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FLUORESCENCE PROBING OF THE FUNCTION-SPECIFIC CYSTEINES OF RAT MICROSOMAL NADPH-CYTOCHROME P-450 REDUCTASE

Jung Ja Lee and Laurence S. Kaminsky

Laboratory of Biochemical and Genetic Toxicology
Wadsworth Center for Laboratories and Research
New York State Department of Health
Albany, New York 12201

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SUMMARY: Titration of NADPH-cytochrome P-450 reductase with a fluorigenic maleimide suggests that approximately four cysteines are initially accessible and in close proximity to four tryptophans. Perturbation of the cysteines and/or tryptophans results in concomitant decreases in enzymic activity. These cysteines were correlated with functional components by binding studies and subsequent tryptic peptide mapping on the acid mobile phase-reverse phase HPLC. Adenine nucleotides and cytochrome c block labelling of the more hydrophilic peptides, while detergents facilitate labelling of the more hydrophobic peptides. The more hydrophobic peptides contain the microsomal binding site of cytochrome P-450. Removal of the prosthetic flavins exposes more cysteines in the more hydrophilic and hydrophobic regions of the peptide map, associating the former with FAD and the latter with FMN binding sites.

In the hepatic microsomal monooxygenase system the reducing equivalents from NADPH are transferred in a cascade (1-3) from FAD to FMN within NADPH-cytochrome P-450 reductase and then to membrane-bound cytochrome P-450. In vitro, cytochrome c can also function as an electron acceptor from the reductase. A role for a charge transfer intermediate involving cysteine as well as other reductase residues has been suggested (2). The two flavin nucleotides and NADPH can cover a considerable extent of the topography of the protein surface, requiring several charged and hydrophobic residues for their non-covalent binding (4).

To gain insight into the interactions of the components of this system, we have used ANM, which becomes fluorescent upon covalently bonding to protein cysteine residues (5,6), as a probe of the cysteine microenvirons as

Abbreviations: ANM, N-(1-anilinonaph-4-yl)maleimide; 2'-AMP, 2-adenosine monophosphate; 2',5'-ADP, 2',5'-adenosine dephosphate.

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well as to provide a label for function-related, cysteine-containing peptide segments of the reductase.

MATERIALS AND METHODS

NADPH-cytochrome P-450 reductase was purified to electrophoretic homogeneity from phenobarbital-induced, Wistar male rat liver microsomes (7) by DE 52 (Whatman) chromatography (8) and 2',5'-ADP Sepharose (P-L Biochemicals) chromatography (7). Portions of reductase were immediately frozen at -80° and, as needed, thawed on ice and dialyzed into 4 changes of 0.05 M potassium phosphate buffer, pH 7.3, 20% glycerol and 0.1 mM EDTA at 4° for 48 h in darkness. ANM (Fluka) in dimethylsulfoxide was added to a series of reductase aliquots. Solutions were kept at 21-24° for 2-2.5 h (5) in darkness. The reaction was terminated by addition of dithiothreitol (100 µM) and each solution was dialyzed into 4 changes of the glycerol buffer at 4° for 48 h in darkness. Reductase samples were incubated with 1/50 (w/w) trypsin (Type III, Sigma) for 24 h, 37°, and aliquots equivalent to 14 µg reductase were injected manually onto an HPLC. Untrypsinized, BSA-ANM (1:1) has a similar retention time to peak 5 at 45.53±.25 min. The HPLC column was a Bakerbond, 4.6 x 250 mm, 330 Å pore, 5 µ spherical particle. Fluorescence was monitored by Waters Detector Model 420 with filters for excitation at 360 nm and emission at 455 nm in a Waters Associates HPLC equipped with a Spectra-Physics 4270 integrator. Solvent A (0.2% trifluoroacetic acid in water) was mixed with solvent B (0.2% TFA in 10% water in acetonitrile) in a linear gradient mixer (Waters Model 660) to 80% B in 1 h at the rate of 1 ml/min.

