Matthews, C. K., and Huennekens, F. M. (1960), *J. Biol. Chem. 235,* 3304.
Palekar, A. G., Tate, S. S., and Meister, A. (1973), *J. Biol. Chem. 248,* 1158.
Schirch, L., (1975), *J. Biol. Chem. 250,* 1939.
Schirch, L., and Diller, A. (1971), *J. Biol. Chem. 246,* 3961.
Schirch, L., and Gross, T. (1968), *J. Biol. Chem. 243,* 5651.
Schirch, L., and Jenkins, W. T. (1964a), *J. Biol. Chem. 239,* 3797.
Schirch, L., and Jenkins, W. T. (1964b), *J. Biol. Chem. 239,* 3801.
Schirch, L., and Mason, M. (1962), *J. Biol. Chem. 238,* 2578.
Schirch, L., and Mason, M. (1963), *J. Biol. Chem. 238,* 1032.
Schirch, L., and Ropp, M. (1967), *Biochemistry 6,* 253.
Schirch, L., and Slotter, R. A. (1966), *Biochemistry 5,* 3175.
Segel, I. H. (1975), in Enzyme Kinetics, New York, N.Y., Wiley, p 274.
Silverstein, E., and Boyer, P. D. (1964), *J. Biol. Chem. 239,* 3908.
Tatum, C. M., Benkovic, P. A., Benkovic, S. J., Potts, R., Schleicher, E., and Floss, H. G. (1977), *Biochemistry 16,* (in press).
Ulevitch, R. J., and Kallen, R. G. (1973), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 32,* 1871.
Wedler, F. C. (1974), *J. Biol. Chem. 249,* 5080.
Wilson, E. M., and Snell, E. E. (1962), *J. Biol. Chem. 237,* 3180.

Enzymatic Oxidation of Cobalt Protoporphyrin IX: Observations on the Mechanism of Heme Oxygenase Action†

Mahin D. Maines* and Attallah Kappas

ABSTRACT: Studies on the enzymatic mechanism of microsomal heme oxygenase were made utilizing various porphyrins and metalloporphyrins of different ring substituents and central metal ions. Co-heme (cobalt protoporphyrin IX) was shown to be a substrate for the enzyme and the product of its oxidative metabolism was identified as the natural bile pigment, biliverdin IXα isomer. Metalloporphyrins, which do not bind molecular oxygen (Ni, Mn, and Sn protoporphyrin IX), were not substrates for heme oxygenase, although they could competitively inhibit oxidation of reactive substrates for the enzyme. The presence of lipophilic substituents on pyrrole rings I and II, as well as a central metal atom, were required for the heme oxidation reaction to occur. The oxidative cleavage of Co-heme displayed typical characteristics of an enzyme-mediated reaction, and the oxidation of this substrate, as well as that of Fe-heme (iron protoporphyrin IX), could be supported with either reduced nicotinamide adenine dinucleotide phosphate or reduced nicotinamide adenine dinucleotide. A hypothesis is proposed on the mode of action of heme oxygenase in which the enzyme and its substrate are considered to form a "transitory" hemoprotein which can activate molecular oxygen for cleavage of the heme tetrapyrrole ring. In this formulation, heme as substrate for heme oxygenase is synonymous with heme as prosthetic group for the enzyme.

Heme oxygenase is an enzyme which catalyzes the oxidative degradation of heme to biliverdin; the latter is subsequently reduced in the cytosol to bilirubin. The microsomal site of heme breakdown was established by Tenhunen et al. (1969) but the mechanism of heme oxidation to biliverdin has remained unclear. Certain analogies between heme degradation and the "mixed function" oxidation of drugs led to the belief that the heme oxygenase system contains cytochrome P 450 as the terminal oxidase (Schmid, 1972), that it has an absolute requirement for NADPH[1] (Tenhunen et al., 1969; Schmid, 1972), and that the central iron atom of heme is indispensable for heme oxidative activity (Tenhunen et al., 1969). Subsequently, studies from this laboratory conclusively dissociated cytochrome P 450 from heme oxidation in liver (Maines and Kappas, 1974; 1975a,b), an organ rich in P-450 content, and, in concurrent studies, the activity of heme oxygenase in spleen was also shown not to be cytochrome P 450 dependent (Yoshida et al., 1974).

