BIOORGANIC CHEMISTRY 19, 66–76 (1991)

Use of Pyrocarbonates for Chemical Modification of Histidine Residues of Horseradish Peroxidase

MARTINE URRUTIGOITY,[1] MICHEL BABOULENE, AND ARMAND LATTES

Laboratoire des IMRCP, UA 470 CNRS, 118 route de Narbonne, 31062 Toulouse Cédex, France

Received July 5, 1990

In an attempt to alter the catalytic properties of horseradish peroxidase (HRP, EC 1.11.1.7), various electrophiles were employed to modify histidine residues in this enzyme. Pyrocarbonates were found to be particularly effective, and their chromatic effect was exploited to determine the number of modified histidine residues directly by uv spectroscopy. We also developed a method for assay of histidines using diethyl pyrocarbonate, which could be extended to determination of these residues in other proteins. We showed that the catalytic activity of HRP was not affected by modification of histine residues, especially His 170, by small-sized substituents not containing reactive groups. On the other hand, electron-rich substituents, especially those with a heteroatom such as sulfur, disrupt the heme structure without producing the catalytic properties of cytochrome P450.

INTRODUCTION

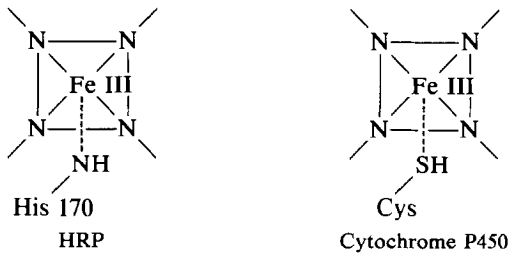
The chemical modification of enzymes has seen considerable development over the past few years (*1, 2*). Most studies have been devoted to modification of the peripheral structure in an attempt to alter the physicochemical characteristics, especially those that would make enzymes more liposoluble. Modified enzymes could then be employed in organic solvents. Among others, a potential application is in ELISA test systems for preparation of conjugates from molecules of biological interest.

Chemical modifications in internal regions has provided a better understanding of the structure of enzymes, especially around the active site (*3*). In this case, modifications are made to the amino acids involved in the catalytic cycle or those close to the active site.

Our main objective was to try and alter the catalytic properties of an enzyme by chemical modification of the amino acid residues involved in the catalytic process. By analogy with the structure of cytochrome P450 (*5*), horseradish peroxidase (HRP, EC 1.11.1.7) an oxidoreductase with a heme-containing prosthetic group (*4*) would appear to be well suited to such an investigation. There is considerable structural similarity between these two enzymes (Scheme 1). It was thus thought worth investigating the effects of changing the heme iron ligand of HRP, notably His 170.

[1] To whom correspondence should be addressed.

0045-2068/91 $3.00



Scheme 1. HRP (left) and cytochrome P450 (right).

We linked carbon chains containing electron rich sites (π system) or heteroatoms such as bromine or sulfur to the imidazole nitrogen of His in HRP. We focused attention on the action of various pyrocarbonates, and devised a method for determination of these novel biocatalysts.

MATERIALS AND METHODS

Materials

HRP (grade II) was from Böehringer. Diethyl pyrocarbonate (DEP), glycoaldehyde, and ethyl and allyl chloroformate were from Fluka. Thiolacetic acid, allyl alcohol, benzyl alcohol, and the other chloroformates were from Aldrich. Ethanol was from Prolabo. Ultraviolet spectra and enzyme activities were recorded on a HP 8450 A spectrophotometer. Temperature and stirring were maintained with an adapted HP 89100 A temperature controller. Isoelectric points were determined using the Pharmacia Phast system with IEF 3-9 Phast Gels for electrofocusing. Proteins were revealed using the silver nitrate procedure recommended by Pharmacia.

IR spectra were recorded on a Perkin–Elmer 685 instrument, and ^{1}H NMR spectra were recorded on a Varian T60 or Bruker AC 80 spectrometer.

Methods

Peroxidation of guaiacol (*6*) and determination of HRP concentration (*7*) have been described in previous publications.

