STUDIES ON THE IDENTITY OF THE HEME-BINDING CYSTEINYL RESIDUE
IN RABBIT LIVER MICROSOMAL CYTOCHROME P-450 ISOZYME 2

Shaun D. Black and Minor J. Coon

Department of Biological Chemistry
Medical School, The University of Michigan
Ann Arbor, Michigan 48109

SUMMARY: The reaction of purified rabbit liver microsomal P-450
Isozyme 2 with 4,4'-dithiobis(2-nitrobenzoate) (DTNB) exhibits first order
kinetics and results in the modification of a single thiol, but causes no
net loss of the native ferrous-carbonyl spectrum.   Inclusion of both
phospholipid and a tight-binding nitrogenous ligand, 1-benzylimidazole, in
the reaction medium produces a burst-phase of DTNB modification, but the
stoichiometry remains one thiol modified per polypeptide chain. The site of
Isozyme 2 rapidly labeled by DTNB and by monobromobimane, a fluorescent
reagent for thiol groups, was shown to be Cys_{152}. Results obtained strongly
suggest that Cys_{152} does not provide the proximal thiolate ligand to the
heme iron atom. Since Cys_{152} represents one of the two highly conserved
cysteine-containing regions in the P-450 cytochromes, it appears likely that
the other region, containing Cys_{436} in this rabbit cytochrome (corresponding
to Cys_{355} in bacterial P-450 cam, Cys_{436} in rat P-450 b or e, Cys_{461} in rat
P-450 c, Cys_{456} in rat P-450 d or mouse Isozyme 3, and Cys_{458} in mouse
Isozyme 1) is the source of the thiolate ligand to the heme.   © 1985 Academic
Press, Inc.

The establishment of the primary structure of P-450 Isozyme 2[*] (1,2)

makes possible the investigation of structure-function correlates for this

mammalian oxygenase (3,4).   It is now generally accepted that P-450

cytochromes contain a proximal or fifth axial thiolate ligand to the heme (5-

7), and that this complex is responsible for the unique spectral and redox

properties observed.   Isozyme 2 contains only four cysteine residues, at

positions 79, 152, 180, and 436, one of which must provide this ligand.

Sequence alignments of cysteine peptides of rabbit Isozyme 2 (1,2) with

those of rabbit Isozyme 4 (8), P-450cam (9,10), rat Isozymes b and e (11-

13), c (14-16), and d (17,18), mouse P_1-450 and P_3-450 (19,20), and bovine C-

21 hydroxylase (21) show that only the regions containing Cys_{152} and

Cys_{436} (in the numbering of Isozyme 2) are conserved.   Although various

laboratories have favored the former (1,14,17,21,22) or latter (2,11) Cys-

containing region as providing the heme ligand, physicochemical evidence is

---

[*] The abbreviations used are: P-450 Isozyme 2, the major phenobarbital-
inducible cytochrome in rabbit liver microsomes; DTNB, 5,5'-dithiobis(2-
nitrobenzoate); CTNB, 2-nitro-4-thiocyanobenzoate; DTPNO, 2,2'-dithio-
bis(pyridine-N-oxide); 2-PDS, 2,2'-dipyridyldisulfide; 4-PDS, 4,4'-di-
pyridyldisulfide; PTH, phenylthiohydantoin; and SDS, sodium dodecyl sulfate.

clearly required to reach a firm conclusion. The thiol modification studies presented here permit one of these Cys residues to be ruled out, thereby providing a tentative assignment of the other as providing the proximal heme ligand.