In further studies on the mechanism of hepatic heme oxygenase, we have explored the substrate specificity and cofactor requirements of the enzyme. The results indicate that microsomal heme oxygenase can catalyze the oxidative cleavage of a non-iron heme substrate—specifically cobalt protoporphyrin IX (Co-heme), and that NADH, as well as NADPH, can serve as electron donor for the reaction. These and other findings reported here provide a basis for interpreting the role of chelated metal in the oxidative metabolism of heme and for defining the possible role of heme as substrate in relation to the catalytic activity of heme oxygenase.

Materials and Methods

Materials. Crystalline cobalt heme (cobalt protoporphyrin IX, Co-heme), Co-heme dimethyl ester, cobalt coproporphyrin III, and cobalt uroporphyrin I were purchased from Porphyrin Products (Salt Lake City, Utah). Iron-heme (iron protopor-

† From The Rockefeller University, New York, New York 10021. *Received August 23, 1976.* This research was supported by United States Public Health Service Grant ES-01055 and by an individual grant from the Scaife Family Trust.

[1] Abbreviations used are: NADP, nicotinamide adenine dinucleotide phosphate; NADPH, reduced NADP; NADH, reduced nicotinamide adenine dinucleotide.

TABLE I: Iron and Cobalt Heme Oxidation by Spleen Microsomal Fractions; Cofactor Requirements and Inhibitory Effects of Various Heme Ligands.[a]

Additions	Activity (nmol of bilirubin mg^{-1}/h^{-1}) (Co-heme)	(Fe-heme)
NADPH-generating system	1.15	6.40
NADPH (2.0 mM)	1.15	4.65
NADH (2.0 mM)	0.92	2.81
NADH (4.0 mM)	1.03	4.07
Cofactors absent	0	0
Na_3N (5 mM)	1.14	2.96
KCN (0.1 mM)	0.55	2.46
CO[b]	0.23	0.59
N_2[b]	0.29	0.59

[a] Pooled microsomal fractions were prepared from rats as described under Materials and Methods. Systems containing microsomal fractions, a NADPH-generating system, biliverdin reductase, and the additions shown above were assayed for heme oxygenase activity as described under Materials and Methods. The final concentration in the assay medium of Co-heme was 150 μM and that of Fe-heme was 20 μM. [b] Gassed for 1-2 min.

phyrin IX, Fe-heme), bilirubin, and cofactors and enzymes (NADH, NADPH, $NADP^+$, glucose 6-phosphate and glucose-6-phosphate dehydrogenase) were purchased from Sigma Chemical Co. Crystalline protoporphyrin IX complexes of Sn, Mn, and Ni were prepared immediately before use. Crystalline-free protoporphyrin IX and mesoporphyrin IX were prepared as described previously (Maines and Kappas, 1975a). The purchased Co-heme was assayed for purity and was found to be slightly contaminated with free protoporphyrin; since this amount of porphyrin would not interfere with the enzymatic assay carried out in the dark, the Co-heme was used without further purification. Tetrapyrrole solutions were prepared immediately before use as described elsewhere (Maines and Kappas, 1975a). Male Sprague-Dawley rats (200-250 g) were utilized.

Tissue Preparation. In all studies it was essential that the tissues have minimal contamination with endogenous Fe-heme; thus, livers, spleens, and kidneys were exhaustively perfused with 0.9% NaCl before use. "Washed" microsomal fractions were prepared as described previously (Maines and Anders, 1973). Washed microsomal fractions were also prepared from bone marrow (femoral aspirates) and peritoneal macrophages.