Chemical Modifications

Protection of lysines. Glycoaldehyde (60 mg) was added to a solution of 40 mg native HRP in 8 ml of 50 mM borate buffer, pH 9.5. After stirring for 1 h at room temperature, 10 mg $NaBH_4$ was added. The mixture was stirred for 90 min at 4°C. The pH was adjusted to 5.0 with 1 N acetic acid, and the solution was then dialyzed for 16 h at 4°C against distilled water. The dialyzed material was freeze-dried.

Modification of histidines. The freeze-dried dialyzed material (8 mg) was dissolved in 4 ml 0.1 M phosphate buffer, pH 6.5. A 50 mM solution of electrophile (pyrocarbonates, allyl chloride, dibromopropane, 3-chloro-1-propanethiol, 2,4′-dibromoacetophenone) was freshly prepared in absolute ethanol. Solutions of acetic anhydride and formaldehyde were made up in the borate buffer. This solution (25 μl) was added to the enzyme preparation. The mixture was allowed to react at room temperature for 60 min with glycoaldehyde and acetic anhydride, for 90 min with the pyrocarbonates and for 3 h with 2,4′-dibromoacetophenone. It was then dialyzed for 16 h at 4°C against 1 mM phosphate buffer, pH 6.5.

Determination of number of histidines modified. Histidines modified with DEP were determined from the difference in absorbance at 240 nm of the modified and unmodified peroxidase using a millimolar coefficient of 3.6 $mM^{-1} \cdot cm^{-1}$ (*8*).

DEP probe. Modified enzyme (2 mg) was dissolved in 2 ml of 0.1 M phosphate buffer, pH 6.5, and 15 μl of 50 mM DEP in absolute alcohol. After stirring for 1 h at room temperature, the solution was dialyzed against 10 mM phosphate buffer.

Preparation of compounds I and II. The formation of compounds I and II was followed from the absorption spectra between 250 and 600 nm. Compound I was produced by addition of 10 eq of H_2O_2 with respect to the heme content of the enzyme, and compound II with 10 eq of ascorbic acid (*9, 10*).

General Synthesis of Pyrocarbonates **1** (*11, 12*)

Carbon dioxide gas is bubbled into a solution of 5 g of sodium alcoholate in 40 ml of the corresponding alcohol (or dry THF for the solid alcohols) for 3–5 h at 25°C. The solid formed is filtered off, washed, and dried. It is then dissolved in 75 ml of anhydrous acetone. Ten grams of the required chloroformate are added while stirring. The mixture is refluxed for 3–5 h. The precipitate is filtered off, and the pyrocarbonate is distilled (see Scheme 2).

Synthesis of Ethyl Propanethiol Pyrocarbonate, **6c**

Three grams of allyl pyrocarbonate, **4c,** is dissolved in 10 ml of anhydrous ether. The mixture is irradiated from an unfiltered 200-W mercury vapor uv lamp via a Muller quartz fiber optic cable. Thioacetic acid (1.3 g) is added dropwise from a syringe. The temperature rises to 36–40°C. The mixture is irradiated for a further 3 h. Compound **5c** is obtained by distillation. $C_9H_{14}O_6S$ (282): bp/2 mm Hg; 90°C; yield, 100%. ^1H NMR ($CDCl_3$ + TMS, δ ppm): 4 (m, 4H, $O-CH_2$); 2.8 (t, 2H, SCH_2); 2.3 (s, 3H, $SCOCH_3$); 1.8 (m, 2H, $C-CH_2-C$; 1.1 (t, 3H, $C-CH_3$). IR (cm^{-1}) : ν C–C (2900); ν O–CO–O–CO–O(1830); ν C=O (1750); ν $CO=CH_3$ (1690).

Anhydrous (2%) HCl followed by 1.25 g of pyrocarbonate **5c** is added to 20 ml of methanol in a 25-ml round-bottomed flask. The mixture is boiled to eliminate the methyl acetate (bp ≈ 37°C) formed. The residual methanol is then evaporated under vacuum leaving compound **6c**. $C_7H_{12}O_5S$ (240): Yield, 70%. ^1H NMR ($CDCl_3$ + TMS, δ ppm): 4 (m, 4H, OCH_2); 2.5 (t, 2H, SCH_2); 1.8 (m, 2H, $C-CH_2-C$); 1.45 (s, 1H, SH); 1.1 (t, 3H, CH_3). IR (cm^{-1}): ν C–C (2900); ν O–CO–O–CO–O (1825); ν C=O (1750).

