Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 1393–1397, March 1982
Biochemistry

Verdohemochrome IXα: Preparation and oxidoreductive cleavage to biliverdin IXα

(myoglobin/hemoglobin/heme ligand/coupled oxidation/sodium ascorbate)

SETSUO SAITO* AND HARVEY A. ITANO†

Department of Pathology, D-006, University of California, San Diego, La Jolla, California 92093

Contributed by Harvey A. Itano, October 28, 1981

ABSTRACT **Several studies have shown that both terminal oxygen atoms of biliverdin are derived from molecular oxygen. Since the conversion of verdohemochrome to biliverdin has been assumed to be hydrolytic, these findings seemed to exclude verdohemochrome as an intermediate in the degradation of heme to biliverdin. Coupled oxidation of myoglobin and ascorbate yielded a pure preparation of verdohemochrome IXα. The structure and ferrous state of this product were determined from its composition, ligand reactions, ^{1}H NMR spectrum, and conversion to biliverdin IXα dimethyl ester. Reaction with ascorbate and $^{18}O_2$ converted this compound to biliverdin that contained an atom of ^{18}O. Successive treatment of verdohemochrome, first oxidation with H_2O_2 and then reduction with phenylhydrazine, yielded the iron complex of biliverdin. These results showed that hydrolysis is not an obligatory step in the conversion of verdohemochrome to biliverdin and, moreover, indicated how heme can be converted, with verdohemochrome as an intermediate, into biliverdin in which the two terminal oxygen atoms are derived from different O_2 molecules.**

Coupled oxidation by O_2 of ferrous protoporphyrin and a reducing agent such as ascorbic acid or hydrazine in the presence of pyridine produces a green compound, the pyridine complex of verdohemochrome. One of the *meso* carbon atoms of the porphyrin ring is expelled as CO and replaced by an oxygen bridge in the process (1). Verdohemochrome is hydrolyzed *in vitro* to biliverdin, and biliverdin is reduced to bilirubin. Hydrolysis of an oxygen-bridged intermediate was, however, excluded as a step in the enzymatic degradation of heme to physiological bile pigments by the finding that both terminal lactam oxygen atoms of bilirubin are incorporated from O_2 without any incorporation of oxygen from water (2). This result contrasted with an earlier report that biliverdin from the coupled oxidation of hemoglobin and ascorbate contains only one atom of oxygen from O_2 (3). More recently, the two terminal oxygen atoms were found to be derived from different molecules of O_2 in the coupled oxidation of octaethylheme and in the physiological formation of bile pigments (4–7). These findings, together with the assumption that the conversion of verdohemochrome or verdohemin to biliverdin requires hydrolysis, have resulted in the rejection of these oxygen-bridged compounds as possible intermediates in heme degradation (2, 4–7).

Biliverdin from the coupled oxidation of pyridine ferrohemochrome is a mixture of the four isomers produced by cleavage at the *meso* carbons of protoporphyrin IX (8); verdohemochrome prepared from heme (9) must also be heterogeneous. In contrast, coupled oxidation produces only biliverdin IXα from myoglobin and only biliverdins IXα and IXβ from hemoglobin (10). Taking advantage of this specificity, we prepared verdohemochrome IXα from myoglobin and a mixture of verdohemochromes IXα and IXβ from hemoglobin. The chemical composition of the pyridine complex of verdohemochrome IXα from myoglobin agreed with an oxygen-bridged structure. We treated verdohemochrome with $^{18}O_2$ and sodium ascorbate to obtain biliverdin in which an atom of oxygen was derived from $^{18}O_2$, and we propose a nonhydrolytic mechanism for the cleavage of verdohemochrome to biliverdin.