RESULTS AND DISCUSSION

The ANM-fluorescence (λ_{ex}:353 nm; λ_{em}:416-426 nm) titration curve showed a break at approximately four equivalents of ANM per reductase (Fig. 1A-a). Excitation spectra of the 420 nm emission of ANM-bound reductase also showed a band at 290 nm. This was largely due to tryptophan residues whose emission at 330 nm overlaps with the absorption band of cysteine-bound ANM. Excitation of the reductase tryptophans (λ_{ex}:290 nm) produced ANM fluorescence nearly as effectively as excitation at the ANM absorption band (Fig. 1A-b) with concomitant quenching of the 330 nm tryptophan fluorescence (Fig. 1A-c). Therefore, approximately four cysteines have similar reactivity/accessibility, and based on fluorescence quenching (44% of the nine tryptophan residues) they reside in close proximity to four tryptophan residues. The difference absorption titration curve (Fig. 1A-d) also indicated a break at approximately four equivalents, and that the reactivity of the ANM accessible cysteines is only 24% per equivalent. The flavin absorption at 450 nm showed negligible depression (Fig. 1A-e).

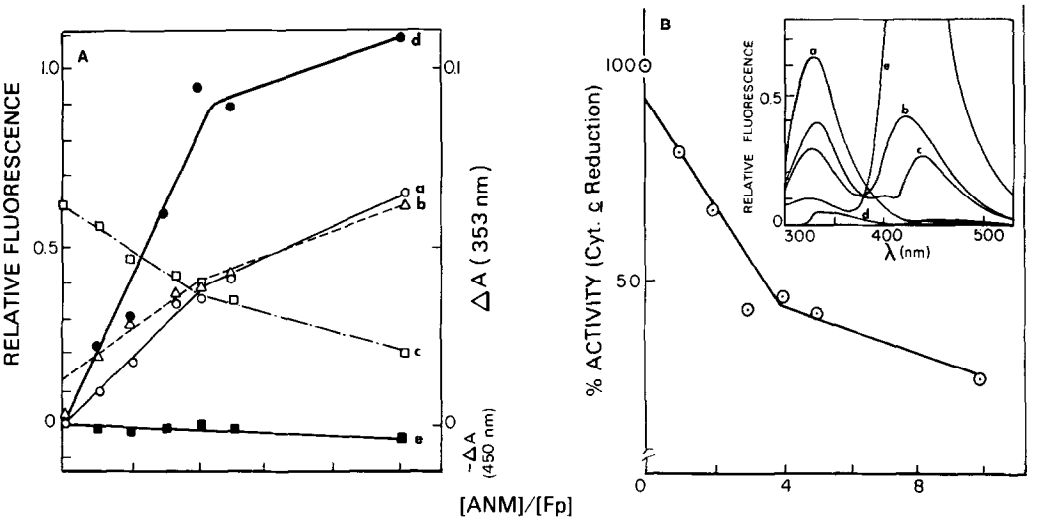

Fig. 1A. Titration of reductase (8.6 μM) with ANM equivalents ([ANM]), monitored by ANM fluorescence. a) ANM fluorescence; λ_{ex}:353 nm, λ_{em}:416-426 nm, red shifting with increasing [ANM]. A Perkin-Elmer Spectrofluorimeter (14) with 3 mm path length quartz cuvettes were used at room temperature. b) ANM fluorescence; λ_{ex}:290 nm, λ_{em}:416-426 nm. c) UV fluorescence of the protein; λ_{ex}:290 nm, λ_{em}:330 nm. d) Difference absorption at 350-354 nm (reference; free reductase) at room temperature in an Aminco DW-2A spectrophotometer; path length: 1 cm. e) Difference absorption at 450 nm showing less than 1% loss in flavin in the initial range. B. Effect of ANM on reductase reduction of cytochrome c (horse heart, Sigma) at 37° determined as described (15) using ANM-bound, dialyzed reductase. Inset: UV-excited fluorescence spectra; λ_{ex}:290 nm, slits: 5 nm, 9.5 μM reductase. a) Free reductase, treated in identical manner with ligand and reagent blanks. b) Reductase-ANM_2: reductase was incubated with ligand blank for 2 h on ice, reacted with 5 [ANM] and dialyzed as described in Fig. 1. c) Effect of 1.7-fold cytochrome c; since the fluorescence was too greatly diminished, 0.17% Lubrol was added with 5 [ANM] which increased the 430 nm band intensity while maintaining the spectral type. d) Only cytochrome c treated as in c). e) Deflavinized, labelled apoprotein, cf. text and Fig. 2E.

