Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 531–535, February 1986
Chemistry

On the mechanism of the chemical and enzymic oxygenations of α-oxyprotohemin IX to Fe·biliverdin IXα

(heme catabolism/porphyrin π-neutral radical)

SEIYO SANO*†, TOYO SANO*, ISAO MORISHIMA‡, YOSHITUGU SHIRO‡, AND YUTAKA MAEDA§

*Department of Public Health, Faculty of Medicine, ‡Division of Molecular Engineering, Graduate School of Engineering, and §Research Reactor Institute, Kyoto University, Kyoto 606, Japan

Communicated by Rudi Schmid, September 3, 1985

ABSTRACT **α-Oxyprotohemin IX, an early intermediate in heme catabolism, was synthesized and its autoxidation to biliverdin IXα was studied. In anaerobic aqueous pyridine, α-oxyprotohemin (hexacoordinated) underwent autoreduction to yield an Fe(II) α-oxyprotoporphyrin π-neutral radical bis(pyridine) complex, which reacted with an equimolar amount of dioxygen to give pyridine·verdohemochrome IXα and CO in 75–80% yield via an intermediate with an absorption maximum at 893 nm. Verdohemochrome IXα did not react with further dioxygen. Reconstituted apomyoglobin·α-oxyprotohemin IX complex (pentacoordinated) reacted with an equimolar amount of dioxygen to form an Fe(II) oxyporphyrin π-neutral radical intermediate, which rearranged to a green compound (λ_{max} 660 and 704 nm) with elision of CO. The green product, which is probably an apomyoglobin·verdoheme π-radical complex, reacted with another equimolar amount of dioxygen to give Fe(III)·biliverdin IXα. Demetallation of this gave biliverdin IXα in overall yield of 70–75%. These results indicate that the sequence of oxyheme autoxidation in the presence of apomyoglobin is α-oxyprotoheme IX $\xrightarrow{O_2,CO}$ verdohemochrome IXα π-radical $\xrightarrow{O_2}$ Fe(III)·biliverdin IXα. A similar mechanism may prevail *in vivo*. The hexa- and pentacoordinated Fe(II) π-radical form of the oxyporphyrin is crucial in triggering the autoxidation of the complex to verdohemochrome IXα. Further oxygenation of verdohemochrome IXα to Fe(III)·biliverdin IXα occurred only in the pentacoordinated apomyoglobin·verdoheme Fe(II) complex.**

Heme catabolism is an important biological process, which is catalyzed by the enzyme heme oxygenase (EC 1.14.99.3). The enzymic reaction has been studied in several laboratories for the past 17 years, and the main biocatabolic pathway has been elucidated (1–3). The first step is assumed to be hydroxylation at the *meso* position to form an α-oxy derivative (4), which is further converted to biliverdin IXα by two molecules of oxygen (2, 5–7). The chemical mechanisms of oxyheme cleavage by molecular oxygen, however, are still undetermined, particularly with respect to the electronic and oxidation states of the intermediates.

In a preliminary communication Sano and Sugiura reported that Fe(II) oxymesoporphyrin·2-methylimidazole complex, a model for the first stable intermediate in the heme oxygenase reaction, showed π-radical character and that this compound could be oxidized stepwise to Fe·mesobiliverdin by two molecules of oxygen (7). The sequence of the chemical reactions was similar to that observed in the reconstituted heme oxygenase system (6). Saito and Itano (8) reported that a verdoheme IXα·2-picoline complex was oxidized to Fe·biliverdin IXα *in vitro* by oxidoreductive cleavage, but the step from α-oxyprotohemin IX to verdohemochrome IXα was not investigated. Previous attempts to synthesize β-oxyprotohemin from pyrroketones were unsuccessful owing to cyclic ether formation between the vinyl side-chain precursor and the *meso* oxygen, and similar results were to be expected in the α series (9). Clezy and Liepa (10) reported synthesis of the α isomer but the yield was low. Recently Jackson *et al.* (11) synthesized the four isomers of *meso*-oxyprotoporphyrin dimethyl esters via direct oxidation of protoporphyrin IX dimethyl ester. We now describe an alternative synthesis of oxyprotohemin isomers, based on earlier coupled oxidation reactions (4, 12), and we present studies on the mechanism of α-oxyprotohemin IX autoxidation and ring-opening in aqueous pyridine and in aqueous myoglobin (Mb) solutions (13).