MATERIALS AND METHODS

P-450 isozyme 2 was purified from liver microsomes of phenobarbital-treated New Zealand White rabbits (4). DTNB, DTPNO, 2-PDS, 4-PDS, and 1-benzylimidazole were obtained from Aldrich. Arg^8-vasopressin, CHAPS, and monobromobimane (Thiolyte-MB) were supplied by Calbiochem-Behring; endo-proteinase-Lys C was from Boehringer-Mannheim, bromelain from Sigma, and α-chymotrypsin from Worthington. For synthesis of dibimane-Arg^8-vasopressin, the peptide disulfide was reduced with a 3-fold molar excess of dithio-threitol for 4 h at 23°C, a 20-fold molar excess of monobromobimane was added, and the mixture was incubated for 24 h at 37°C. The product was separated from reactants by reversed-phase HPLC. Other materials were described previously (1,4).

The concentration of P-450 isozyme 2 was measured in the Fe^{II}-CO state with use of ϵ 451nm= 110 mM^{-1}cm^{-1} (4). Stoichiometry measurements employed the following molar extinction coefficients: thiolate anions of DTNB and CTNB, $\Delta\epsilon$ 412nm= 13,600 (23); thiopyridone products of DTPNO, 2-PDS, and 4-PDS, ϵ 333nm= 8,750 (determined in present work), ϵ 343nm= 7,000 (24) and ϵ 324nm= 19,800 (24), respectively. Spectral measurements were made with a Varian-Cary 219 instrument with a 1.0-cm pathlength. In all experiments sample and reference cells had equal concentrations of thiol reagent.

For modification studies with DTNB and CTNB, a 10-μM solution of P-450 in 0.1 M KPi buffer, pH 7.4, was incubated with a 100-fold molar excess of thiol reagent at 30°C. When present, DTPNO, 2-PDS, or 4-PDS were added at 50-, 50-, or 25-fold molar excess, respectively. In experiments with dilauroylglyceryl-3-phosphorylcholine or 1-benzylimidazole, these had final concentrations of 30 μg/ml and 100 μM, respectively; 1-benzylimidazole was added from a 50 mM stock solution prepared in buffered 50% aqueous methanol. When present, CHAPS was at a final concentration of 1% (w/v). Modification studies with monobromobimane were carried out as with DTNB but reaction was at 37°C and the P-450 concentration was 37 μM. Denaturing conditions were achieved with 2% SDS (w/v), 4 M urea, or 3.8 M guanidine. Proteolytic digestion mixtures were at 37°C and contained protease and P-450 at a 1:50 (w/w) ratio. Limited endoproteinase-Lys C digestions (1,25) required 3 h, while extensive digestion with this protease, chymotrypsin, or bromelain required 24 h.

HPLC was performed with a Vydac C-18 column (5 μm, 4.5 x 250 mm) and Beckman 340 system having a model 160 or 165 absorbance detector and Schoeffel model GM 770 fluorescence detector. Gradients from water to 3:1 (v/v) CH$_3$CN:2-propanol, 0.1% trifluoroacetic acid throughout, were at a rate of 1% per ml. After loading samples at 0% organic solvent, gradients began at 50% for intact P-450 and for limited endoproteinase Lys-C digestion mixtures, or at 20% for small peptides. Peptides were repurified with a Waters μBondapak phenyl column (10 μm, 3.9 x 300 mm) and the same mobile phase and gradient as for small peptides. Peptide bond absorbance in eluates was measured at 214 nm; fluorescence was measured as emission > 418 nm with excitation at 395 nm, the absorption maximum of R-S-bimane. Peptide sequencing and amino acid analysis by HPLC of phenylthiocarbamyl derivatives were by the method of Tarr (26); PTH amino acids were analyzed and quantified by the method of Black and Coon (27).

