bimane-labelled reductase (A₄₃₅/mg of unmodified reductase is set equal to 100%). The results relate to reductase modified with monobromobimane for 24 h. All values given represent medians of triplicate determinations. Values in parentheses in the Table represent activity (%).



Reaction no. of flow sheet	Number of thiol groups modified/mol of reductase after:				Thiol groups reactive with Nbs ₂ /mol of bimane-labelled reductase after:				Total number of thiol groups modified/mol of reductase	Flavin content in bimane-labelled reductase
	1 h	3 h	5 h	24 h	1 h	3 h	5 h	24 h		
I	0.59 (35)	0.94 (28)	1.41 (20)	2.49 (6)	2.60	2.27	1.80	0.44	2.93-3.21	100
II	0.19 (118)	0.52 (107)	0.67 (118)	1.30 (82)	2.87	2.67	2.51	1.80	3.06-3.19	77
III	0.65 (67)	1.34 (37)	1.76 (26)	3.07 (10)	2.40	1.60	1.31	0	2.94-3.07	82
IV	0.27 (97)	0.59 (95)	0.88 (95)	1.50 (81)	2.94	2.35	2.09	1.52	2.94-3.20	87
V	1.64 (32)	2.74 (21)	3.09 (16)	3.90 (2)•	-	-	-	-	-	47



Fig. 2. Digestion of (un)modified NADPH-cytochrome *P*-450 reductase by intrinsic proteinase activity

Modified NADPH-cytochrome *P*-450 reductase was obtained by reaction with 1.5 mM-thiol-specific reagent for 24 h at 4°C. (Un)modified NADPH-cytochrome *P*-450 reductase (10 µg) was incubated in 0.1 M-NH₄HCO₃ buffer, pH 8.2, for 24 h at 4°C. Leupeptin (0.02 µg) was added. The separating gel used was 20% acrylamide containing 0.1% (w/v) NaDodSO₄. For further details see the Materials and methods section. Explanation of lanes: 1, unmodified reductase; 2, reductase modified by monobromobimane in the presence of leupeptin; 3, reductase modified by monobromobimane; 4, reductase modified by monobromobimane in the presence of NADP⁺ (1.5 mM); 5, reductase modified by Nbs₂ in the presence of leupeptin; 6, reductase modified by Nbs₂; 7, reductase modified by Nbs₂ in the presence of NADP⁺ (1.5 mM); 8, protein standard mixture.

determinations, the expected stoichiometry of 1.1 ± 0.1 thiol groups protected/mol of reductase is indeed found for the reaction with 0.3 mM-monobromobimane, whereas the number of cysteine residues masked by NADP⁺ is 1.5 ± 0.1 for the modification with 1.5 mM-monobromobimane (4°C).

Isolation of bimane-modified CNBr peptides of the NADPH-cytochrome *P*-450 reductase

The bimane group was specifically introduced at the critical cysteine residue of the NADPH-cyto-

Table 2. Stability of *S*-bimane-*L*-cysteine (0.031 mM) against chemical cleavage reagents

The reactants were dissolved in: *, 70% (v/v) formic acid; †, 80% (v/v) acetic acid. The reaction was allowed to proceed at 30°C.

Reagent	Concentration (mM)	Reaction time (h)	<i>S</i> -bimane- <i>L</i> -cysteine determined by h.p.l.c. (%)
CNBr*	3–16	24	97–100
<i>O</i> -Iodosobenzoic acid†	0.031	24	100
	0.062	24	70
	0.312	0.2	0
BNPS-skatolet†	0.062	24	95
	0.625	24	72
	3.125	24	30
	7.812	24	10

Table 3. CNBr peptides obtained from NADPH-cytochrome *P*-450 reductase

Pool	Designation of peptides	Apparent molecular weight of peptides (kDa)	<i>N</i> -Termini found
I	–	1–>15	Gly
II	CB 1–CB 13	13.0	Ala, Val, Gly
III	CB 2	11.0	Val
IV	CB 14–CB 15	5.8	Ile, Gly
V	CB 16–CB 12	3.0	Ile, Pro, Phe, Gly, Ala, Leu, Val, Lys
VI	CB 13–CB 14	1.6	Pro, Ala, Gly
VII	–	–	–

chrome *P*-450 reductase by differential labelling: after masking the accessible but non-essential thiol groups by 1 mM-Nbs₂ in the presence of 1 mM-NADP⁺ (Lazar *et al.*, 1977), the (Nbs)₂-reductase synthesized was alkylated by 1.5 mM-monobromobimane (4°C) after removal of NADP⁺ by gel filtration. The cleavage of the mono-*S*-bimane-derivative of the NADPH-cytochrome *P*-450 reductase has been performed by CNBr treatment, since the stability of *S*-bimane-*L*-cysteine against this reagent could be shown (Table 2). The mixture of CNBr-cleaved peptides obtained can be resolved on NaDodSO₄/polyacrylamide-gel electrophoresis into seven peptide-containing zones. The CNBr-cleaved peptides were fractionated on Sephadex G-75 prepared in 10% (v/v) acetic acid and collected in seven pools as indicated by the bars in Fig. 3. The peptides present in each pool were characterized by their approximate molecular weights, their *N*-termini (Table 3) and in the case of homogeneity by amino acid analysis. The CNBr peptides in pools I–III were only soluble in acidic solutions with a pH below 3.0. Peptide CB 2 was collected in pool III as a