MATERIALS AND METHODS

Protohemin IX chloride was obtained from Sigma, and crystalline μ-oxo-bis(protoporphyrin IX dimethyl ester) Fe(III) was prepared (14). Pyridine was distilled first from NaOH, then from potassium permanganate. Mb (type III from horse heart) and biliverdin IXα were purchased from Sigma; the latter was purified as the dimethyl ester according to the method of Bonnett and McDonagh (15) and O'Carra and Colleran (16) and ε at 660 nm = 15.6 $mM^{-1}·cm^{-1}$ in $CHCl_3$ was used for estimation of biliverdin IXα. Apomyoglobin (apoMb) prepared by Teale's method (17) was dissolved in 0.1 M Tris·HCl buffer, pH 7.0. Aluminum oxide 90 and silica gel 60 precoated TLC plates from Merck (Darmstadt, F.R.G.), silica gel C-300 for chromatography from Wako, and HPLC columns (Water Cosmosil 5SL) from Nakarai Chemical (Kyoto, Japan) were used. Melting points (uncorrected) were determined on a hot-stage apparatus. Elemental analysis was done in the Faculty of Pharmaceutical Science, Kyoto University. Electronic spectra were measured with a Union Giken SM 401 spectrophotometer fitted with a stopped-flow rapid-scan analyzer and computer. ESR spectra were recorded with a JEOL JES-3X spectrometer operating with 100-kHz magnetic field modulation. Proton NMR spectra (300 MHz) were recorded with a Nicolet NT-300 spectrometer equipped with a 1280 computer system. For preparation of the α-oxyprotohemin·bis(pyridine) and apoMb complexes, a specially designed Thunberg-type reaction vessel connected to a high-vacuum line was used (18). Equimolar amounts of α-oxyprotohemin and apoMb (0.1 mM) were incubated at 37°C for 20 min under strictly anaerobic conditions. Oxygen was added to reaction mixtures by injecting a calculated volume of an aqueous solution through a septum. Carbon monoxide was determined quantitatively with a Dräger tube (19).

Synthesis of the Four Benzoyloxyprotohemin Isomers. μ-Oxo-bis(protoporphyrin IX dimethyl ester)·Fe(III) (125 μmol, 162.5 mg) was dissolved in a mixture of 1,2-dichloroethane (2400 ml) and pyridine (250 ml) and the solution was stirred while a stream of argon was passed

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

†To whom reprint requests should be addressed.

through sintered glass into the solution at room temperature. After 2 hr, the solution was heated to reflux under argon and a solution of ascorbic acid (380 mg) in deoxygenated water (3.8 ml) was added. After 2 min, deoxygenated hydrogen peroxide solution (780 μmol in 35 ml of water) was added to the stirred mixture and vigorous stirring at 60°C under argon was continued. After 3 min, benzoyl chloride (12.4 mmol, 1.43 ml) was added, and stirring was continued for 1 hr under argon at room temperature. The reaction mixture was washed anaerobically with 0.2% $FeSO_4$ (300 ml), saturated $NaHCO_3$ solution (three times with 500 ml), and water (three times with 500 ml). The organic phase was evaporated under reduced pressure, and the residue was dissolved in a small amount of $CHCl_3$ and chromatographed on an alumina column (4 × 15 cm) with chloroform/methanol (200:1, vol/vol). The main fraction was collected and evaporated to dryness under reduced pressure. Demetallation was carried out according to the method of Morell or Grinstein (20), and the porphyrin esters were purified by chromatography on an alumina column with $CHCl_3$ as eluent, followed by further chromatography on a silica column (2 × 130 cm) with CH_2Cl_2/acetone (990:10, vol/vol) as eluent. The four isomers eluted as four fractions in the order γ, δ, β, and α. Each isomer was rechromatographed on a new silica column and then crystallized from chloroform/methanol. The overall yield of individual isomers was 12–15%.

α-Benzoyloxyprotoporphyrin IX dimethyl ester: mp 241–248°C; electronic spectra, λ_{max} ($CHCl_3$) 410 nm (ε 174,900), 506 (19,500), 540 (6780), 578 (6350), 632 (2140); ^1H NMR (C^2HCl_3) δ 9.96, 9.82, 9.69 (each 1 H, s; β, δ, γ *meso* proton, respectively), 8.5 (2 H, m; benzyl *ortho*), 8.0 (1 H, q; 4 position CH=CH_2), 7.82, 7.7 (3 H, m; benzyl *meta* and *para*), 7.6 (1 H, q; 2 position CH=CH_2), 6.15 (2 H, t; 4 position CH=CH_2), 5.61, 5.36 (2 H, d; 2 position CH=CH_2), 4.25 (4 H, t; two $CH_2CH_2CO_2CH_3$), 3.64 (6 H, s; two OCH_3), 3.45 (9 H; three CH_3), 3.18 (3 H; CH_3; and 4 H, m; two $CH_2CH_2CO_2CH_3$), −3.86 (2 H, two NH). Analysis: Found C, 72.3%; H, 5.79%; N, 7.70%; calculated for $C_{43}H_{42}O_6N_4$: C, 72.6%; H, 5.91%; N, 7.89%. β-Benzoyloxyprotoporphyrin IX dimethyl ester: mp 246–248°C; Found: C, 72.0%; H, 5.88%; N, 7.70%. γ-Benzoyloxyprotoporphyrin IX dimethyl ester: mp 249–251°C; Found: C, 72.4%; H, 5.84%; N, 7.72%. δ-Benzoyloxyprotoporphyrin IX dimethyl ester: mp 235–237°C; Found: C, 72.5%; H, 5.80%; N, 7.85%. The β, γ, and δ isomers were also identified by ^1H NMR. Iron was inserted into each benzoyloxyprotoporphyrin IX dimethyl ester as previously described (21) and the chloride salt was crystallized from ethyl acetate. The pyridine hemochrome of the α isomer showed absorption maxima ($CHCl_3$) at 422 (ε 153,600), 524 (17,500), and 557 nm (19,400). Alkaline hydrolysis of each benzoyloxyprotohemin dimethyl ester to the oxyprotohemin was carried out according to the method of Sano *et al.* (18). The oxyprotohemin was transferred to a mixture of ethyl acetate (50 ml) and glacial acetic acid (1 ml) under argon, and the ethyl acetate layer was washed three times with water and evaporated to dryness under reduced pressure, and the residue was used immediately. *meso*-Deuterated α-oxyprotohemin was prepared similarly from iron μ-oxo *meso*-deuterated protoporphyrin IX dimethyl ester made according to the method of Kenner *et al.* (22).