MATERIALS AND METHODS

Myoglobin, type III from horse heart, was purchased from Sigma. Washed erythrocytes from 25 ml of human blood were lysed with the addition of distilled water to a total volume of 100 ml. The lysate was centrifuged at 12,000 × *g* for 90 min. The resulting supernatant solution was dialyzed against potassium phosphate buffer, pH 6.8, and applied to a CM-52 cellulose (Whatman) column (7 cm inside diameter × 30 cm) equilibrated with the same buffer. The column was eluted with this buffer until the eluate was clear and then with 1 M potassium phosphate buffer, pH 6.8, to elute oxyhemoglobin (950 mg in 380 ml). $^{18}O_2$ gas (99 atom %) and $H_2{}^{18}O$ (99 atom %) were purchased from Prochem (Summit, NJ). Tosylmethyl isocyanide ($TosCH_2NC$), purchased from Aldrich, was recrystallized from ethanol. Uniplate (silica gel G; Analtech) was used for analytical and preparative TLC. Electronic absorption spectra were recorded on a Cary model 17 spectrophotometer. Mass spectra were obtained on an LKB type 9000 spectrometer at an ionizing energy of 70 eV or 20 eV by the direct inlet method. Proton NMR spectra of samples in C^2HCl_3 solution containing internal tetramethylsilane were recorded with a custom-designed 360-MHz spectrometer with 200–400 pulses in the Fourier-transform mode. Elemental analyses were performed by V. Tashinian, Micro-lab, Department of Chemistry, University of California, Berkeley. Mass spectra of biliverdin dimethyl ester from the $^{18}O_2$ and $H_2{}^{18}O$ experiments were taken by Sherri Ogden, Mass Spectrometry Laboratory, Department of Chemistry, University of California, Berkeley.

RESULTS

Verdohemochrome from the Coupled Oxidation of Myoglobin and Hemoglobin with Ascorbate. A mixture of 5 g of metmyoglobin in 500 ml of 0.1 M potassium phosphate buffer, pH 7.4, and 1.0 g of sodium ascorbate in 10 ml of the same buffer was incubated aerobically for 8 hr at 37°C. The reaction mixture was mixed with 100 ml of pyridine and extracted with two 400-

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Abbreviation: $TosCH_2NC$, tosylmethyl isocyanide [(*p*-tolylsulfonyl)methyl isocyanide].

* Present address: Faculty of Pharmaceutical Science, Josai University, Saitama 350-02, Japan.
† To whom reprint requests should be addressed.

ml portions of chloroform. The extracts were combined, washed with distilled water, dried over Na_2SO_4, and filtered. The filtrate was evaporated to obtain a syrupy residue, which was purified by preparative TLC (20 cm × 20 cm × 2 mm silica gel; pyridine/$CHCl_3$/H_2O, 6:4:1‡). The major green zone was scraped from the plate and suspended in 150 ml of pyridine. After 2 ml of 6 M HCl was added dropwise under nitrogen at 0°C, the suspension was filtered with suction. Chloroform (200 ml) was added to the filtrate, and the resulting solution was washed with distilled water, dried over Na_2SO_4, and filtered. The filtrate was evaporated to a syrupy residue, which was dissolved in 2 ml of benzene. Ligroin was added dropwise to the benzene solution to the point of precipitation, and the solution was allowed to stand overnight at 4°C. The precipitate was collected by suction filtration to obtain the chloride of the monopyridine complex of verdohemochrome (68 mg, 33% yield).

Analysis. Calculated for $C_{38}H_{36}ClFeN_5O_5$: C, 62.18; H, 4.94; Cl, 4.83; Fe, 7.61; N, 9.54. Found: C, 62.06; H, 5.03; Cl, 4.70; Fe, 7.25; N, 9.70. Electronic absorption spectra, λ_{max} (pyridine) 398 nm (ε_{mM}40.4), 495 (4.6), 532 (7.8), 660 (21.7); (pyridine/CN^-) 396 (34.1), 505 (3.5), 535 (5.2), 665 (20.2); (pyridine/$TosCH_2NC$) 396 (36.4), 495 (4.6), 532 (7.8), 654 (21.5); (pyridine/CO) same as in pyridine. Similar spectra, including failure to bind CO, were observed in 3-picoline and in 1-methylimidazole. (2-picoline) 401 (37.9), 495 (7.1), 533 (8.5), 670 (20.4); (2-picoline/CN^-) 400 (36.9), 500 (9.4), 533 (8.4), 663 (20.4); (2-picoline/$TosCH_2NC$) 380 (37.4), 495 (7.1), 533 (8.0), 640 (23.2); (2-picoline/CO) 405 (35.5), 500 (6.2), 543 (9.4), 628 (16.6). Similar spectra, including binding of CO, were observed with 10% 2-methylimidazole in ethanol and with 10% 1,2-dimethylimidazole in ethanol.