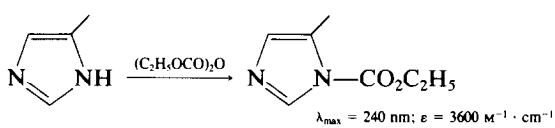
RESULTS AND DISCUSSION

To our knowledge, there are no reports on the chemical modification of histidine residues of HRP despite the role attributed to His 170 in enzyme activity (*13*a). In the absence of X-ray structural information, it is known, however, that HRP possesses three histidine residues (*13*b). In order to avoid interfering reactions, the ε-NH_2 groups of lysines were blocked by treatment with glycolaldehyde and reduction with $NaBH_4$ (*14*). In the pH range used in our study (6 to 6.5), the ε-NH_2 groups of lysines are relatively unreactive compared to the imidazoyl nitrogen atoms of histidine (pK_a histidine = 6.6). Blocking the lysine ε-NH_2 groups by glycolaldehyde/$NaBH_4$ led to a slight reduction in enzyme activity ($\leq$5%). A number of electrophiles were investigated for the attack on the imidazole ring (*15*).

Modification by Halogen Derivatives

The reactivity of imidazoyl nitrogen atoms in the protein structure is reduced by a variety of steric and electronic effects, and by the presence of hydrophilic and hydrophobic regions within the enzyme. We used four reagents in the first series of experiments: dibromopropane, chloropropanethiol, allyl chloride, and 2,4′-dibromoacetophenone. However, in most cases, we were unable to evaluate the progress of the reaction. Only the modification with 2,4′-dibromoacetophenone produced a characteristic absorption (λ_{max} = 265 nm; ε = 17,600 $M^{-1} \cdot cm^{-1}$) (*16*), enabling assay of the modified histidine residues. The absence of chromorphoric or chromogenic groups in the other reagents effectively prevented evaluation of the extent of the modification. Determination of residual histidines using the method described by Pauly (*17*) was unsatisfactory, and so we developed a new technique for assay of histidines in proteins using diethyl pyrocarbonate (DEP) as probe.

Assay of histidine using the DEP probe. Diethyl pyrocarbonate is known to be highly reactive toward histidines (*18, 19*). In the pH range 5.5 to 7, it attacks the imidazoyl nitrogen, producing a carbethoxy-histidine which has a characteristic absorption at 240 nm.



Reacting DEP with HRP gives rise to an absorption band at 240 nm, indicating that it had in fact reacted with the histidine residues in the enzyme. DEP was found to react with all the histidine residue on HRP, and the native enzyme could be regenerated by treatment with hydroxylamine (*20*).

We used three characteristics to develop an assay for histidines in modified HRP. The proteins recovered after treatment of HRP with the four previously mentioned electrophiles were reacted with DEP. The presence in the uv spectrum

TABLE 1

Modification of Histidine Residues of HRP

Treatment	DEP probe	Conclusion
HRP + $(CH_3CO)_2O$	No peak at 240 nm	All His modified
HRP + $Br(CH_2)_3Br$	Peak at 240 nm	His unmodified
HRP + $ClCH_2CH{=}CH_2$	Peak at 240 nm	His unmodified
HRP + $Cl(CH_2)_3SH$	Peak at 240 nm	His unmodified
HRP + $BrC_6H_4COCH_2Br$	No peak at 240 nm	All His modified
HRP + HCHO	No peak at 240 nm	All His modified

of a band at 240 nm indicated that some of the histidine residues had not reacted (Table 1).

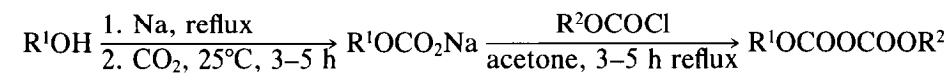
The results were in agreement with those obtained using 2,4′-dibromoacetophenone. The DEP probe demonstrated the reactivity of agents such as acetic anhydride or formaldehyde (*15*). It also showed that the halogenated alkyl derivatives did not react with the imidazoyl nitrogen atom of histidines.