RESULTS AND DISCUSSION

Some Physicochemical Properties of α-Oxyprotoheme IX in Aqueous Pyridine. When we dissolved α-oxyprotohemin IX in aqueous pyridine (60% pyridine, vol/vol) under anaerobic conditions we obtained an electronic spectrum with absorption maxima at 425 nm and a diffuse band in the visible region with weak shoulders at 540, 616, and 636 nm (Fig. 1, spectrum a). The ESR spectrum at 77 K exhibited a strong and axially

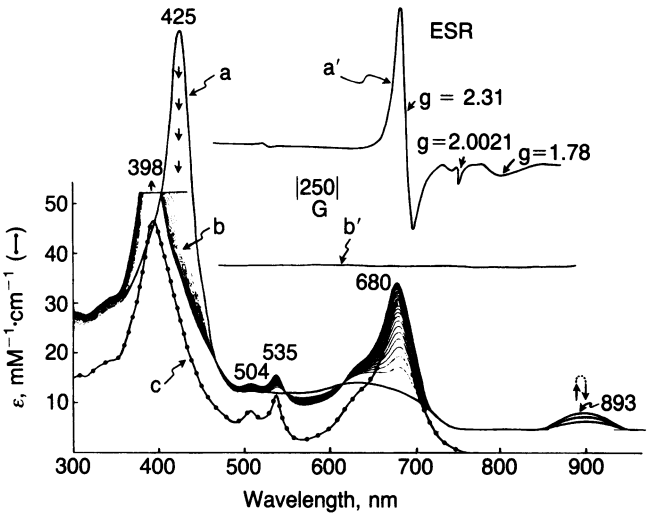

FIG. 1. Conversion of α-oxyprotoheme·bis(pyridine) complex to verdohemochrome IXα. Spectra a and a', electronic (a) and ESR (77 K) (a') spectra of α-oxyprotoheme (0.42 mM) dissolved in oxygen-free pyridine/ethanol/water (5:2.3:2.2, vol/vol). Spectra b, electronic spectra observed during oxygenation of the same solution with dioxygen (1 equivalent); spectrum b', ESR spectrum of verdohemochrome IXα; spectrum c, electronic spectrum of purified pyridine verdohemochrome IXα in $CHCl_3$.

symmetric signal with $g_\perp = 2.30$, $g_\parallel = 1.78$ (Fig. 1, spectrum a') contaminated with a small amount of radical ($g = 2.0021$). This unusual ESR spectrum was identical to that of the oxymesoheme bis(pyridine) complex previously reported (18), which was considered to have the Fe(I) oxidation state. The NMR spectrum of this complex at room temperature showed paramagnetic perturbations, with three sharp proton resonances located well upfield at 110 and 140 ppm. These were readily assigned to the three *meso* protons (Fig. 2, spectrum a). This assignment was substantiated by the NMR spectrum of the corresponding *meso*-deuterium-labeled compound (Fig. 2, spectrum b). The unexpectedly large upfield shift for the *meso* protons cannot be explained as arising solely from iron-centered paramagnetism but must be due to extensive electron spin delocalization in the porphyrin π system (23, 24). Therefore the oxyheme·pyridine complex probably exists in the porphyrin π-neutral radical form as shown in *Scheme 1* (**1a** → **2a**) in Fig. 3. In this molecule, Fe(III) is reduced to Fe(II) intramolecularly by transfer of one electron from the enolate anion $\rangle$—O^- (4, 19, 25) to the iron even in the absence of any extraneous reducing agent (18). To further characterize the electronic state of this compound, we examined the temperature dependence of the NMR spectrum. The methyl and vinyl side proton resonances showed unusual non-Curie law behavior (Fig. 2 *Inset c*). Although the *meso*-proton resonances appeared to follow the normal Curie law, their chemical shifts did not approach the normal diamagnetic region when extrapolated to infinite temperature (Fig. 2 *Inset c'*). These unusual temperature dependences cannot be accounted for if the pyridine complex is in a single spin state. Therefore different spin states may be thermally mixed in this compound. Hence, α-oxyprotohemin IX in pyridine at room temperature is best described as Fe(II) oxyprotoporphyrin π-neutral radical mixed with Fe(I) species (see *Scheme 1*, **2a** ⇄ **2'a**). From the ESR and NMR observations, the Fe(I) species predominated at 77 K, with the Fe(II) π-neutral radical species increasing with increasing temperature. A similar equilibrium has been observed between a Ni(III) porphyrin and the corresponding Ni(II) porphyrin π-radical (26, 27). The Mössbauer spectrum of α-oxyprotoheme·bis(pyridine) complex showed no paramagnetic hyperfine interaction, and the quadrupole splitting (ΔE_Q