Verdohemochrome (15 mg) obtained from myoglobin was dissolved together with 40 mg of $TosCH_2NC$ (11) in 30 ml of $CHCl_3$ under N_2. This mixture was cooled at −70°C for 2 min and then mixed with excess diazomethane ethyl ester solution. Soon after the addition of diazomethane, the mixture was evaporated to obtain a syrupy residue. The residue showed a major bluish green spot (R_F 0.69 in $CHCl_3$/MeOH, 22:3) accompanied by many minor spots (R_F 0.97, pink; R_F 0.93–0.82, brown; R_F 0.75, brown; R_F 0.42, blue) by TLC. The major bluish green pigment was isolated by preparative TLC (20 cm × 20 cm × 2 mm silica gel; $CHCl_3$/MeOH, 22:3) followed by TLC (20 cm × 20 cm × 0.5 mm silica gel in the same solvent system). Verdohemochrome dimethyl ester di-$TosCH_2NC$ complex (8.8 mg, 41% yield) was obtained as a bluish green oil. This oil showed only one spot on TLC, but its NMR spectrum showed that it had a small amount of impurity. The signals of the major component were assignable: NMR (C^2HCl_3) δ 9.23, 9.02, and 8.92 (each 1H, s, *meso* proton), 7.65 (2H, m, CH=CH_2 × 2), 7.09 (4H, *ortho* protons of tolyl group of $TosCH_2NC$ × 2), 6.67 (4H, *meta* protons of tolyl group of $TosCH_2NC$ × 2), 5.98 (4H, m, CH=CH_2 × 2), 3.78 (4H, m, Ar—CH_2 × 2), 3.68 (6H, s, OCH_3 × 2), 3.18 (4H, m, CH_2CO × 2), 3.10 (CH_3), 3.07 (CH_3), 3.05 (CH_3), 3.03 (CH_3), 2.37 (10H, s, CH_3 and CH_2 of $TosCH_2NC$ × 2). Verdohemochrome (28 mg, 65% yield) was obtained from the protoheme of hemoglobin by the coupled oxidation of 950 mg of human oxyhemoglobin in 500 ml of 0.1 M potassium phosphate buffer, pH 7.4, with 790 mg of sodium ascorbate in 10 ml of the same buffer.

Hydrolytic Conversion of Verdohemoglobin to Biliverdin. Hydrolytic reactions of verdohemochrome were observed spectrophotometrically. Addition of water to a solution of verdohemochrome in 2-picoline resulted in a rapid change in spectrum to that of iron biliverdin (12). In contrast, when water was added to a solution of verdohemochrome in pyridine, the spectrum remained unchanged. Addition of KOH resulted in a rapid change in spectrum in 2-picoline, a slow change in pyridine, and no change in 2-picoline/$TosCH_2NC$. The changes in electronic absorption spectrum that occurred when verdohemochrome was converted to biliverdin by the addition first of KOH and then of HCl are shown in Fig. 1.