NADPH-dependent reduction of cytochrome c by ANM-modified reductase showed a similar pattern of change, i.e., linear initial decrease to approximately 50% of the activity at the breaking point (Fig. 1B). These accessible cysteines are thus involved in the reductase function with cytochrome c.

Tryptic digestion of the ANM-modified reductase and analysis by C_{18}-reverse phase HPLC with fluorescence detection revealed four major peptides with comparable fluorescence intensities, which increased proportionately with increasing equivalents up to four ANM per reductase without any changes in the fluorescent peptide map. Above four equivalents, the increases in the major peaks 1, 3, 5, and 6 were less marked, and two minor peaks (peaks 2 and 4) began to increase in intensity (Fig. 2A).

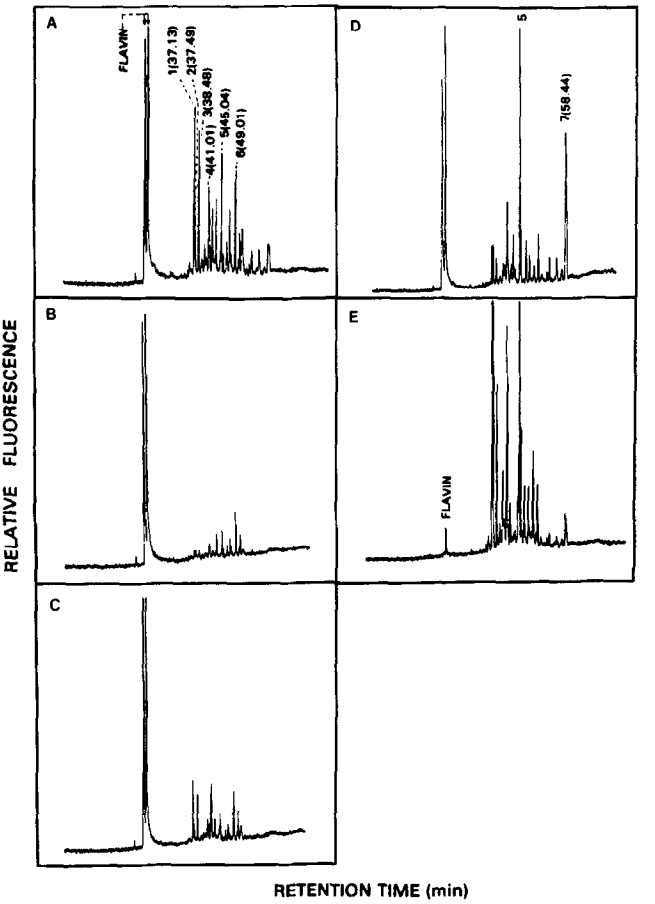

Fig. 2. C_{18}-reverse phase HPLC of trypsinized, ANM-labelled reductase.
A. Reductase-ANM_5. cf. Fig. 1B, inset b. The first 2 peaks are the flavin nucleotides as confirmed by fluorescence spectra of the fractions.
B. Effect of 27-fold NADPH on reductase-ANM_5.
C. Effect of 1.7-fold cytochrome c on reductase-ANM_5. The cytochrome c itself does not contribute to the peptide map.
D. 2'-AMP-bound/deoxycholate-solubilized, labelled reductase, cf. text. Peak 7 is labelled 4-fold of peak 5 when the reductase preparation is new, and converts to peak 5 upon frozen storage, producing a constant value of label on peak 5.
E. Labelled peptides of deflavinized apoprotein, cf. text. When normalized to peak 5 in Fig. 2D, the number of cysteines in the peaks are: 1.1/peak 1, 1.7/peak 2, 0.72/peak 3, 0.84-0.84/peak 4 (unresolved double peak), 2.0/peak 5, 0.45/peak 6, 0.33/peak 7. Residual flavin: 4.0%.