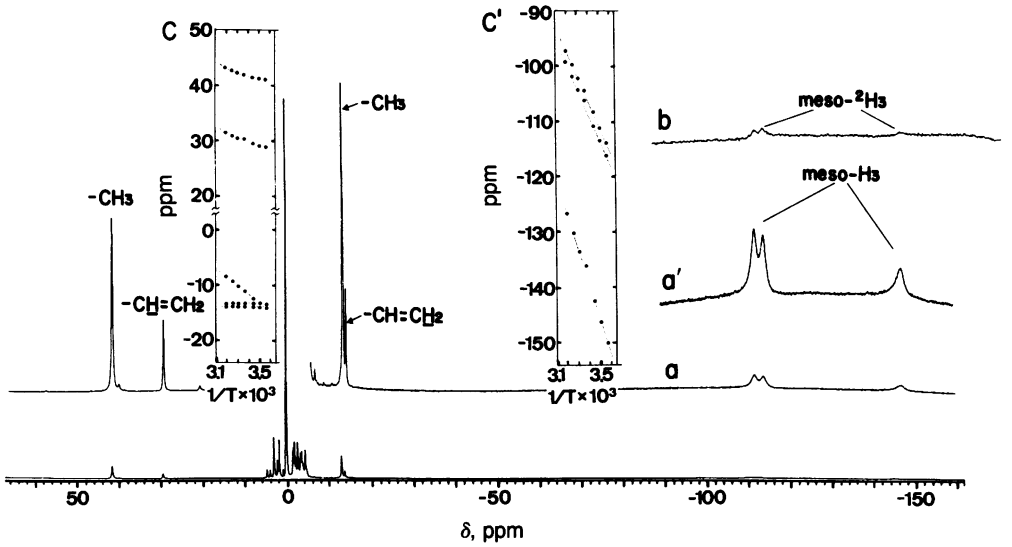

FIG. 2. ^{1}H NMR of α-oxyprotoheme·bis(pyridine) π-neutral radical (5 mM) in oxygen-free deuterated pyridine/deuterated ethanol/deuterium oxide at room temperature; spectrum a is portions of the unlabeled spectrum with the gain increased by a factor of 10; spectrum a' has the gain increased by another factor of 5; spectrum b, ^{1}H NMR of *meso*-deuterated α-oxyprotoheme·bis(pyridine) π-neutral radical (gain same as for x). c and c', Curie law plots for various peaks in the α-oxyprotoheme·bis(pyridine) π-neutral radical. *T*, temperature in K.

= 1.35 mm·s^{-1} at 77 K) and isomer shift (δFe = 0.21 mm·s^{-1} at 77 K) resembled the corresponding parameters seen with ferrous low-spin compounds (28). The Mössbauer data indicate a d^6 rather than a d^7 structure and are inconsistent with the Fe(I) assignment. However, this discrepancy might be due to uptake of one electron by the ligand (29, 30).

Oxygenation of α-Oxyprotoheme IX in Aqueous Pyridine. The α-oxyprotoheme IX·bis(pyridine) π-neutral radical reacted with 1 equimolar amount of dioxygen. Intermediate A was produced initially (λ_{max} = 893 nm) within 3 min, and this rapidly gave way to verdohemochrome IXα in a yield of 75–80% (Fig. 1, spectrum a yielding spectra b). The half-time of verdohemochrome IXα formation was 8 min. Addition of more dioxygen produced no more formation of and no change in verdohemochrome IXα. Carbon monoxide was quantitatively liberated during the conversion of the 893-nm compound to verdohemochrome IXα. Verdohemochrome IXα gave no ESR signal (Fig. 1, spectrum b') and its Mössbauer data were ΔE_Q = 1.2 mm·s^{-1} at 77 K and δFe = 0.35 mm·s^{-1} at 77 K, indicating that it is a Fe(II) low-spin compound. Verdohemochrome IXα was extracted into chloroform and purified by TLC (silica gel) (31). The electronic spectrum of the purified product is shown in spectrum c in Fig. 1. Verdohemochrome IXα (or derived verdohemin IXα) was further converted to biliverdin IXα dimethyl ester by the usual work-up procedure (21), and the latter was identified by comparison with an authentic sample and on the basis of its electronic spectrum, λ_{max} ($CHCl_3$) = 380 and 660 nm, and TLC (15, 16) and HPLC (11) behavior.

Autoxidation of α-oxyprotohemin IX to verdohemochrome IXα in aqueous pyridine in the presence of oxygen is shown in *Scheme 1*. The initial oxygenation involves the Fe(II)·bis(pyridine) π-neutral radical (**2a** in *Scheme 1*), not the Fe(II)·bis(pyridine) complex (λ_{max} = 428, 535, and 550 nm; $\varepsilon_\beta > \varepsilon_\alpha$). Dioxygen probably attacks a pyrrole carbon atom adjacent to the *meso*-oxy bond of the π-neutral radical to give a dioxygen adduct (4, 32) (**3a** in *Scheme 1*). Subsequent rearrangement by an unknown mechanism gives verdohemochrome IXα and CO (*Scheme 1*, **3a** → **4a**). The structure of intermediate A is not yet known, but it may be an isoporphyrin type of compound (e.g., **3a**), judging from its absorption maximum at 893 nm.