Modification Using Pyrocarbonates

From the results of these preliminary experiments, it appeared that pyrocarbonates were the most promising reagents for chemical modification of histidine residues in HRP. They have the required reactivity, and after reaction, the modified species can be identified by its absorption at 240 nm. We therefore synthesized a number of pyrocarbonates according to the reactions in Scheme 2 (*11*, *12*).

The compounds **4** were characterized from their IR and ^1H NMR spectra and by elemental analysis (*12*).

Under the conditions described in Scheme 2, pyrocarbonates attacked the imidazoyl nitrogen of histidines as indicated by the characteristic absorption of carbalkoxy histidine (Table 2).



2 **4**

1–4	R^1	R^2	bp (°C/mbar)
a	C_2H_5	C_6H_5	80/2
b	C_2H_5	$CH_2C_6H_5$	90/2
c	C_2H_5	$CH_2CH{=}CH_2$	80/2
d	C_2H_5	CH_2CH_2Br	85/2
e	$CH_2C_6H_5$	$CH_2C_6H_5$	95/2
f	$CH_2CH{=}CH_2$	$CH_2CH{=}CH_2$	80/2 [65/0.05 Ref. (11)]

Scheme 2. Synthesis of pyrocarbonates **4**.

TABLE 2

Modification of HRP by Pyrocarbonates **4**

$R^1OCOOCOOR^2$ (**4**)	*N*-Acetyl histidines + **4** λ (nm)	**HRP** + **4** λ (nm)	**HRP** + **4** + DEP λ (nm)	No.[a]
a	258	258	258	3
b	258	258	258	3
c	256	256	256	3
d	260	260	260	3
e	260	260	260	3
f	242	242	245	3

[a] Number of histidine residues modified in HRP evaluated from change in DO after addition of DEP.

The absorption bands for the modified enzymes (HRP+**4**) and those treated with DEP (HRP+**4**+DEP) recorded between 200 and 600 nm were obtained by difference spectrometry (i.e., with the native enzyme in the reference beam). The spectra of *N*-acetyl histidines + **4** between 200 and 400 nm were recorded directly.

The addition of DEP to the products resulting from chemical modification of HRP (HRP+**4**) did not affect the uv spectra of these products nor those produced by treatment of *N*-acetyl histidines with compounds **4.** This indicated that the histidine residues had reacted. Analysis of the extinction coefficients confirmed that all three histidine residues had in fact reacted.

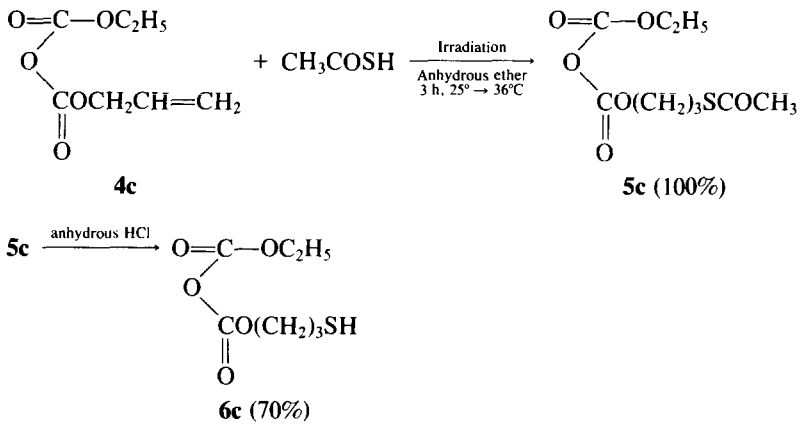
In the case of the reaction with the mixed pyrocarbonates (**4a** to **4d**), it is not obvious which functional group had reacted. In fact we observed a band at 260 nm rather than the characteristic uv absorption at 240 nm of the carbethoxy derivatives of histidine. This indicated that the functional group (bearing R^2) was linked to the histidine residues of HRP, demonstrating the accessibility of histidine residues to pyrocarbonates. This approach thus shows promise for chemical modification of enzymes such as HRP. For the synthesis of mixed pyrocarbonates, ethanol is used as the starting alcohol rather than alcohols with other functional groups that would be required for synthesis of the symmetric pyrocarbonates, thus avoiding competing reactions from bases during formation of the alcoholate (cf. Scheme 2). This is a further reason for employing mixed pyrocarbonates.