Heme Oxygenase Assay. The following method was designed to measure microsomal heme oxidation activity. To 0.7 ml of microsomal fraction, containing protein ranging from 2-3 mg/ml for spleen, 6-9 mg/ml for liver and kidney, and 1-1.5 mg/ml for macrophages and bone marrow, were added 300 μg of partially purified biliverdin reductase prepared from rat cytosol, and the following constituents to obtain the indicated final concentrations in the assay medium $NADP^+$, 4 mM; glucose 6-phosphate, 0.8 mM; glucose-6-phosphate dehydrogenase, 0.5 unit; $MgCl_2$, 1 mM, and potassium phosphate buffer, 0.09 M; plus various concentrations of the metalloporphyrins as indicated in appropriate experiments. The final volume of the incubation mixture was 1.5 ml. The usual duration of incubation was 10 min at 37 °C in complete dark under air. The reaction was terminated by freezing. Thereafter the bilirubin formed was extracted by repeated treatment with $CHCl_3$, and the combined $CHCl_3$ layers were evaporated to a small volume and read against an appropriate blank containing all incubation mixture constituents, except the heme compounds. The extinction coefficient of 58 mM^{-1} cm^{-1} between 452-530 nm was used for the calculation of results. Extraction of bilirubin by $CHCl_3$, as in this procedure, permits assay of heme oxygenase activity in considerably smaller amounts of tissues than methods previously utilized (Maines and Kappas, 1975b) and prevents spectral interference in the Soret region, of the substrate porphyrin compound with that of the product, bilirubin.

Identification of Biliverdin as the Degradation Product of Co-heme Oxidation. A combination of the methods described by Nichols (1971) for extraction and by O'Carra and Colleran (1970) for chromatography was used and the procedures were carried out in the minimum amount of light possible; the pigments were also extracted with least exposure to an oxidizing atmosphere. Preparative-size incubation mixtures containing all constituents of the incubation mixture, except biliverdin reductase, were used: one contained Co-heme as substrate, and the other contained Fe-heme. The incubation mixtures were then poured into a glass-stoppered tube containing acetone to obtain a 1:1, v/v, ratio, with the addition of ascorbic acid (10 mg/ml). The mixture was vigorously mixed, and 1 volume of ethyl ether was then added. The mixture was again vigorously mixed and centrifuged. The top ether layer was aspirated and mixed with 0.5 volume of 2 M HCl and recentrifuged, and the bottom layer was collected and its absorption spectrum recorded. The biliverdin was then extracted into 0.5 volume of $CHCl_3$. The spectrum was again recorded and the $CHCl_3$ was evaporated under N_2, redissolved in methanol and then methylated. The bile pigment was then chromatographed in the dark on a Merck Silica Gel G plate (0.25-mm thickness, activated for 1 h at 100°C) utilizing the solvent system heptane-methyl ethyl ketone-glacial acetic acid (10:5:1). Biliverdin extracted, as above, from 18 day old chick bile was used as the standard.

Results

Co-heme was oxidized by microsomal fractions from spleen and other tissues, such as kidney, liver, and bone marrow. Macrophages had a trace amount of Co-heme oxidative activity. The activities expressed as nmol of bilirubin mg of $protein^{-1}$ h^{-1} formed under standard conditions using 100 μM Co-heme were 0.53-1.2 for spleen, 0.16-0.22 for kidney, 0.04-0.08 for liver, 0.01-0.02 for bone marrow and <0.01 for macrophages. The microsomal fraction was the most active cell fraction for the metabolism of Co-heme; no activity was detected in the cell sap.

For oxidation of Co-heme, the spleen microsomal fraction was able to utilize NADPH or a NADPH-generating system, as well as NADH (Table I). These requirements were the same as those observed for Fe-heme oxidation, although at lower concentrations of NADH Fe-heme oxidation was 60% of that observed for equimolar concentrations of NADPH. The utilization of NADH as cofactor in the oxidation of heme was unexpected, since this reaction was reported to have an absolute requirement for NADPH (Tenhunen et al., 1969; Schmid, 1972); however, this finding was confirmed with different preparations of NADH in repeated experiments with spleen (and liver) microsomes.