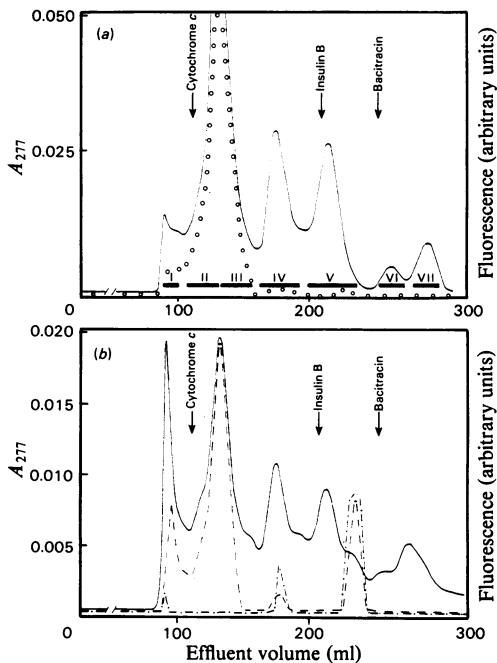


Fig. 3. Gel chromatography of *S*-bimane-labelled NADPH-cytochrome *P*-450 reductase on Sephadex G-75 column (1.7 cm \times 130 cm)

Fractions (3.8 ml) were collected at a flow rate of 14 ml/h and pooled as indicated by the bars. For further details see the text and in particular the Materials and methods section. —, A_{277} Fluorescence of peptides at 475 nm (λ excitation = 395 nm): $\circ \circ \circ \circ$, from mono-*S*-bimane-labelled NADPH-cytochrome *P*-450 reductase (0.9 mol of *S*-bimane-L-cysteine/mol of reductase; peptides prepared from 32 nmol of NADPH-cytochrome *P*-450 reductase); $\cdots \cdots$, from bis-*S*-bimane-labelled NADPH-cytochrome *P*-450 reductase (1.6 mol of *S*-bimane-L-cysteine/mol of reductase, 95% of the activity of the unmodified enzyme); $----$, from tris-*S*-bimane-labelled NADPH-cytochrome *P*-450 reductase (3.1 mol of *S*-bimane-L-cysteine/mol of reductase). Peptides of bis-*S*-bimane- and tris-*S*-bimane-labelled NADPH-cytochrome *P*-450 reductase were prepared from 10 nmol of modified NADPH-cytochrome *P*-450 reductase.

homogeneous peptide. Peptide CB 1 present in pool II could not be obtained in pure form by gel filtration on Sephadex G-75 or ion-exchange chromatography (DE-32; SP-Sephadex). The *N*-terminus of the trypsin-solubilized NADPH-cytochrome *P*-450 reductase from pig liver has been determined to be isoleucine in agreement with the results published for the large proteolytic fragment arising by trypsin cleavage of the detergent-solubilized NADPH-cyto-

Table 4. Amino acid composition of CB 2 from NADPH-cytochrome *P*-450 reductase

All values given are medians of five determinations.

Residue	Content (mol %)
Asx	8.49 \pm 0.46
Thr	3.53 \pm 0.22
Ser	6.95 \pm 0.55
Glx	14.58 \pm 0.14
Gly	8.21 \pm 0.42
Ala	8.84 \pm 0.31
Pro	3.27 \pm 0.60
Val	5.97 \pm 0.26
Ile	4.16 \pm 0.13
Leu	11.79 \pm 0.93
Tyr	5.16 \pm 0.20
Phe	2.43 \pm 0.37
His	4.15 \pm 0.39
Lys	4.94 \pm 0.62
Arg	7.53 \pm 0.32