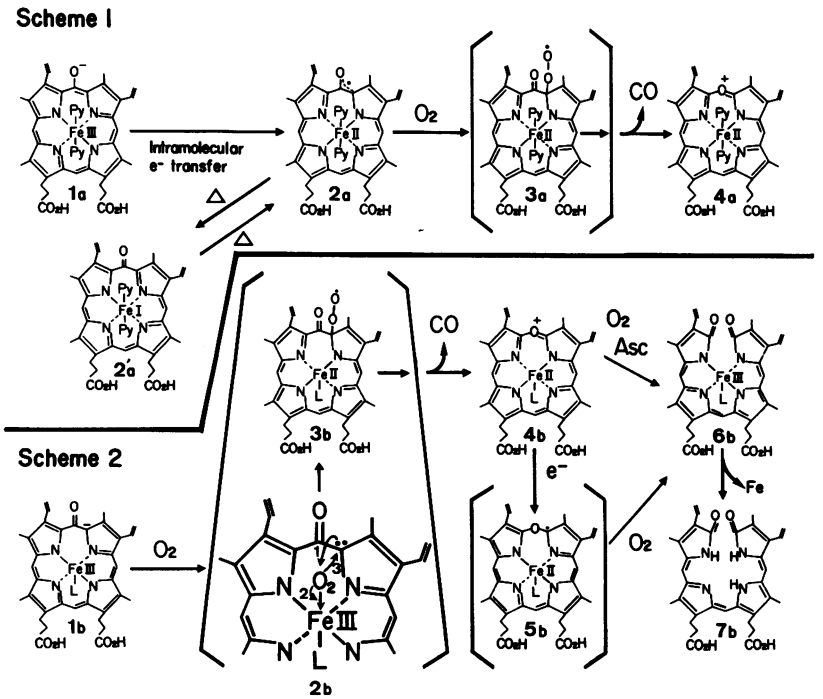

FIG. 3. Possible reactions. *Scheme 1*. Formation of pyridine (Py) verdohemochrome IXα (**4a**) from α-oxyprotohemin IX (**1a**). Structure **2a** is Fe(II)·bis(pyridine) π-neutral radical, formed at room temperature from **1a** by intramolecular electron transfer; **2'a** is Fe(I)·bis(pyridine) complex observed at 77 K. Structure **3a** is the dioxygen adduct. *Scheme 2*. Formation of biliverdin IXα (**7b**) from apoMb·α-oxyprotohemin complex (**1b**); L is a histidine residue of apoMb. Structure **2b** is intermediate BI; **4b** or **5b** is intermediate BII; **6b** is Fe(III)·biliverdin IXα; and **7b** is biliverdin IXα. In the reaction **1b** → **2b**, oxygen oxidizes the porphyrin enolate anion (*1*), giving O_2^-—Fe(III) π-radical (*2*). Internal electron rearrangement of this gives O_2—Fe(II) π-radical (*3*). Finally, O_2 attacks the π-radical to give **3b**. It is not certain at the present stage whether a one-electron reduction path (**4b** to **5b**) or a two-electron reduction (**4b** to **6b**; Asc, ascorbate) is involved in the ring opening of the verdoheme intermediate **4b**.

Table 1. Absorption spectra of the four isomers of pyridine-verdohemochrome IX in $CHCl_3$/pyridine (7:3, vol/vol) and R_f values

Isomer		Absorption maxima, nm (relative peak intensity)					R_f*
α		398 (1.0)	504 (0.182)	535 (0.23)	624sh (0.27)	680 (0.67)	0.806
	ε, $mM^{-1}\cdot cm^{-1}$	46.6	8.5	10.6	12.4	31.2	
β		397 (1.0)	502 (0.16)	533 (0.25)	620sh (0.27)	669 (0.64)	0.755
γ		396 (1.0)	495 (0.21)	530 (0.30)	605sh (0.26)	654 (0.62)	0.689
δ		397 (1.0)	500 (0.16)	534 (0.22)	610sh (0.25)	664 (0.76)	0.739
α†		398 (1.0)	495 (0.11)	532 (0.19)		660 (0.53)	
	ε, $mM^{-1}\cdot cm^{-1}$	40.4	4.6	7.8		21.7	
γ‡		399 (1.0)	495 (0.36)	531 (0.35)	605 (0.23)	657 (0.53)	

sh, Shoulder.
*Silica gel, pyridine/$CHCl_3$/H_2O (6:4:1, vol/vol) (31).
†Pyridine-verdohemochrome IXα obtained from coupled oxidation of Mb with ascorbate (8) (solvent, pyridine).
‡The γ isomer prepared from γ-oxyprotohemin synthesized from pyrrole (33).

Oxidation of β-, γ-, and δ-oxyprotoheme in aqueous pyridine gave the corresponding pyridine verdohemochromes, and the electronic spectra and R_f values are compiled in Table 1. The four isomers have different absorption maxima in the visible region and different R_f values on TLC. The absorption maximum of the γ isomer agreed with that previously reported (33). However, the spectral values of the α isomer were not in agreement with those reported (8) for material prepared by coupled oxidation of Mb with ascorbate followed by addition of pyridine. The reason for the discrepancy is unknown.

Oxygenation of α-Oxyprotohemin IX Incorporated into apoMb. The complex prepared under strictly anaerobic conditions showed absorption maxima at 410, 590, and 640 nm (Fig. 4, spectrum a) and its ESR spectrum had a prominent signal at $g = 6.3$, which was attributed to a high-spin Fe(III) species (Fig. 4, spectrum a').