RESULTS AND DISCUSSION

Modification of P-450 by DTNB and Related Thiol Reagents: When isozyme 2 was incubated in the absence of denaturants with a large excess of DTNB, a stoichiometry of 1.06 ± 0.09 thiol modified per polypeptide chain was
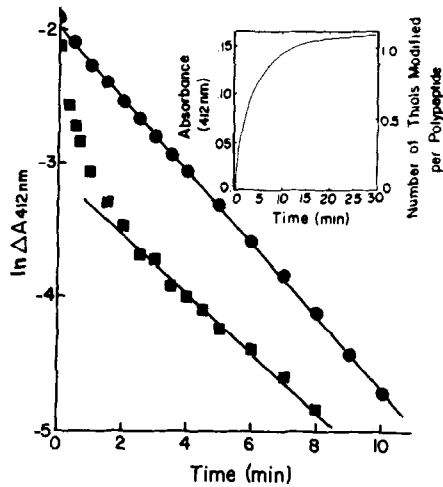
Fig. 1   Kinetics of modification of isozyme 2 by DTNB: effect of phospholipid and 1-benzylimidazole.   ●, Logarithmic plot of data shown in inset, with phospholipid present; the stoichiometry was 1.09 thiol modified per polypeptide.   ■, Logarithmic plot of data obtained in the presence of phospholipid and 1-benzylimidazole; the stoichiometry was 1.15 thiol modified per polypeptide.

observed as shown by the example in Fig. 1 (inset). No additional modification was observed, even after 10 h of reaction. However, in experiments in which agents such as SDS, urea, or guanidine were present, 4.0 ± 0.31 thiols per polypeptide were derivatized, in agreement with the number of Cys residues in the primary structure (1,2). In the absence of denaturants, reaction of isozyme 2 with four additional reagents, DTPNO, CTNB, 2-PDS, and 4-PDS, resulted in stoichiometries of 0.5, 0.7, 0.9, and 1.8 thiols modified per polypeptide, respectively, thus showing the limited accessibility of thiols in the protein. None of the reagents examined caused significant loss of the native ferrous-carbonyl spectrum, thereby indicating that the rapidly modified Cys residue(s) does not function as the proximal heme ligand.

The modification of isozyme 2 by DTNB proceeds as a pseudo first-order process, as shown in Fig. 1. Althought not definitive, these data suggest that the stoichiometry of one thiol per polypeptide may correspond to the modification of a single highly reactive or accessible Cys residue, rather than the fractional labelling of multiple residues. Limited proteolysis of the modified protein (data not shown) indicated that the site of modification was limited to the NH_2-terminal half of the molecule which contains Cys_{79}, Cys_{152}, and Cys_{180}; Cys_{436} was not modified by DTNB. Although the rate constant, order of reaction, and extent of modification were unchanged by the addition of phospholipid, when both phospholipid and a nitrogenous heme ligand, 1-benzylimidazole ($K_d = 1$ μM in the presence of

phospholipid (28)), were included in the reaction mixture a pronounced burst phase of sulfhydryl modification was seen (Fig. 1). The initial rate was 10-fold greater than in the absence of a distal ligand, but the stoichiometry remained one thiol modified per polypeptide chain. To investigate whether this ligand-induced burst was a property of the individual P-450 molecules or due to cooperative interactions within the aggregate (29), the modification by DTNB with and without 1-benzylimidazole present was carried out in the presence of 1% CHAPS, which brings the cytochrome to an essentially monomeric state (K. Inouye and M. J. Coon, unpublished results). Although the rate constant was slightly lower in the presence of the zwitterionic detergent, the same general behavior as in Fig. 1 was observed (data not shown). These results suggest that protein-protein interactions are not responsible for the ligand-induced burst of modification, and that this effect is due to an intrinsic property of the protein molecule, possibly a conformational change.

To identify which Cys residue provides the rapidly modified thiol, DTNB-modified isozyme 2 was freed of excess reagents, digested extensively with endoproteinase-Lys C, and fractionated by HPLC with monitoring at 214 nm for peptide bonds and 340 nm for bound thionitrobenzoate, and the single modified peptide was isolated and sequenced. The site of modification was shown to be Cys_{152}. However, because of the possibility of thiol-disulfide interchange, this result needed to be confirmed with use of a non-transferable thiol label as described below.