chrome *P*-450 reductase (rat and rabbit liver) (Black & Coon, 1982). However, trypsin cleaves NADPH-cytochrome *P*-450 reductase (pig liver) bound to endoplasmic reticulum at two sites, since in some preparations glutamate is found as (additional) *N*-terminus (Lumper *et al.*, 1980). The *N*-terminal region of the proteolytic fragment obtained from detergent-solubilized NADPH-cytochrome *P*-450 reductase contains methionine at position 17 (rat) or 18 (rabbit) (Black & Coon, 1982). On the basis of these sequences, we have tentatively identified the CNBr peptide (M_r approx. 1500) with *N*-terminal isoleucine present in pool V as the *N*-terminal CNBr peptide of the trypsin-solubilized NADPH-cytochrome *P*-450 reductase (pig liver). As shown in Fig. 3, most of the fluorescence (85%) of mono-*S*-bimane-labelled NADPH-cytochrome *P*-450 reductase was found in pools II and III obtained by gel filtration of the CNBr peptides. NaDodSO₄/polyacrylamide-gel electrophoresis revealed that the fluorescent component present in pools II and III is peptide CB 2 (Fig. 3). The amino acid analysis of the fluorescent peptide CB 2 is listed in Table 4. Modification of the NADPH-cytochrome *P*-450 reductase with 1.5 mM-monobromobimane at pH 8.2 (4°C; 24 h) results in the formation of an enzymically inactive reductase containing 3.2 \pm 0.2 mol of *S*-bimane-L-cysteine/mol (Table 1). The distribution of the *S*-bimane-derived fluorescence on the CNBr peptides obtained from this derivative of the reductase is shown in Fig. 3. In addition to the peptide CB 2 the bimane-label is located on peptides present in the pools IV and VI. By fractional labelling with monobromobimane (1.5 mM) in the presence of 2 mM-NADP⁺ (pH 8.2; 4°C; 24 h) bis(*S*-bimane)-reductase is synthesized, which shows the original activity

of the unmodified enzyme, and is thought to be modified at the accessible but non-essential cysteine residues (1.5 ± 0.2 mol of *S*-bimane-L-cysteine/mol of reductase; Table 1). In agreement with this supposition peptide CB 2 obtained from bis(*S*-bimane)-reductase contains no bimane-label (Fig. 3). The fluorescence originating from the bimane groups at the two blocked cysteine residues in the bis(*S*-bimane)-reductase is exclusively located on peptides present in pools IV and VI of the CNBr digest (Fig. 3). Our experiments give therefore direct evidence that the inactivation of NADPH-cytochrome *P*-450 reductase by thiol-specific reagents is caused by the modification of one special cysteine residue located within fragment CB 2 of the NADPH-cytochrome *P*-450 reductase.

We thank Professor Dr. S. Stirn and Dr. D. Linder for generous support with amino acid analysis. We express gratitude to Professor Dr. G. Gundlach for use of the Perkin-Elmer MPF-2A instrument. The excellent technical assistance of Mrs. M. Wagner and Mr. F. Busch is gratefully acknowledged.

References

- Black, Sh. D. & Coon, M. J. (1982) *J. Biol. Chem.* **257**, 5929–5938
- Brocklehurst, K. (1979) *Biochem. J.* **181**, 775–778
- Chen, R. (1976) *Hoppe-Seyler's Z. Physiol. Chem.* **357**, 873–886
- Fahey, R. C., Newton, G. L., Dorian, R. & Kosower, E. M. (1981) *Anal. Biochem.* **111**, 357–365
- Gray, W. R. (1972) *Methods Enzymol.* **25**, 121–138
- Iyanagi, T., Makino, N. & Mason, H. S. (1974) *Biochemistry* **13**, 1701–1710
- Kitz, R. & Wilson, I. B. (1962) *J. Biol. Chem.* **237**, 3245–3249
- Kosower, E. M., Pazhenchevsky, B. & Hershkowitz, E. (1978) *J. Am. Chem. Soc.* **100**, 6516–6518
- Kosower, E. M., Pazhenchevsky, B., Dodiuk, H., Kanety, H. & Faust, D. (1981) *J. Org. Chem.* **46**, 1666–1673
- Kosower, N. S., Kosower, E. M., Newton, G. L. & Ranney, H. M. (1979) *Proc. Natl. Acad. Sci.* **76**, 3382–3386
- Kuhn, R. W., Walsh, K. A. & Neurath, H. (1974) *Biochemistry* **13**, 3871–3877
- Lazar, T., Ehrig, H. & Lumper, L. (1977) *Eur. J. Biochem.* **76**, 365–371
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Lumper, L., Busch, F., Dzelic, S., Henning, J. & Lazar, T. (1980) *Int. J. Peptide Protein Res.* **16**, 83–96
- Nishibayashi-Yamashita, H. & Sato, R. (1970) *J. Biochem. (Tokyo)* **67**, 199–210
- Nisimoto, Y. & Shibata, Y. (1982) *J. Biol. Chem.* **257**, 12532–12539
- Oakley, B. R., Kirsch, D. R. & Morris, N. R. (1980) *Anal. Biochem.* **105**, 361–363
- Schenkman, J. B. & Cinti, D. L. (1978) *Methods Enzymol.* **52**, 83–88
- Swank, R. T. & Munkres, K. D. (1971) *Anal. Biochem.* **39**, 462–477
- Yoshinaga, T., Sassa, Sh. & Kappas, A. (1982) *J. Biol. Chem.* **257**, 7786–7793