(i) *First step of oxygenation with dioxygen (1 equivalent) (Fig. 4, spectra a to c, and Fig. 3,* Scheme 2*).* The solution containing the complex was allowed to come into contact with a trace of air (much less than 1 equivalent of oxygen) and frozen immediately. In the ESR spectrum the $g = 6.3$ signal had decreased and a major free radical species with $g = 2.004$ (line width = 10 G) had appeared (Fig. 4, spectrum b') (intermediate BI). The electronic spectrum of this intermediate had a broad new peak at 660 nm (Fig. 4, spectrum b). Further addition of dioxygen up to 1 equivalent resulted in formation of a new intermediate BII, with absorption maxima at 408, 540sh, 660, and 704 nm (Soret intensity slightly decreased). During formation of this intermediate CO was quantitatively released and the ESR signal at $g = 6.3$ from the first intermediate BI completely disappeared (Fig. 3, spectrum c'), indicating formation of a Fe(II) species. Concomitantly the major free radical signal at $g = 2.004$ decreased and a new radical peak at $g = 2.0012$ grew (Fig. 4, spectrum c').

The observed ESR changes can be explained according to the mechanism shown in *Scheme 2* of Fig. 3. Oxygen removes one electron from the enolate anion to give O_2^- (**2b**, step 1) and the π-neutral radical. The O_2^- then binds to the ferric ion (**2b**, step 2) to form an $Fe^{2+}\cdot O_2$ complex, which accounts for the decrease in the ESR signal at $g = 6.3$ (Fig. 4, spectra b' to c'). The bound dioxygen then migrates from Fe(II) to the pyrrole α carbon (**2b**, step 3), where radical addition takes place (**3b**). On this basis the first intermediate, BI, is assigned an apoMb·Fe(II) porphyrin π-neutral radical structure, which is consistent with the ESR signal at $g = 2.004$. Rearrangement of the subsequent dioxygen adduct (**3b**) would give the second intermediate, BII (**4b** or **5b**) (see below). The half-time for formation of BII was 100–120 ms. Since oxygen could not be replaced by hydrogen peroxide and ascorbate was not used, reductant is unnecessary for the first oxygenation step (up to **4b**).

(ii) *Oxygenation of intermediate BII to Fe(III)·biliverdin IXα (Fig. 4, spectra c to e, and* Scheme 2*).* Addition of a second equivalent of dioxygen to the intermediate BII caused a gradual decrease in the visible absorption spectrum and after 30 min yielded a brown solution with λ_{max} 402, 500–540 (plateau), 705 (plateau), and 878 nm (Fig. 4, spectrum d), which resembled that previously attributed to Fe(III)·biliverdin IXα (34) (Soret band decreased in intensity). The reaction proceeded more rapidly in the presence of ascorbate, and in either case CO was not formed. During this second oxidation step the ESR signal at $g = 2.0012$ disappeared, with no

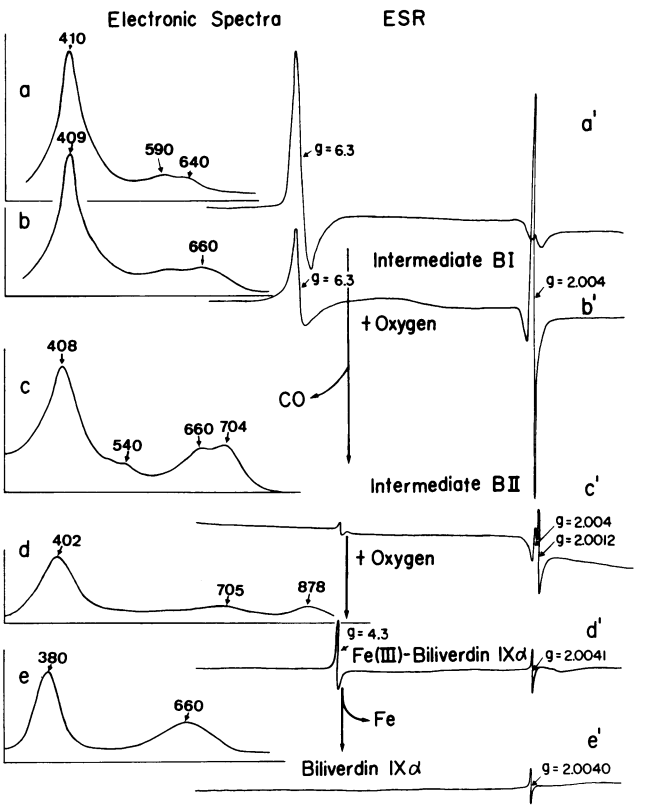

FIG. 4. Electronic (a, b, c, and d) and ESR (*a'*, *b'*, *c'*, and *d'*) spectra observed during the stepwise stoichiometric oxygenation of apoMb α-oxyprotohemin complex (0.1 mM for electronic spectra, 1–2 mM for ESR; all ESR spectra at 77 K). Spectra a and a', apoMb·α-oxyprotohemin complex in 0.1 M Tris·HCl buffer, pH 7.0; spectra b and b', solutions for a and a' after admitting a trace of air by diffusion; spectra c and c', solutions for a and a' after oxygenation with 1 equivalent of dioxygen; spectra d and d', solutions for c and c' after oxygenation with another equimolar amount of oxygen. Spectra e and e', biliverdin IXα after demetallation of solutions for d and d' with acid ($CHCl_3$).

change in the small signal at $g = 2.004$, which originates from Fe·biliverdin IXα (Fig. 4, spectrum d'). The appearance of another ESR signal at $g = 4.3$ in Fe·biliverdin IXα (Fig. 4, spectrum d') suggested that a nonplanar Fe(III) type of complex (35), unstable to acid, may also be formed. Acid treatment of the final solution gave biliverdin IXα in 75–77% overall yield (Fig. 4, spectrum e). These stoichiometric titration experiments support the two-oxygen-molecule mechanism of Brown and King (2).