Modification with Monobromobimane and Localization of the Rapidly Modified Thiol: Monobromobimane has proven to be of great utility in protein modification studies since it is apparently specific for cysteine thiols and the thioether adduct is non-transferrable, stable, fluorescent over a broad pH range, and non-photolabile (30,31). When isozyme 2 was incubated at 37°C under non-denaturing conditions with a 10-fold molar excess of monobromobimane, HPLC of aliquots removed at various times showed the gradual incorporation of label such that by 12 h all four Cys residues were modified, as seen in Fig. 2. This was in contrast to the behavior seen with DTNB where only a single thiol is accessible. HPLC analyses of limited endoproteinase-Lys C digests of the P-450 at various points in the reaction showed rapid bimane incorporation into the NH_2-terminal portion of the protein containing Cys_{79}, Cys_{152}, and Cys_{180}, and slow reaction with the COOH-terminal half containing Cys_{436}. An examination of the P-450 spectrum during the reaction, as seen in the figure, showed a gradual loss of the native Fe^{II}-CO absorption band, but with kinetics much slower than for gross incorporation of the bimane label. Of interest, the rate approximately mirrored that of Cys_{436} modification. A control experiment with isozyme 2
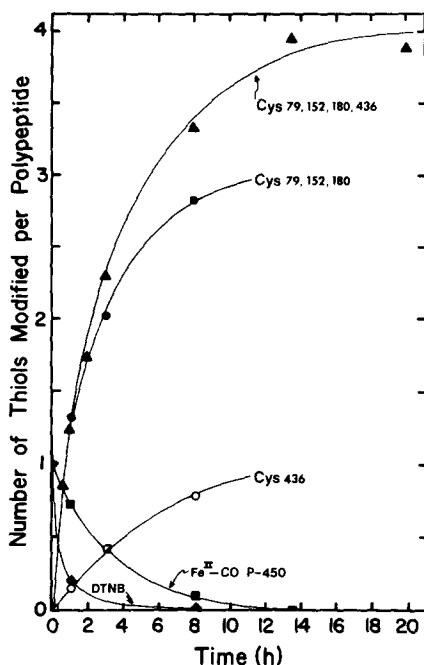
Fig. 2. Modification of isozyme 2 by monobromobimane. Number of thiols modified per polypeptide chain under non-denaturing conditions, ▲. Aliquots removed at various times were submitted to reversed-phase HPLC with dual absorbance and fluorescence detection. Fluorescent label/peptide bond ratios were calculated at each time point and were scaled to a maximum yield of four total thiols; in experiments not shown, DTNB titrations under denaturing conditions showed the validity of this scaling. Aliquots of the original incubation mixture were dialyzed to remove excess reagent and then digested briefly with endoproteinase-Lys C to separate the NH_2-terminal and COOH-terminal halves of the cytochrome by scission of the Lys_{274}-Asp_{275} bond. HPLC analysis and the scaling of fluorescence to absorbance ratios for NH_2-terminal (Cys_{79}, Cys_{152}, Cys_{180}) and COOH-terminal (Cys_{436}) halves of the protein were as above, but to 3 thiols, ●, and 1 thiol, ○, respectively. Results of ferrous-carbonyl spectral determinations were scaled to correspond to one total thiol, ■ , on the assumption that the P-450 spectrum is related to a single iron-sulfur bond. Aliquots of the digestion mixture were dialyzed and then titrated under non-denaturing conditions to assess the equivalents of DTNB-accessible thiol remaining, ◆.

at 37°C in the absence of monobromobimane showed no more than 10% loss of P-450 in 12 h, thus indicating that the loss was due to modification by this agent and not simply thermal denaturation. Titration of dialyzed bimane-modified samples by DTNB showed that the thiol accessible to the latter reagent was lost rapidly during monobromobimane treatment with little loss of the P-450 spectrum. Thus, these data suggest that both DTNB and monobromobimane rapidly modify the same Cys residue in the P-450.