Co-heme degradative activity of microsomes was completely destroyed by heating (60 °C, 5 min). The oxidation of both Co- and Fe-heme was inhibited by CO, KCN, and N_2. In contrast to Fe-heme oxidation, which was inhibited by sodium azide

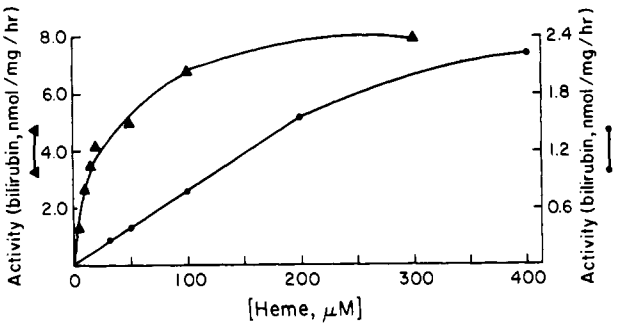

FIGURE 1: Splenic heme oxidation reaction rate as a function of substrate concentrations. Standard assay system was used for Co-heme or Fe-heme oxidation activity utilizing final concentrations of the heme compounds indicated: Fe-heme (▲); Co-heme (●).

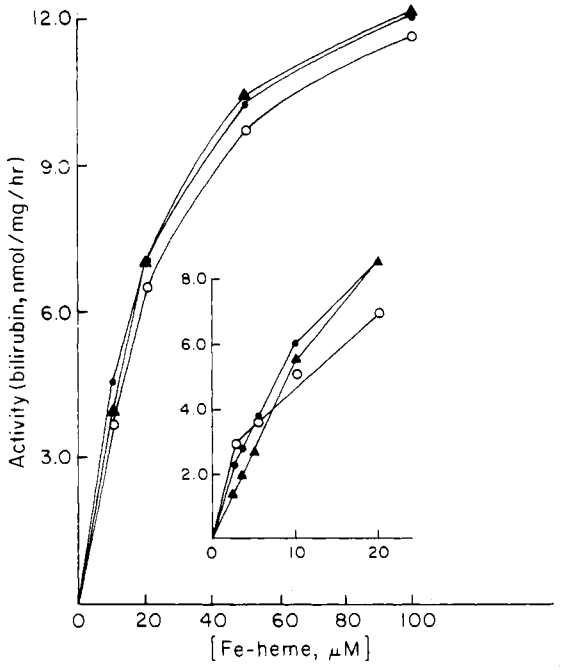

FIGURE 2: Effect of cobalt heme on iron heme oxidation by the splenic microsomal enzyme. Fe-heme oxidation was measured in the presence of two different concentrations of Co-heme. Fe-heme (▲); + 75 μM Co-heme (●); + 150 μM Co-heme (○). The experiments shown above were carried out on two different occasions.

(Na_3N), the oxidation of Co-heme was not affected by this heme ligand up to 5 mM.

Figure 1 shows the comparative oxidation rates of Fe-heme and Co-heme by spleen microsomes. The oxidation of Fe-heme was linear with respect to concentration of substrate up to 10–15 μM; for Co-heme, this activity continued at a linear rate up to 150–200 μM. The apparent K_m for Fe-heme was approximately 17×10^{-6} M and for Co-heme, 125×10^{-6} M. Heme oxidation activity for Co-heme was a protein- and time-dependent function (data not shown). This activity was linear with time for 10–12 min. The same kinetic characteristics were observed for Fe-heme oxidation under the conditions described (data not shown). The data shown in Figure 2 suggest that the substrate-binding site for both Co-heme and Fe-heme may be the same. This figure shows Fe-heme oxidation in the presence of two different concentrations of Co-heme, 75 and 150 μM. Control values for the oxidation of these two concentrations of Co-heme were 0.83 and 1.43 nmol mg^{-1} h^{-1}, respectively. At low concentrations of Fe-heme (<10 μM),