(iii) *Structure of intermediate BII.* Intermediate BII had ESR signals at $g = 2.0012$ and 2.004, consistent with a porphyrin free radical species. Anaerobic addition of pyridine to BII, to a final concentration of 20–30%, followed by extraction with chloroform, gave a compound that was identical with pyridine verdohemochrome IXα in electronic spectrum and TLC behavior. When the chromophore was dissociated from BII by Teale's procedure (pH 3.5 under CO) the resulting green pigment showed absorption maxima at 504, 546, and 628 nm (methyl ethyl ketone/$CHCl_3$, 1:1, vol/vol), identical with those of synthetic α-verdoheme IXα·CO chloride complex. Acid treatment (6 M HCl) of BII under anaerobic conditions gave biliverdin IXα in 70–75% yield. Similar treatment of synthetic verdoheme chloride gave biliverdin IXα immediately. These observations suggested that BII is a monoligated complex of apoMb and verdoheme. To confirm this, α-verdohemin chloride was prepared according to the method of Jackson *et al.* (21) and reduced with ascorbate. Combination of this with apoMb gave a complex with a spectrum resembling that of BII (Fig. 5, spectrum f). This complex underwent oxidation with 1 equivalent of dioxygen and ascorbate or hydrogen peroxide to give Fe(III)·biliverdin IXα (Fig. 5, spectrum g) which was then demetallated to biliverdin IXα (Fig. 5, spectrum h). This result is consistent with the oxygenation of the α-verdoheme·2-picoline complex to Fe(III)·biliverdin IX reported by Saito and Itano (8). Thus, we conclude that the chromophore of intermediate BII is most likely monoligated verdoheme Fe(II) or its one-electron π-radical reduced form (*Scheme 2*, **4b** or **5b**). It is not certain at present whether a one-electron (*Scheme 2*, **4b** → **5b** → **6b**) or two-electron reduction path (**4b** → **6b**) is involved.

Interestingly, biliverdin IXα was formed in 70% yield from either BII (one-oxygen product) or FE(III)·biliverdin IXα (two-oxygen product).

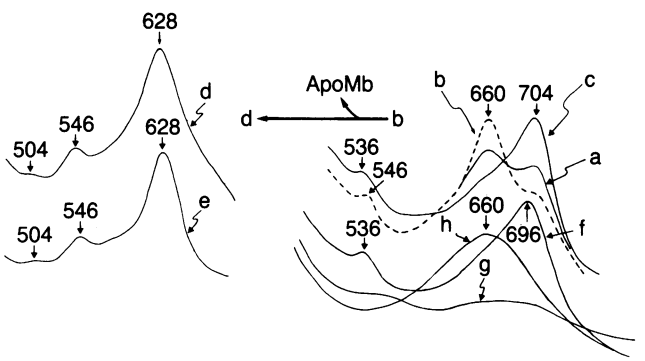

FIG. 5. Electronic absorption spectra. Spectrum a, intermediate BII; spectrum b, BII·CO complex; spectrum c, solution for a after removal of CO under reduced pressure; spectrum d, verdoheme IXα·CO chloride derived from solution b; spectrum e, synthetic α-verdoheme·CO chloride; spectrum f, ApoMb·verdoheme IXα complex; spectrum g, Fe(III)·biliverdin IXα obtained by oxygenation of solution f; spectrum h, biliverdin IXα from demetallation of solution g.

It is not yet known to what extent the reactions in the apoMb model system mimic those that occur in the heme oxygenase system. There are slight discrepancies between the spectral properties of intermediate BII (λ_{max} 660 and 704 nm) of the Mb system and the 638/688-nm intermediate of the heme oxygenase system (36). These differences could be due to differences in the protein environment between the two systems.

In summary, the present studies suggest that the sequence of heme catabolism *in vivo* is protoheme IX $\xrightarrow{O_2}$ α-oxyprotoheme IX $\xrightarrow[CO]{O_2}$ verdohemochrome IXα π-radical $\xrightarrow{O_2}$ Fe(III)·biliverdin IXα $\xrightarrow{Fe}$ biliverdin IXα. Hexa- or pentacoordinate Fe(II) complexes of oxyprotoporphyrin IX π-radical, as previously speculated by Schmid and McDonagh (37), play a key role in triggering autocatalytic oxygenation to verdohemochrome IXα. Further oxygenation of verdohemochrome to Fe·biliverdin IXα complexes occurs only with pentacoordinate verdohemochrome complexes. Thus, the old suggestion that verdohemochrome is an intermediate of heme catabolism *in vivo*, proposed by Lemberg (4) and later by Saito and Itano (8) and Lagarias (38), seems to be confirmed by the present study.

We are grateful to Drs. Y. Orii, S. Kawanishi, and H. Ohya for helpful discussions and to Dr. K. Matushita (JEOL) and Dr. T. Iwashita (Santory Institute) for recording the ^1H NMR spectrum of porphyrin. We thank the Ministry of Education and Culture of Japan for a research grant for S.S. (Grant 58440039) and the Fujiwara Foundation of Kyoto University for partial support of the work.