Verdohemochrome (30 mg, 41 μmol) obtained from myoglobin was dissolved in 50 ml of ethanol, and 50 ml of 6 M HCl was added at 0°C under N_2. The mixture was allowed to stand overnight at 4°C, poured into ice water (100 ml), and then allowed to stand for 2 hr at 0°C while being bubbled with N_2. The solution was extracted with two 150-ml portions of chloroform, and the combined extracts were washed with distilled water, dried over Na_2SO_4, filtered, and evaporated to dryness. The residue was dissolved in 50 ml of 5% H_2SO_4 in methanol at 0°C and allowed to stand overnight at 4°C. This solution was poured into 50 ml of ice water, and the mixture was extracted with two 50-ml portions of chloroform. The combined extracts were washed with distilled water, dried over Na_2SO_4, filtered, and evaporated to dryness (19.1 mg, 76% yield). Biliverdin IXα dimethyl ester (13.9 mg, 56% yield) was isolated from the residue by preparative TLC (20 cm × 20 cm × 1 mm silica gel G; *n*-heptane/ethyl methyl ketone/acetic acid, 10:5:1). Mass spectrum (70 eV) *m/e* (rel. intensity) 610 (M^+, 100), 595 (9.0), 463 (9.5), 410 (8.5), 330 (5.6), 326 (9.0), 313 (14.5), 311 (10.4), 305 (9.3), 300 (21.5), 298 (6.0), 285 (6.0), 268 (6.0), 225 (14.5); (20 eV) *m/e* (rel. intensity) 610 (M^+, 86.4), 523 (6.1), 326 (21.0), 315 (13.5), 311 (15.0), 300 (100), 287 (5.0), 285 (9.0), 283 (9.0), 259 (6.0), 227 (30.0); NMR (C^2HCl_3) δ 8.61 (3H, broad s, NH × 3), 6.67 (1H, s, γ *meso* proton), 6.62 and 6.50 (each 1H, dd, J_1 = 18 Hz, J_2 = 12 Hz, CH=CH_2), 6.12, 5.98, 5.61, and 5.38 (each 1H, dd, J_1 = 12 Hz, J_2 = 1.5 Hz, CH=CHH), 6.00 and 5.96 (each s, β and δ *meso* protons), 3.68 (6H, s, OCH_3 × 2), 2.93 (4H, Ar—CH_2 × 2), 2.55 (4H, CH_2CO × 2), 2.16 (CH_3), 2.06 (CH_3), 2.11 (CH_3), 1.85 (CH_3).

Verdohemochrome (30 mg, 41 μmol) from hemoglobin was hydrolyzed and esterified in the same manner. The product showed two blue spots (R_F 0.32 and 0.50 *n*-heptane/ethyl

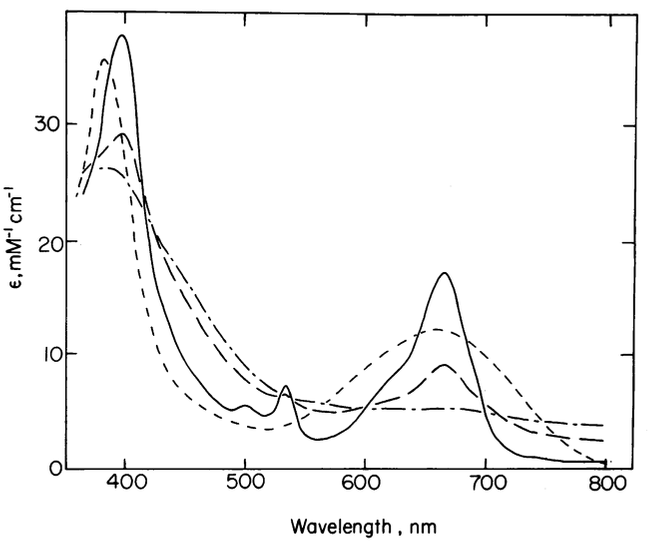

FIG. 1. Conversion of verdohemochrome to biliverdin by hydrolysis. Electronic absorption spectrum of 0.15 μmol of verdohemochrome in 5 ml of 2-picoline (——). Spectra after successive addition of 2 μl of 0.15 M aqueous KOH (— — —), 5 μl of 0.15 M aqueous KOH (—·—), and 5 ml of 6 M HCl (– – –).