The electron donor to the reductase, NADPH, blocked virtually all of the cysteines from ANM labelling (Fig. 2B), consistent with its ability to block inhibitory effects of various cysteine reagents (9). The fact that NADPH, despite its relatively small size, blocks most of the cysteines from reaction with five equivalents of ANM, suggests that the purified reductase conformation is tightly contracted. Apparently, when the purified reductase

preparation was dialyzed into buffer, release of the large amount of adsorbed deoxycholate used in the purification generated sufficient positive entropy to drive the reductase into intermolecular aggregation and/or intramolecular phase inversion.

Cytochrome c blocked ANM reaction with the reductase in a similar manner (Fig. 2C). Still more definitive evidence is that cytochrome c, when bound to the reductase before ANM reaction, caused resolution of the broad ANM fluorescence band into 400 nm and 430 nm bands with selective quenching of the 400 nm band (Fig. 1B, inset - cf. a,b,c,d). This is a consequence of the split cytochrome c circular dichroic bands in this region (10) interacting with bound ANM on the reductase.

In microsomes, the reductase interacts with cytochromes P-450 and b_5, and with the membrane lipids, which are associated with its hydrophobic surface while its hydrophilic surface is free to be adsorbed to 2',5'-ADP Sepharose. When the hydrophobic surface was purified of the cytochromes by detergents and the reductase was eluted from the column-bound affinity nucleotide with 2'-AMP, its microsomal conformation must have been largely preserved. When this reductase in the elution buffer containing 5 mM 2'-AMP and 0.15% deoxycholate (7) was reacted with 5 ANM equivalents, dialyzed and trypsinized, the more hydrophilic peptides, peaks 1 and 3, were not labelled, while the more hydrophobic peptide, peak 5, was highly labelled (Fig. 2D) and a large, relatively more hydrophobic, compound peak, peak 7, appeared. Peak 7 converted to peak 5 on freezing, storage, and thawing of the detergent-solubilized reductase. Peak 5 was, therefore, attached to a very hydrophobic peptide, possibly the one with no cysteine which was identified at the amino terminus of the reductase from rabbit (11), and which was susceptible to protease cleavage (11,12). The cleaved reductase does not bind to or reduce cytochrome P-450. Thus, peak 7, which incorporates peak 5, probably represents the microsomal binding sites of cytochromes P-450, as well as b_5 and the membrane phospholipids.

In an attempt to assess whether FAD and FMN binding sites in the reductase contain cysteine residues, we determined the effect of removal of the flavins on ANM reactions with the reductase. The following experimental protocol was followed: Five equivalents of ANM were reacted with the reductase, which was then dialyzed, and an aliquot was hydrolyzed with trypsin producing the peptides shown in Fig. 2A. Another aliquot was treated with guanidine hydrochloride (1 M), and after 2 min, was reacted with a further five ANM equivalents and then dialyzed. The tryptic peptide maps of the two samples were identical with the exception that a previously weakly labelled peak, peak 2, became equivalent to peak 5 (Fig. 2E), which, in turn, showed a doubling of the fluorescence intensity relative to that in the 2'-AMP-bound/deoxycholate-solubilized preparation shown in Fig. 2D. Since NADPH binds close to FAD in the hydrophilic region, peak 2 (both of its cysteines buried) is associated with the FAD binding site. Conversely, peak 5, which contains the cytochrome P-450 binding site involving the exposed cysteine, must bind FMN involving the buried cysteine. Most of the tryptophan fluorescence is quenched upon deflavinizing and ANM binding (Fig. 1B-inset e). A strong cooperativity is suggested since the peaks approach maximal fluorescence upon deflavinizing.

After this work was concluded, the complete amino acid sequence of the rat reductase was published (13), confirming the salient features previously determined to be similar to rabbit reductase. Although identification of each labelled peptide awaits completion of our amino acid analysis, an interesting correlation of our function-specified cysteines with those in the amino acid sequence emerges if we align peak 7 with the hydrophobic amino terminus, and the more hydrophilic peaks 1, 2, and 3 with the carboxy terminal half of the reductase. It may be noted that the flavin-binding amino residue for each flavin, determined from a homology computation (13), is located close to two cysteines in each half of the protein polypeptide.

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