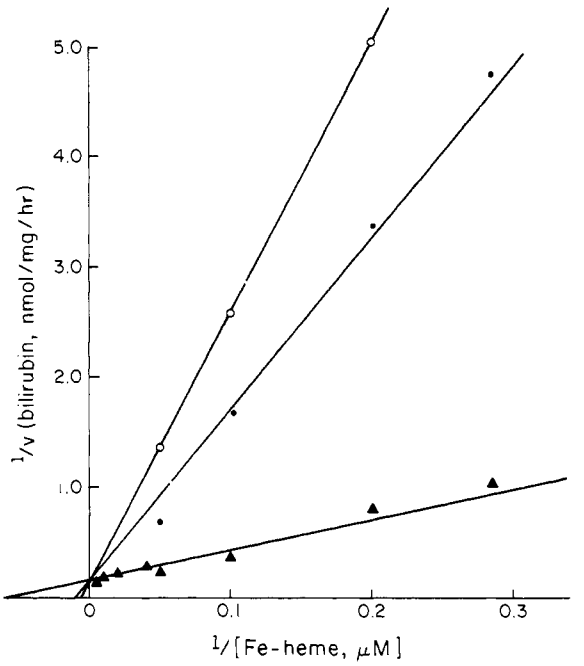

FIGURE 3: Kinetics of inhibition of Fe-heme oxidation by Ni-heme. Fe-heme oxidation was measured in the presence of two different concentrations of Ni-heme. The assay was carried out as described under Materials and Methods. Fe-heme (▲); + 50 μM Ni-heme (●); + 100 μM Ni-heme (○).

product formation was additive when Co-heme (at both concentrations) was simultaneously present in the incubation mixture (Figure 2, inset). However, at higher concentrations of Fe-heme (10–20 μM) there was less additive effect seen with a Co-heme concentration of 75 μM; with the higher concentration of Co-heme (150 μM), the oxidation of Fe-heme at concentrations >10 μM was slightly inhibited. At an Fe-heme concentration exceeding 20 μM, both concentrations of Co-heme were somewhat inhibitory to the oxidation of Fe-heme.

The specificity of microsomal heme oxygenase for Co-porphyrins as a function of substituents on pyrrole rings I and II was investigated by testing cobalt coproporphyrin III and cobalt uroporphyrin I, and for substituents on rings III and IV by testing cobalt protoporphyrin IX dimethyl ester as possible substrates. The substrate specificity of the enzyme in relation to the metal moiety of heme was investigated by utilizing protoporphyrin IX complexes of Sn, Ni, and Mn. The requirement of the oxygenase for a central metal in the tetrapyrrole nucleus was also studied by utilizing free protoporphyrin IX and free mesoporphyrin IX as test substrates. It was found that, with the exception of cobalt protoporphyrin IX dimethyl ester, which was oxidized at a very low rate, none of the tetrapyrroles or metalloporphyrins mentioned, at concentrations of 50–100 μM, were oxidized by spleen microsomes (data not presented). The very low rate of Co-heme dimethyl ester oxidation might reflect its great hydrophobicity, which would cause it to adhere to cell membranes, preventing it from reaching the enzymatic site.

The specificity of the enzyme binding site for heme substrates was tested in other experiments in which the effect of a nonmetabolizable heme, such as Ni-heme and Mn-heme, on the oxidation of the reactive hemes, Fe-heme and Co-heme, was investigated. Ni-heme competitively inhibited the oxidation of both substrates when present in the assay medium in concentrations of 50 and 100 μM; the results of experiments obtained with Fe-heme are presented in Figure 3. Mn-heme

(concentration 50 μM) completely inhibited the oxidation of Fe- and Co-heme (data not shown). These findings imply that reactive (Co-heme, Fe-heme) as well as nonreactive (Ni-heme, Mn-heme) heme compounds bind to the oxygenase at common sites but only those capable of activating molecular oxygen (Co-heme, Fe-heme) can be degraded by the enzyme.