‡ All reagent percentages and solvent ratios are vol/vol.

methyl ketone/acetic acid, 10:5:1) on TLC, which were identified by TLC, NMR, and mass spectrum as biliverdin IXα dimethyl ester (8.6 mg, 34% yield) and biliverdin IXβ dimethyl ester (4.6 mg, 18% yield). Biliverdin IXβ dimethyl ester: NMR (C^2HCl_3) δ 8.60 (3H, broad s, NH × 3), 6.80, 6.19, and 6.05 (each s, *meso* proton), 6.69 and 6.50 (each 1H, dd, J_1 = 17.5 Hz, J_2 = 12 Hz, C*H*=CH_2), 6.12, 5.45, 5.44, and 5.39 (4H, CH=CH_2 × 2), 3.68 (OCH_3), 3.65 (OCH_3), 2.85 (4H, Ar—CH_2 × 2), 2.63 (2H, CH_2CO), 2.50 (2H, CH_2CO), 2.30 (CH_3), 2.20 (CH_3), 2.18 (CH_3), and 1.77 (CH_3). Mass spectrum (70 eV) *m/e* (rel. intensity) 610 (M^+, 100), 594 (18.5), 579 (6.2), 537 (9.5), 532 (5.5), 387 (6.0), 360 (47.5), 287 (28.0), 266 (34.0), 251 (25.0), 240 (16.5), 225 (10.5), 213 (8.0); (20 eV) *m/e* (rel. intensity) 610 (M^+, 100), 387 (12.5), 360 (26.5), 287 (5.0), 226 (22.5), 251 (10.0), 240 (7.5), 225 (5.0).

Nonhydrolytic Conversion of Verdohemochrome to Biliverdin. Rapid changes in absorption spectrum, shown in Fig. 2, followed the aerobic addition of sodium ascorbate or phenylhydrazine to a solution of verdohemochrome in 2-picoline. These changes occurred very slowly in pyridine and did not occur in 2-picoline in the presence of $TosCH_2NC$. No change was observed when only ascorbate, phenylhydrazine, or O_2 was added to a 2-picoline solution of verdohemochrome. When H_2O_2 was added, the spectrum changed immediately. Another rapid change in spectrum followed the anaerobic addition of ascorbate or phenylhydrazine; the final spectrum was that of iron biliverdin. These results are shown in Fig. 3. Changes in spectra were very slow when H_2O_2 was added to verdohemochrome in pyridine. No change in spectrum occurred when H_2O_2 was added to a solution of verdohemochrome and $TosCH_2NC$ in 2-picoline.

Verdohemochrome IXα (10 mg, 13.6 μmol) in 10 ml of 2-picoline under N_2 was injected into a container of $^{18}O_2$ (100 μl) cooled to −70°C. The reaction mixture was warmed to room temperature, and 41 μmol of sodium ascorbate in 100 μl of H_2O/2-picoline (1:4) was added. The mixture was shaken vigorously and, after 15 min, was poured into 20 ml of ice-cold 6 M HCl under N_2; this mixture was extracted with two 30-ml portions of chloroform. The combined extracts were washed with distilled water, dried over Na_2SO_4, filtered, and evaporated to dryness. The residue was dissolved in 5% H_2SO_4 in methanol (20 ml) under N_2 and allowed to stand overnight at 4°C. The reaction mixture was poured into 10 ml of ice water, and this mixture was extracted with two 25-ml portions of chloroform. The combined extracts were washed with distilled water, dried over Na_2SO_4, filtered, and evaporated to dryness. Biliverdin IXα dimethyl ester (4.9 mg, 59%) was isolated and purified by TLC (20 cm × 20 cm × 1 mm; *n*-heptane/methyl ethyl ketone/acetic acid, 10:5:1). The product was identified as the IXα isomer by mass spectrometry. In duplicate experiments, peaks at *m/e* 610 and 612 were present in ratios of 53:47 and 52:48. Thus, more than half of the biliverdin contained ^{16}O. To determine the amount of hydrolysis attributable to the small amount of water used with 2-picoline to dissolve sodium ascorbate, the conversion was carried out in $^{16}O_2$ with ascorbate in $H_2{}^{18}O$/2-picoline. Sodium ascorbate (41 μmol) in 100 μl of $H_2{}^{18}O$/2-picoline (1:4) was added to verdohemochrome IXα (10 mg, 13.6 μmol, in 10 ml of 2-picoline) under air. Biliverdin IXα dimethyl ester (3.5 mg, 42%) was obtained as described. The mass spectrum of this product showed peaks at *m/e* 610 and 612 in the ratio 75:25. Water used as solvent for sodium ascorbate thus accounted for 25% of the total biliverdin produced. The $^{18}O_2$ experiments showed that hydrolysis with $H_2{}^{16}O$ accounted for 53% of the product. Since 25% was due to water in the ascorbate solution, 28% must have been due to water from other sources.