To identify the site of rapid bimane labelling, isozyme 2 was modified for 30 min; at this point, about 0.60 of the accessible thiol had been modified compared to about 0.65 of all thiols, with only negligible loss (~5%) of the native P-450 spectrum. This preparation was freed of excess reagent and portions were digested with either chymotrypsin or bromelain to produce small fragments, thus insuring comparable and high yields of polar
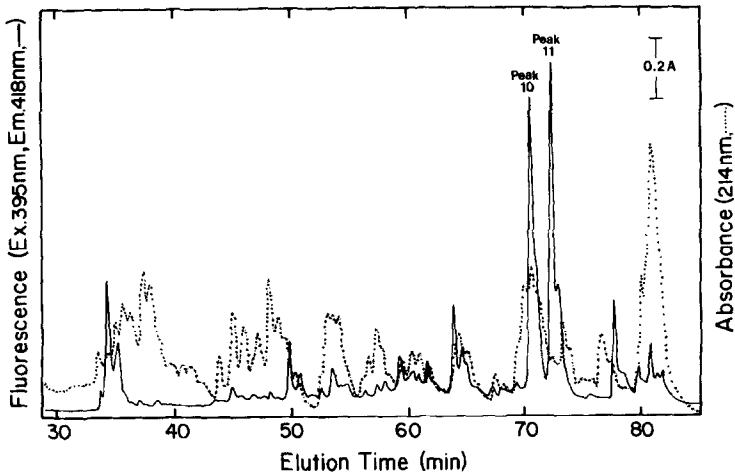
Fig. 3. HPLC of a chymotryptic digest of Isozyme 2 modified with monobromobimane for 30 min at 37°C. The digestion mixture (equivalent to 7 nmol of starting Isozyme 2) was analyzed by reversed-phase HPLC with absorbance and fluorescence detection.

and nonpolar Cys peptides during purification. Reversed-phase HPLC of the chymotryptic digestion is shown in Fig. 3. Two major fluorescent peptides, in peaks 10 and 11, were observed along with a few minor components, whereas in the analysis of the bromelain digest (data not shown) three major fluorescent peptides, in peaks 5,7, and 9, were found. A control experiment in which DTNB-modified isozyme 2 was labeled for 2 h at 37°C with monobromobimane, digested with either chymotrypsin or bromelain, and then analyzed by HPLC, showed that only the major fluorescent peaks described above were absent. These data provided further proof that DTNB and monobromobimane modify a common site. After rechromatography, the peptides were submitted to sequence analysis with the results shown in Table I. All peptides could be placed uniquely within the primary structure of Isozyme 2. Bromelain peptides 5,7, and 9 all proved to have a COOH-terminal S-bimane-Cys_{152}, although they differ in the site of cleavage at the NH_2-terminus. Similarly, chymotryptic peptides 10 and 11 had a COOH-terminal S-bimane-Cys_{152}-Leu and differed at the NH_2-terminus by the sequence Gly-Lys-Arg_{140}. Thus, these findings corroborate those obtained with DTNB and show that Cys_{152} is the rapidly modified thiol in P-450 Isozyme 2.

Since our results show no significant loss of the native P-450 spectrum during modification of Cys_{152} in Isozyme 2, this residue is highly unlikely to provide the proximal thiolate ligand to the heme. This and other laboratories (cf. 8,13) have noted in comparisons of sequence information on cysteine peptides from various P-450's that only two regions of high conservation occur, one near the NH_2-terminus (containing Cys_{152} in Isozyme 2) and the other near the COOH-terminus (Cys_{436} in Isozyme 2). Because heme ligands are expected to be highly conserved as in other heme proteins (32),