Special Case of Ethyl Propanethiol Pyrocarbonate **6c**

This compound was prepared by radical addition of thioacetic acid to allyl pyrocarbonate **4c** according to Scheme 3.

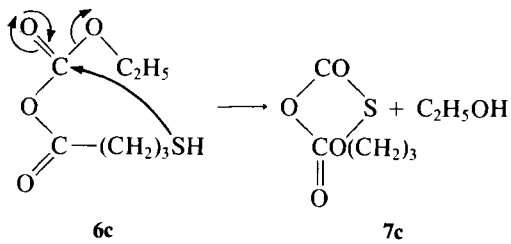
The compounds **5c** and **6c** were characterized from their ir and ^1H NMR spectra and by elemental analysis.

The nucleophilic character of the thiol group of compound **6c** gives rise to a spontaneous attack on the ethyl carbonate group, producing compound **7c** via an intramolecular transesterification.



Scheme 3. Synthesis of ethyl propanethiol pyrocarbonate **6c**.

Compound **6c** cannot therefore be stored for long periods of time. Under the conditions described above, uv spectroscopy showed that compounds **5c** and **6c** reacted with the three histidine residues on HRP.



Enzymatic Parameters of the Modified HRP

The UV spectra and the usual enzyme parameters were measured for the modified enzymes. The results are shown in Table 3 and Fig. 1.

It can be seen from these results that modifications of histidine residues by linkage to small-sized groups such as DEP or formaldehyde which are unlikely to have electronic interactions with the active site, had little influence on the enzymatic activity. We did not observe such a strong hydrogen donor effect of His 170 and His 42 to neighboring Arg 183 and Asp 43 as reported by other workers (*21, 22*). However, the alterations in structure of HRP by reactive groups such as 2,4′-dibromoacetophenone and the pyrocarbonates **4c, 5c,** and **6c** indicate that the histidines appeared to have a stabilizing effect on the ligands of heme iron, as found by Thanabal *et al.* (*23*).

Marked changes in properties were observed after modification of HRP by the pyrocarbonates **6c.** There was an increase in specific activity of the enzyme (3.5 to 5 U), and a loss of stability (Table 4).

TABLE 3

Enzyme Parameters of Modified HRP

Reagent	p*I*	Specific activity (U/nmol heme)	RZ[a]	Yield in hemoprotein[b]
None[c]	4.6	3.2	2.5	
HCHO	—	2.7	2.3	80
$BrC_6H_4COCH_2Br$	4	2	1	65
DEP	3.5	2.7	2.3	80
4c	3.5	1.8	1.5	70
5c	3.5	1.8	0.9	60
6c	—	5	3.6	140

[a] Reinheitszahl = A_{402} nm/A_{280} nm.
[b] Determined from ratio final A_{402} nm/initial A_{402} nm
[c] Unmodified HRP.

This marked loss of activity was matched by a change in spectral properties of the enzyme. There was a progressive fall in intensity of the characteristic Soret band of hemoprotein (Fig. 2).

Similar behavior was noted for the intermediate compounds I and II in the catalytic cycle of HRP (Scheme 4 and Table 5) (*24*).

This cycle is akin to that currently accepted for peroxidases (*25*). However, the modifications of the active site do not confer the oxidizing properties of cyto-