The end product of Co-heme oxidation by splenic microsomes was identified as biliverdin of the IXα configuration on the basis of (1) its visible absorption spectrum, (2) its chromatographic behavior, and (3) its conversion to bilirubin in the presence of the substrate-specific enzyme (O'Carra and Colleran, 1969), biliverdin IXα reductase. When the product of the incubation of Co-heme with splenic microsomes in the absence of biliverdin reductase was compared with that produced from Fe-heme under the same conditions, it was found that the $CHCl_3$ extracts of both incubation mixtures exhibited identical absorption spectra. Furthermore, these spectra were identical with that obtained from the biliverdin of extracted avian bile. These spectra displayed a Soret absorption band at 383 nm with a second smaller aborption at 678 nm. The thin-layer chromatograms of all three preparations each exhibited only a single major spot at identical distances from the origin. When the incubation mixture contained biliverdin reductase, the $CHCl_3$ extracts of each exhibited the typical absorption spectrum of bilirubin whether the substrate was Fe-heme or Co-heme.

Discussion

It has been stated that a central iron atom is "indispensable" (Tenhunen et al., 1969) for heme oxygenase activity and, on that basis, it has been generally accepted that Fe-heme is the only metalloporphyrin substrate for this microsomal enzyme system. In the present report, however, evidence is provided that heme oxygenase can oxidize Co-heme, as well as Fe-heme as substrate; this finding together with the results of studies with other porphyrins and metalloporphyrins indicate that, while a central metal is necessary for the oxidation of heme compounds, this metal requirement is not exclusively limited to iron.

The oxidation of Fe-heme by microsomal fractions has the characteristics of an enzyme-catalyzed reaction; the microsomal oxidation of Co-heme displays similar characteristics. The same splenic enzyme appears to oxidize both Co-heme and Fe-heme compounds, although the enzyme has a considerably greater affinity for the Fe-heme as compared with the Co-heme complex.

It has been shown that the oxidation of heme (Fe-heme) in liver (Maines and Kappas, 1974) and spleen (Yoshida et al., 1974) does not require the involvement of cytochrome P 450 as an oxygenase; however, the possibility that heme oxidation is mediated by a hemoprotein other than cytochrome P 450 has not been ruled out (Maines and Kappas, 1975b). In the past, criteria such as the inhibition of heme oxidation by KCN, Na_3N and CO have been taken as evidence for the involvement of hemoproteins in heme degradation (Tenhunen et al., 1969). As we have shown here, the oxidation of Co-heme is also inhibited by certain heme ligands (KCN, CO) as effectively as is that of Fe-heme. However, Fe-heme oxidation was also found to be substantially inhibited by Na_3N, whereas the oxidation of Co-heme was not inhibited at high concentrations of this ligand. This finding strongly suggests that the observed inhibition of Fe-heme oxidation is a consequence of the ligand binding with the substrate rather than with a hemoprotein enzyme.

These data, together with those relating to the oxidation of Co-heme and the results of the studies with other porphyrin compounds, permit certain inferences to be drawn concerning the characteristics and mode of action of microsomal heme oxygenase.

Firstly, the enzyme protein is not an iron hemoprotein. Secondly, in contrast to earlier reports that heme oxygenase can utilize only NADPH as the sole source of reducing equivalents for the reaction, the enzyme system in our experiments could utilize either NADH or NADPH as an electron donor for heme degradation. The isolated enzyme from liver, similarly, can utilize either NADH or NADPH as cofactor for heme oxidation in the presence of the appropriate flavoprotein reductase, NADH-cytochrome b_5 reductase or NADPH-cytochrome c reductase, respectively (Maines et al., manuscript in preparation), and, as with microsomes, NADPH, supports heme degradation approximately twice as effectively as does NADH. Thirdly, as noted, the iron atom of the tetrapyrrole is not indispensable for heme oxygenase activity and can be replaced, at least, by cobalt. Finally, it appears evident that lipophilic substituents on pyrrole rings I and II, e.g., methyl and vinyl groups, are an essential feature for oxidation of the heme molecule. The view that such lipophilic side chains are necessary for the binding of the metalloporphyrin to the enzyme is consistent with the suggestion made earlier by O'Carra and Colleran (1969) and O'Carra (1975).