TABLE I

Manual Sequence Analysis of Bimane-Labelled Cysteine Peptides

Peptide	Cycle	Residue Identified	Yield (pmol)	Fluorescence detected Residue	Fluorescence detected Peptide	Sequence of peptide
Bromelain 5.1	1	Ala	133	−	+	ARC_{152}
	2	Arg_a	43	−	+	
	3	Cys_a	19	+	−	
Bromelain 7.1	1	Glu	28	−	+	$EARC_{152}$
	2	Ala	36	−	+	
	3	Arg_a	19	−	+	
	4	Cys_a	6	+	−	
Bromelain 9.1	1	Glu	22	−	+	$EEARC_{152}$
	2	Glu	15	−	+	
	3	Ala	23	−	+	
Chymotrypsin 10.1	1	Gly	263	−	+	$GKRSVEERIQEEARC_{152}L$
	2	Lys	93	−	+	
	3	Arg	87	−	+	
	4	Ser	138	−	+	
	5	Val	137	−	+	
	6	Glu	82	−	+	
	7	Glu	102	−	+	
	8	Arg	51	−	+	
	9	Ile	55	−	+	
	10	Gln	90	−	+	
	11	Glu	57	−	+	
	12	Glu	77	−	+	
	13	Ala	64	−	+	
	14	Arg_a	13	−	+	
	15	Cys_a	14	+	±	
	16	Leu	25	±	±	
Chymotrypsin 11.1[b]	1	Ser	150	−	+	$SVEERIQEEARC_{152}L$
	2	Val	199	−	+	
	3	Glu	150	−	+	
	4	Glu	160	−	+	
	5	Arg	46	−	+	
	6	Ile	69	−	+	
	7	Gln	55	−	+	

[a] S-Bimane-Cys was somewhat unstable in the acidic conversion medium and was identified as two peaks in the PTH amino acid analysis by HPLC. The first peak was eluted after PTH-Glu-Me ester and the second before PTH-Met. This was confirmed through sequence analysis of bimane-modified Arg-vasopressin. S-Bimane-Cys was quantified as the sum of the areas of the two peaks on the assumption that both have the same extinction coefficient as PTH-Nle (the internal standard) at 254nm.

[b] The amino acid composition of this peptide was determined to be A,C,E_4,I,L,R_2,S,V, in excellent agreement with that expected from the sequence.

only one candidate apparently remains, that is, the Cys residue in position 436 of rabbit isozyme 2 or rat isozymes b and e, position 355 of P-450cam, position 461 of rat P-450 c, position 456 of rat P-450 d or mouse P_3-450, and position 458 of mouse P_1-450. Recently, Morohashi et al. (33) published the deduced amino acid sequence of bovine P-450$_{scc}$ which contains only one of the conserved Cys-containing regions, equivalent to Cys_{436} in isozyme 2, a finding in agreement with the conclusions reached in the present work.