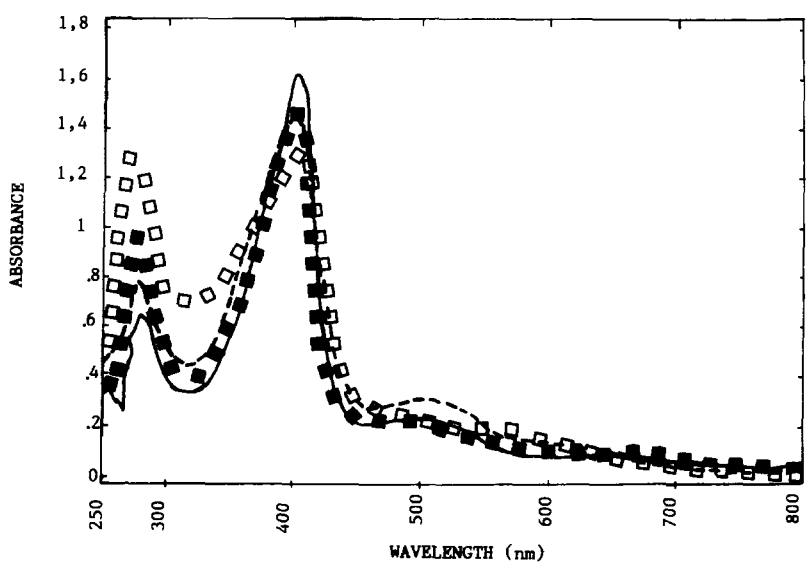


Fig. 1. Ultraviolet spectra of modified HRP. (---) HRP-glycol; (□) HRP-thioacetate; (■) HRP-allyl; (—) HRP-thiol.

TABLE 4

Temporal Stability of Modified HRP

Enzyme	Specific activity (U/nmol heme)		
	0 h	24 h	48 h
HRP-glycol	2.7	2.7	2.7
HRP-DEP	2.7	2.7	2.6
HRP-5c	1.8	1.8	1.6
HRP-6c	5	2	0.7

TABLE 5

Ultraviolet Absorption Bands of Compounds I and II

Enzymes	Compound I	Compound II
HRP	406 nm	418 nm
HRP + BrC_6H_4CO	412 nm	420 nm
HRP + 4f	402 nm	404 nm
HRP + 5c	404 nm	404 nm
HRP + 6c	404 nm	404 nm

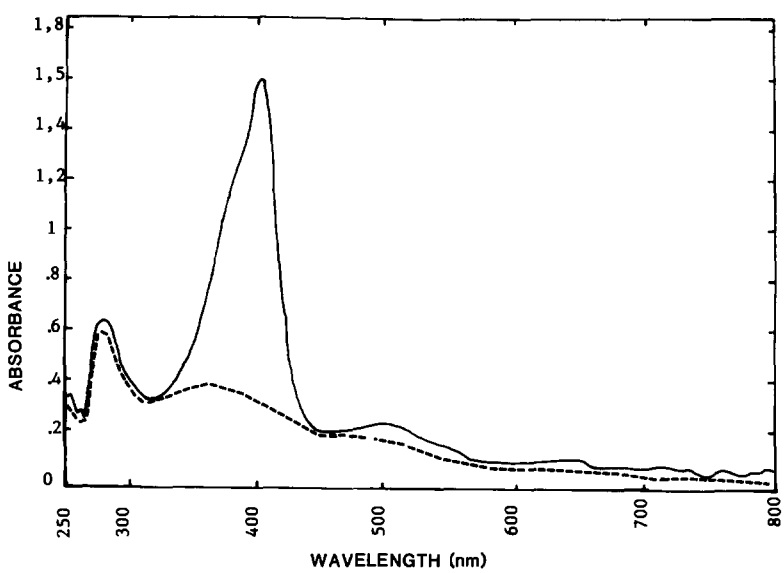
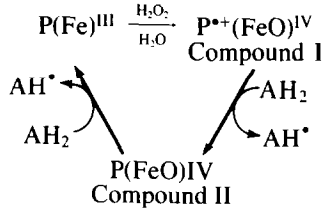


Fig. 2.



Scheme 4. Catalytic cycle of HRP.

chrome P450 on the new biocatalyst (oxidation of cinnamic to coumaric acid (*26*)), and carbon monoxide was not found to inhibit its activity (*27*).

CONCLUSION

The three histidine residues in horseradish peroxidase were shown to be readily modified using the highly reactive diethyl pyrocarbonate. The modifications were evaluated from the change in uv absorption due to the carbethoxy chromophore. We also developed a spectroscopic method for determination of histidine residues in modified HRP using DEP as probe. Experiments with this probe are being conducted with other enzymes to find out if it has general applicability.

Symmetrical and mixed pyrocarbonates were synthesized and coupled to the histidine results of HRP. Although steric factors had little influence (cf. HRP + DEP and HRP + $BrC_6H_4COCH_2Br$), electronic effects and the chemical reactivity of the added group led to loss of enzyme activity. Although there is some doubt on the role of His 170 as hydrogen donor, our results indicated that it stabilizes the ligands of heme iron. Our results provide an indication of the type of modification which should alter the properties of the enzyme without loss of activity. Work is in progress with reagents of different chain length and differing chemical reactivities.