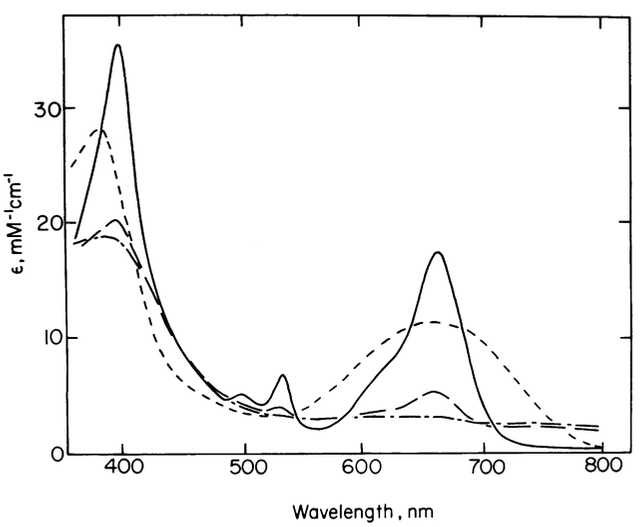

FIG. 2. Conversion of verdohemochrome to biliverdin by ascorbate/O_2. Electronic absorption spectrum of 0.13 μmol of verdohemochrome in 5 ml of 2-picoline (——). Spectra 1 (—— ——) and 5 min (—·—) after addition of 2 μl of 0.2 M aqueous sodium ascorbate and after addition of sodium ascorbate followed by addition of 5 ml of 6 M HCl (– – –).

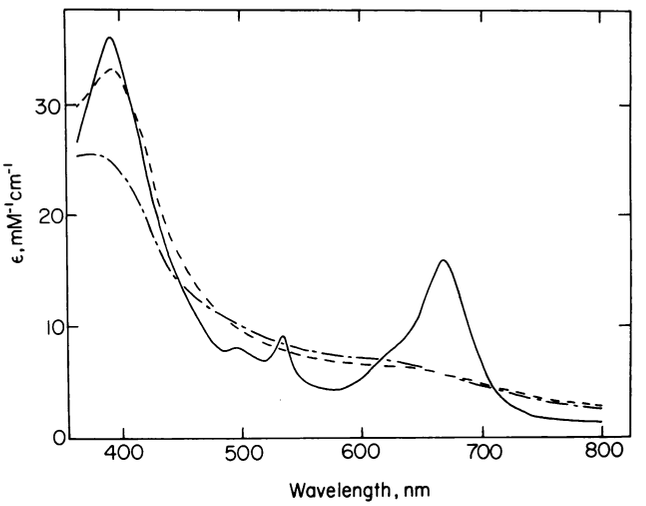

FIG. 3. Conversion of verdohemochrome to biliverdin by H_2O_2/phenylhydrazine. Electronic absorption spectrum of 0.22 μmol of verdohemochrome in 5 ml of 2-picoline (——). Spectra after addition of 10 μl of 0.035 M aqueous H_2O_2 (– – –) and after addition of H_2O_2 followed by addition of 10 μl of 0.07 M aqueous phenylhydrazine HCl (—·—). Acidification with HCl produced biliverdin (spectrum not shown).