REFERENCES

1. Tarr, G. E., Black, S. D., Fujita, V. S., and Coon, M. J. (1983) Proc. Natl. Acad. Sci. USA 80, 6552-6556.
2. Heinemann, F. S., and Ozols, J. (1983) J. Biol. Chem. 258, 4195-4201.
3. Haugen, D. A., van der Hoeven, T. A., and Coon, M. J. (1975) J. Biol. Chem. 250, 3567-3570.
4. Haugen, D. A., and Coon, M. J. (1976) J. Biol. Chem. 251, 7929-7939.
5. White, R. E., and Coon, M. J. (1980) Ann. Rev. Biochem. 49, 315-356.
6. Hahn, J. E., Hodgson, K. O., Andersson, L. A., and Dawson, J. H. (1982) J. Biol. Chem. 257, 10934-10941.
7. Champion, P. M., Stallard, B. R., Wagner, G. C., and Gunsalus, I. C. (1982) J. Am. Chem. Soc. 104, 5469-5472.
8. Fujita, V. S., Black, S. D., Tarr, G. E., Koop, D. E., and Coon, M. J. (1984) Proc. Natl. Acad. Sci. USA 81, 4260-4264.
9. Haniu, M., Armes, L. G., Tanaka, M., Yasunobu, K. T., Shastry, B. S., Wagner, G. C., and Gunsalus, I. C. (1982) Biochem. Biophys. Res. Commun. 105, 889-894.
10. Haniu, M., Armes, L. G., Yasunobu, K. T., Shastry, B. A., and Gunsalus, I. C. (1982) J. Biol. Chem. 257, 12664-12671.
11. Fujii-Kuriyama, Y., Mizukami, Y., Kawajiri, K., Sogawa, K, and Muramatsu, M. (1982) Proc. Natl. Acad. Sci. USA 79, 2793-2797.
12. Yuan, P.-M., Ryan, D. E., Levin, W., and Shively, J. E. (1983) Proc. Natl. Acad. Sci. USA 80, 1169-1173.
13. Gotoh, O., Tagashira, Y., Iizuka, T., and Fujii-Kuriyama, Y. (1983) J. Biochem. (Tokyo) 93, 807-817.
14. Haniu, M., Yuan, P.-M., Ryan, D. E., Levin, W., and Shively, J. E. (1984) Biochemistry 23, 2478-2482.
15. Yabusaki, Y., Shimizu, M., Murakami, H., Nakamura, K., Oeda, K., and Ohkawa, H. (1984) Nucl. Acids Res. 12, 2929-2938.
16. Sogawa, K., Gotoh, O., Kawajiri, K., and Fujii-Kuriyama, Y. (1984) Proc. Natl. Acad. Sci. USA 81, 5066-5070.
17. Haniu, M., Ryan, D. E., Levin, W., Shively, J. E. (1984) Proc. Natl. Acad. Sci. USA 81, 4298-4301.
18. Kawajiri, K., Gotoh, O., Sogawa, K., Tagashira, Y., Muramatsu, M., and Fujii-Kuriyama, Y. (1984) Proc. Natl. Acad. Sci. USA 81, 1649-1653.
19. Kimura, S., Gonzalez, F. J., and Nebert, D. W. (1984) Nucl. Acids Res. 12, 2917-2928.
20. Kimura, S., Gonzalez, F. J., and Nebert, D. W. (1984) J. Biol. Chem. (In press).
21. Yuan, P.-M., Nakajin, S., Haniu, M., Shinoda, M., Hall, P. F., and Shively, J. E. (1983) Biochemistry 22, 143-149.
22. Black, S. D., Tarr, G. E., and Coon, M. J. (1982) J. Biol. Chem. 257, 14616-14619.
23. Ellman, G. L. (1959) Arch. Biochem. Biophys. 82, 70-77.
24. Grassetti, D. R., and Murray, J. F., Jr. (1967) Arch. Biochem. Biophys. 119, 41-49.
25. Black, S. D., Tarr, G. E., Fujita, V. S, Yasunobu, K. T., and Coon, M. J. (1983) Fed. Proc. 42, 1774.
26. Tarr, G. E. (1985) in Microcharacterization of Polypeptides: A Practical Manual (J.E. Shively, ed.) Humana Press, Clifton, N.J., in press.
27. Black, S. D., and Coon, M. J. (1982) Anal. Biochem. 121, 281-284.
28. White, R. E., and Coon, M. J. (1982) J. Biol. Chem. 257, 3073-3083.
29. French, J. S., Guengerich, F. P., and Coon, M. J. (1980) J. Biol. Chem. 255, 4112-4119.
30. Kosower, N. S., Kosower, E. M., Newton, G. L., and Ranney, H. M. (1979) Proc. Natl. Acad. Sci. USA 76, 3382-3386.
31. Vogel, F., and Lumper, L. (1982) Biochem. J. 215, 159-166.
32. Dayhoff, M. O., Eck, R. V., and Park, C. M. (1972) in Atlas of Protein Sequence and Structure (Dayhoff, M. O., ed) National Biomedical Research Foundation, Washington, D.C.
33. Morohashi, K., Fujii-Kuriyama, Y., Okada, Y., Sogawa, K., Hirose, T., Inayama, S., and Omura, T. (1984) Proc. Natl. Acad. Sci. USA 81, 4647-4651.