REFERENCES

1. FEENEY, R. E., YAMASAKI, R. B., AND GEOGHEGAN, K. F., (1980) *in* Modification of Proteins, (Feeney, R. E., and Whitaker, J. R., eds.), Advances in Chemistry Ser. 198, Amer. Chem. Soc., Washington, D.C.
2. COHEN, L. A., (1970) *in* The Enzymes (Boyer, P. D., ed.) 3rd ed. Vol. 1, Academic Press, New York.
3. EYZAGUIRRE, J., (Ed) (1987) Chemical Modifications of Enzymes, pp. 9–22, Halstead Press, New York.
4. SAUNDERS, B. C., (1973) *Inorg. Chem.* **2,** 988–1021.
5. DAWSON, J. H., AND MANSANORI, S. (1987) *Chem. Rev.* **87,** 1255–1276.
6. SAUTIMONE, H. (1975) *Canad. J. Biochem.* **53,** 649–651.
7. OHLSSON, P. J., AND PAUL, K. G. (1976) *Acta Chem. Scand. Ser. B Org. Chem.* **30**(4) 373–375.
8. ERECINSTA, M., OSHINO, N., LOH, P., AND BROCK LE HURST, T. E. (1973) *Biochim. Biophys. Acta* **292,** 1–5.

9. ASAD, R., VANNGARD, T., AND DUNFORD, H. B. (1975) *Biochim Biophys. Acta* **391,** 259–264.
10. MARTIN DAVIES, D. M., JONES, P., AND MANTLE, D. (1976) *J. Biochem.* **157,** 247–253.
11. SENNYEY, G., BARCELO, G., AND SENET, J. P. (1987) *Tetrahedron Lett.* **28,** 5809–5810 and references cited therein.
12. Physicochemical details of these syntheses are to be published.
13. (a) DUNFORD, H. B. (1982) *Adv. Inorg. Biochem.* **4,** 41–68. (b) WELINDER, K. G. (1976) *FEBS Lett.* **72,** 19–22.
14. GEOGHEGAN, K. F., BARRA, D. M., AND FEENEY, R. E. (1979) *Biochemistry* **18,** 5392–5399.
15. FLUKA (Biochemika) (Ed.) (1986) Protein Modification, pp. 33–36, Fluka.
16. YANG, C. C., AND KING. (1980) *Biochim. Biophys. Acta* **614,** 373–376.
17. GREENSTEIN, J. P., AND WINITZ, M. (1961) Chemistry of the Amino Acids, Vol. III p. 1971, Wiley, New York.
18. MELCHIOR, W. B., AND FARHNEY, D. (1970) *Biochemistry* **9,** 251–255.
19. HOLBROOK, J., INGRAM, V. (1973) *J. Biochem.* **131,** 729–738.
20. AWASTHI, Y. C., BHATNAGAR, A., SINGH, V. S. (1987) *Biochem. Biophys. Res. Commun.* **143**(3) 965–970.
21. SAKURADA, J., TAKAHASHI, S., AND HOSOYA, T. (1986) *J. Biol. Chem.* **261,** 9657–9662.
22. THANABAL, V., DE ROPP, J. S., AND LA MAR, G. N. (1988) *J. Amer. Chem. Soc.* **110,** 3027–3035.
23. THANABAL, V., DE ROPP, J. S., AND LA MAR, G. N. (1987) *J. Amer. Chem. Soc.* **109,** 7516–7525.
24. SERIS, J. L. (1985) Enzymes d'Oxydo-Réduction. II Oxydases et Peroxydases, Rapport du Centre de Recherches de Lacq (S.N.E.A.) France, No. 8875.
25. GASPAR, TH., PENEL, C., THORPE, T., GREPPIN, H. Peroxidases 1970–1980: A Survey of Their Biochemical and Physiological Roles in Higher Plants, Université de Genève, Centre de Botanique (1982), and references cited therein.
26. RUSSEL, D. W. (1971) *J. Biol. Chem.* **246**(12), 3870–3878.
27. SOUPPE, J. (1986) Enzymes d'Oxydo-Réduction. II Cytochrome P450, Rapport du Centre de Recherches de Lacq (S.N.E.A.) France, No. 9148.