DISCUSSION

Structure of Verdohemochrome. Structural formulas and reaction pathways to be discussed below are shown in Fig. 4. Structure V has been proposed for verdohemochrome, and structure IV with ferric instead of ferrous iron has been proposed for verdohemin (13). The composition of verdohemochrome prepared by the coupled oxidation of myoglobin and ascorbate corresponded to that of the monopyridine monochloride of IV. Hydrolytic conversion of this compound to biliverdin IXα dimethyl ester placed its oxygen bridge at the α-*meso* position. Cyanide ion is a ligand of ferric as well as ferrous porphyrins, but CO and isocyanides bind only ferrous porphyrins (14). The ligand-binding reactions of our preparation of verdohemochrome therefore indicated that its iron atom is in the

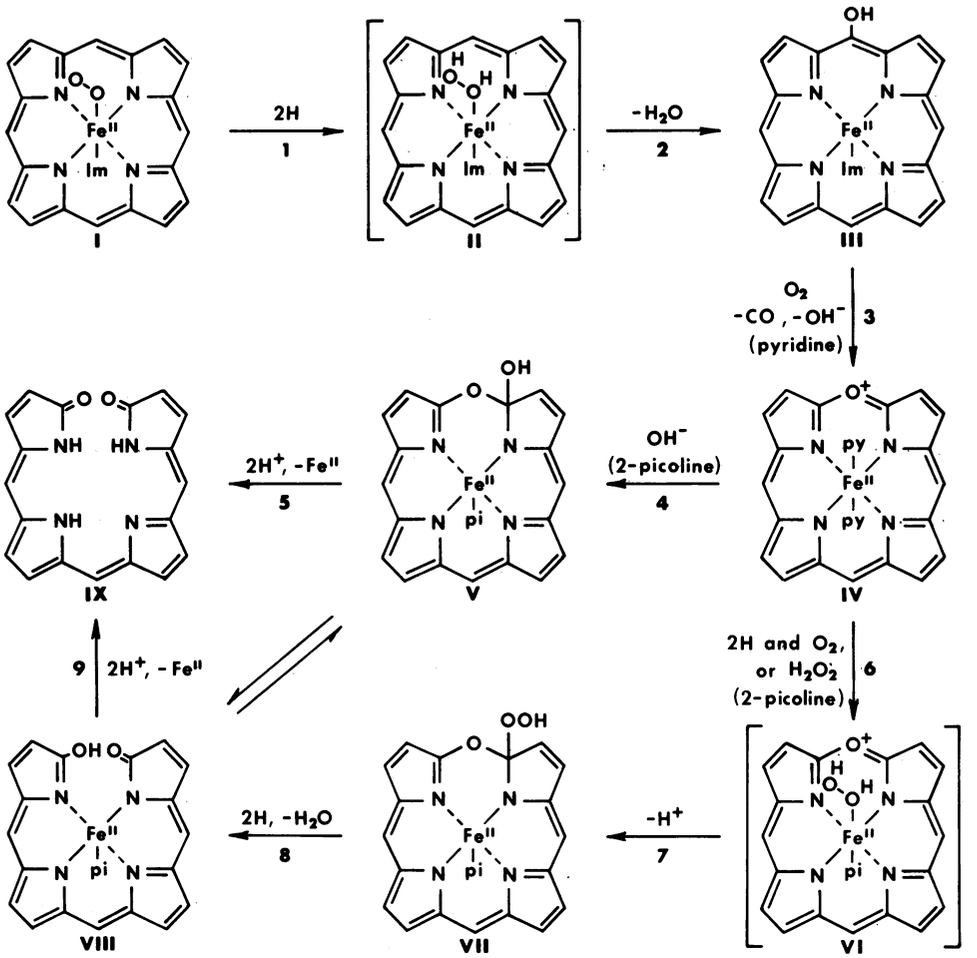

FIG. 4. Formation of biliverdin IXα from verdohemochrome IXα. The side chains of protoporphyrin IX can be located in relation to the α-*meso* position of the oxygen bridge of verdohemochrome IXα (**IV**). Verdohemochrome IXα, prepared as the chloride of its monopyridine complex by coupled oxidation of myoglobin (**I**) and ascorbate by O_2, is present as the dipyridine complex in pyridine solution and as the mono-2-picoline complex in 2-picoline solution. Addition of KOH to verdohemochrome produced an intermediate suggested to be **V**, the hemiketal of the iron complex (**VIII**) of biliverdin. Hydrolytic cleavage of verdohemochrome to biliverdin (**IX**) was completed by the addition of HCl (Fig. 1). Ascorbate, phenylhydrazine, or O_2 alone did not react with verdohemochrome, but ascorbate and O_2 produced iron biliverdin, acidification of which released biliverdin (Fig. 2). Addition of H_2O_2 to verdohemochrome produced an intermediate suggested to be **VII**, which was converted to iron biliverdin by phenylhydrazine or ascorbate (Fig. 3). The intermediates represented by **VIII** and **V** may both be the same equilibrium mixture of iron biliverdin and its hemiketal. Im, proximal imidazole in the heme crevice of myoglobin; py, pyridine; pi, 2-picoline; 2H, ascorbate in reaction **1** and ascorbate or phenylhydrazine in reactions **6** and **8**.