Hypothesis on the Mode of Action of Heme Oxygenase. On the basis of the findings in these experiments, a plausible hypothesis for the degradation of heme compounds can be formulated. The catalytic sites of microsomal heme oxygenase consist of hydrophobic regions which can accommodate two rings of a tetrapyrrole nucleus possessing lipophilic substituents on adjacent pyrroles, such as the vinyl groups on rings I and II of heme; these hydrophobic regions contain coordinating sites for the central metal atom of the metalloporphyrin. In this form, the substrate heme and the enzyme protein form a "transitory" hemoprotein. The binding site for heme permits reactivity of the microsomal flavoproteins, NADPH-cytochrome c reductase, as well as NADH-cytochrome b_5 reductase, with the hemoprotein formed by the enzyme-substrate complex, thus allowing utilization of either nucleotide. Following the formation of this hemoprotein, an electron-transport chain is created with the hemoprotein serving as its own terminal oxygenase. It is evident that the "transitory" hemoprotein need not be an Fe-heme hemoprotein, since, as these studies show, it may be a Co-heme hemoprotein as well. The function of the hemoprotein complex would be to bind and activate molecular oxygen, which is then utilized for the oxidative cleavage of the α-methylene bridge of the tetrapyrrole ring and the formation of water.

Although the mechanism formulated here constitutes a "mixed-function" type of oxidative reaction, it differs from that involved in the metabolism of drugs in that the "oxygenase" is not a preformed microsomal hemoprotein, rather it is the substrate heme-enzyme complex itself which serves to activate molecular oxygen. O'Carra and Colleran (1969) have also proposed the possible function of substrate heme as oxygen activator in its own catabolism; however, they assumed that the apoprotein moiety of cytochrome P 450 or its denatured form P 420 serves as the binding site for the substrate heme, i.e., that exogenous heme replaces the rapidly turning-over heme moiety of P 450 (P 420), obviating the need for a specific enzyme-catalyzing heme degradation. However, previous studies from this laboratory on hepatic heme oxygenase activity in whole animals, evidence obtained from cultured avian embryo hepatocytes, which contain negligible amounts of cyto-

chrome P 450 (Maines and Sinclair, 1976), and studies with heme oxygenase isolated from liver microsomes (Maines et al., manuscript in preparation) support the idea that heme oxygenase is a discrete enzyme protein whose prosthetic group is synonymous with its substrate.

Acknowledgment

We are indebted to Mrs. Ilona Scher for her able and devoted technical assistance.

References

Maines, M. D., and Anders, M. W. (1973), *Mol. Pharmacol. 9,* 219–228.
Maines, M. D., and Kappas, A. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 4293–4297.
Maines, M. D., and Kappas, A. (1975a), *J. Biol. Chem. 250,* 2363–2369.
Maines, M. D., and Kappas, A. (1975b), *J. Biol. Chem. 250,* 4171–4178.
Maines, M. D., and Kappas, A. (1976), in Porphyrins in Human Diseases, Doss, M., Ed., Basel Switzerland, S. Karger Publishing Co., pp 43–52.
Maines, M. D., and Sinclair, P. (1976), *J. Biol. Chem.* (in press).
Nichols, A. W. (1971), *Biochim. Biophys. Acta 244,* 595–605.
O'Carra, P. (1975), in Porphyrins and Metalloporphyrins, Smith, K. M., Ed., Amsterdam, Elsevier, pp 123–153.
O'Carra, P., and Colleran, E. (1969), *FEBS Lett. 5,* 295–298.
O'Carra, P., and Colleran, E. (1970), *J. Chromatogr. 50,* 458–468.
Schmid, R. (1972), *N. Engl. J. Med. 287,* 703–709.
Tenhunen, R., Marver, H. S., and Schmid, R. (1969), *J. Biol. Chem. 244,* 6388–6394.
Yoshida, T., Takahashi, S., and Kikuchi, G. (1974), *J. Biochem.(Tokyo) 175,* 1187–1191.