1. Schmid, R. & McDonagh, A. F. (1979) in *The Porphyrins*, ed. Dolphin, D. (Academic, New York), Vol. 7, pp. 257–292.
2. Brown, S. B. & King, F. G. (1978) *Biochem. J.* **170**, 297–311.
3. Kikuchi, G. & Yoshida, T. (1980) *Trends Biochem. Sci.* **5**, 323–325.
4. Lemberg, R. (1956) *Rev. Appl. Chem.* **6**, 1–23.
5. Kondo, T., Nickolson, D. C., Jackson, A. H. & Kenner, G. W. (1971) *Biochem. J.* **121**, 601–607.
6. Yoshida, T., Noguchi, M., Kikuchi, G. & Sano, S. (1981) *J. Biochem.* **90**, 125–131.
7. Sano, S. & Sugiura, Y. (1982) *J. Chem. Soc. Chem. Commun.*, 750–752.
8. Saito, S. & Itano, H. A. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 1393–1397.
9. Carr, R. P., Jackson, A. H., Kenner, G. W. & Sach, G. S. (1971) *J. Chem. Soc. C*, 487–502.
10. Clezy, P. S. & Liepa, A. J. (1970) *Aust. J. Chem.* **23**, 2477–2488.
11. Jackson, A. H., Nagaraja Ras, K. R. & Wilkins, M. (1982) *J. Chem. Soc. Chem. Commun.*, 794–796.
12. Bonnett, R., Dimsdale, M. L. & Sales, K. D. (1970) *J. Chem. Soc. Chem. Commun.*, 962–963.
13. O'Carra, P. & Colleran, E. (1969) *FEBS Lett.* **5**, 295–298.
14. Fleischer, E. B. & Srivastava, T. S. (1969) *J. Am. Chem. Soc.* **91**, 2403–2405.
15. Bonnett, R. & McDonagh, A. F. (1973) *J. Chem. Soc. Perkin Trans. 1*, 881–888.
16. O'Carra, P. & Colleran, E. (1970) *J. Chromatogr.* **50**, 458–468.
17. Teale, F. W. J. (1959) *Biochim. Biophys. Acta* **35**, 543.
18. Sano, S., Sugiura, Y., Maeda, Y., Ogawa, S. & Morishima, I. (1981) *J. Am. Chem. Soc.* **103**, 2888–2890.
19. Fuhrhop, J. H., Besecke, S., Subramanian, J., Mengersen, C. & Riesner, D. (1975) *J. Am. Chem. Soc.* **97**, 7141–7152.
20. Fuhrhop, J. H. & Smith, K. M. (1975) in *Porphyrins and Metalloporphyrins*, ed. Smith, K. (Elsevier, New York), pp. 800–802.
21. Jackson, A. H., Kenner, G. W. & Smith, K. H. (1968) *J. Chem. Soc.* 302–310.
22. Kenner, G. W., Smith, K. M. & Sutton, N. J. (1973) *Tetrahedron Lett.* **16**, 1303–1306.
23. Morishima, I., Shiro, Y. & Takamuki, Y. (1983) *J. Am. Chem. Soc.* **105**, 6168–6170.
24. Morishima, I., Takamuki, Y. & Shiro, I. (1984) *J. Am. Chem. Soc.* **106**, 7666–7672.
25. Clezy, P. S. (1979) in *The Porphyrins*, ed. Dolphin, D. (Academic, New York), Vol. 2, pp. 103–130.
26. Dolphin, D., Niem, T., Felton, R. H. & Fujita, I. (1975) *J. Am. Chem. Soc.* **97**, 5288–5290.
27. Wolberg, A. L. & Manassen, J. (1970) *J. Am. Chem. Soc.* **92**, 2982–2991.
28. Maeda, Y. (1979) *J. Phys. (Paris) Colloq. C2.* Suppl. to 3, **40**, 514–522.
29. Oosterhuis, W. T. & Lang, G. (1969) *J. Chem. Phys.* **50**, 4381–4387.
30. Taube, R., Drevs, H., Fluck, E., Kuhu, P. & Brauch, K. F. (1969) *Z. Anorg. Allg. Chem.* **364**, 297–315.
31. Levin, E. Y. (1966) *Biochemistry* **5**, 2845–2852.
32. Anan, F. K. & Mason, H. S. (1961) *J. Biochem.* **49**, 765–767.
33. Jackson, A. H., Jenkins, R. M., Jones, M. & Matin, S. (1983) *Tetrahedron* **39**, 1849–1858.
34. Yoshida, T. & Kikuchi, G. (1978) *J. Biol. Chem.* **253**, 4230–4236.
35. Beinert, H., Gelder, B. F. & Hansen, R. E. (1968) in *Structure and Function of Cytochromes*, eds. Okunuki, K., Kamen, M. D. & Sekuzu, I. (Univ. Tokyo Press, Tokyo), pp. 141–146.
36. Yoshida, T. & Noguchi, M. (1984) *J. Biochem.* **96**, 563–570.
37. Schmid, R. & McDonagh, A. F. (1975) *Ann. N.Y. Acad Sci.* **244**, 533–552.
38. Lagarias, J. C. (1982) *Biochim. Biophys. Acta* **717**, 12–19.