Mechanism of Lysozyme Catalysis: Role of Ground-State Strain in Subsite D in Hen Egg-White and Human Lysozymes†

Melvin Schindler, Yehudah Assaf, Nathan Sharon, and David M. Chipman*

ABSTRACT: The association constants for the binding of various saccharides to hen egg-white lysozyme and human lysozyme have been measured by fluorescence titration. Among these are the oligosaccharides GlcNAc-β(1 → 4)-MurNAc-β(1 → 4)-GlcNAc-β(1 → 4)-GlcNAc, GlcNAc-β(1 → 4)-MurNAc-β(1 → 4)-GlcNAc-β(1 → 4)-*N*-acetyl-D-xylosamine, and GlcNAc-β(1 → 4)-GlcNAc-β(1 → 4)-MurNAc, prepared here for the first time. The binding constants for saccharides which must have *N*-acetylmuramic acid, *N*-acetyl-D-glucosamine, or *N*-acetyl-D-xylosamine bound in subsite D indicate that there is no strain involved in the binding of *N*-acetyl-D-glucosamine in this site, and that the lactyl group of *N*-acetylmuramic acid (rather than the hydroxymethyl group) is responsible for the apparent strain previously reported for binding at this subsite. For hen egg-white lysozyme, the dependence of saccharide binding on pH or on a saturating concentration of Gd(III) suggests that the conformations of several of the complexes are different from one another and from that proposed for a productive complex. This is supported by fluorescence difference spectra of the various hen egg-white lysozyme–saccharide complexes.

Human lysozyme binds most saccharides studied more weakly than the hen egg-white enzyme, but binds GlcNAc-β(1 → 4)-MurNAc-β(1 → 4)-GlcNAc-β(1 → 4)-MurNAc more strongly. It is suggested that subsite C of the human enzyme is "looser" than the equivalent site in the hen egg enzyme, so that the rearrangement of a saccharide in this subsite in response to introduction of an *N*-acetylmuramic acid residue into subsite D destabilizes the saccharide complexes of human lysozyme less than it does the corresponding hen egg-white lysozyme complexes. This difference and the differences in the fluorescence difference spectra of hen egg-white lysozyme and human lysozyme are ascribed mainly to the replacement of Trp-62 in hen egg-white lysozyme by Tyr-63 in the human enzyme. The implications of our findings for the assumption of superposition and additivity of energies of binding in individual subsites, and for the estimation of the role of strain in lysozyme catalysis, are discussed.

The hypothesis that the active sites of enzymes are complementary in structure to the transition states of the reactions which they catalyze (Haldane, 1930; Fersht, 1974; Jencks, 1975; Pauling, 1948) is now widely accepted. A corollary to this hypothesis is that there must be strain in the interaction between an enzyme and the ground state of its substrate(s), although this strain need not necessarily be steric and need not lead to distortion of either the enzyme or the substrate in the complex formed between them (Fersht, 1974; Jencks, 1975). The role of strain in the lysozyme-catalyzed hydrolysis of oligosaccharides has been under active investigation ever since the proposal by Phillips and his co-workers of a model for a hen egg-white lysozyme (HEWL[1])–substrate complex (Blake et al., 1967; Phillips, 1966). According to this model, the enzyme

† From the Department of Biophysics, Weizmann Institute of Science, Rehovoth, Israel (M.S. and N.S.), and the Department of Biology, Ben Gurion University of the Negev, Beersheva, Israel (Y.A. and D.M.C.). *Received October 5, 1976.* This investigation was supported in part by a grant from the National Institutes of Health (GM 19143) to N.S.

[1] Abbreviations used are: HEWL, hen egg-white lysozyme; HL, human lysozyme; Mes, 4-morpholineethanesulfonic acid; TLC, thin-layer chromatography; GlcNAc, *N*-acetyl-D-glucosamine; MurNAc, *N*-acetylmuramic acid; XylNAc, 2-acetamido-2-deoxy-D-xylose. All oligosaccharides referred to are linked β(1 → 4).