ferrous state. The di-TosCH_2NC complex of verdohemochrome dimethyl ester gave the ^1H NMR spectrum expected of a low-spin ferrous complex. A neutral structure such as **V** is excluded by the presence of a chloride in pyridine–verdohemochrome. Two chlorides, presumably counterions to the central ferric atom and the ring oxonium atom, were present in a preparation of verdohemin IXβ dimethyl ester derived from the mesoheme **IX** (15), and one chloride was present in zinc oxaoctaethylporphyrin (16). The presence of only one chloride in our product is thus in accord with the ferrous state of **IV**. In summary, according to its elemental analyses, ligand-binding properties, and the product of its hydrolysis, this compound has structure **IV**, in which an oxygen bridge has replaced the α-*meso* carbon of protoporphyrin IX. Dissolved in pyridine, it binds two molecules of pyridine; in the solid state, it contains one molecule of pyridine. Its electronic absorption spectrum is that of verdohemochrome (9). Structures **IV** and **V** are not related to each other by an acid–base equilibrium because successive addition of base and then acid to **IV** failed to restore **IV** but instead produced biliverdin (**IX**). We suggest that the addition of hydroxide to **IV** results in **V** and that the latter is the hemiketal of ferrous biliverdin (**VIII**), not a neutral form of verdohemochrome (**IV**).

Reactions of Verdohemochrome with Ligands. Excess pyridine prevents the binding of CO by ferrous heme, owing to the presence of pyridine on both the fifth and the sixth coordination sites (17). Binding of CO by verdohemochrome likewise is prevented by pyridine, 3-picoline, and 1-methylimidazole. The affinities of CN^- and TosCH_2NC must be sufficiently high that these ligands can displace these bases from the sixth coordination site. On the other hand, verdohemochrome formed complexes with CO as well as with CN^- and TosCH_2NC in the presence of 2-picoline, 2-methylimidazole, and 1, 2-dimethylimidazole. The common structural feature of this group of bases is a methyl group on the carbon atom adjacent to a nitrogen atom. A bulky group in this position results in hindrance to the binding of a second molecule of the same base to ferrous porphyrin compounds (18); consequently the sixth coordination site is open for occupancy by CO.

Other reactions were also influenced by ligand solvents. Both hydrolysis and reaction with H_2O_2 were rapid in 2-picoline and

very slow or blocked in pyridine. Both reactions were blocked by $TosCH_2NC$, a strong ligand that displaces pyridine from verdohemochrome in pyridine solution. Thus, ligands that bind both the fifth and the sixth coordination sites of verdohemochrome and prevent the binding of CO also inhibit or block reactions that result in the formation of biliverdin from verdohemochrome.

Conversion of Verdohemochrome to Biliverdin. Reactions **1–3** in Fig. 4 were previously proposed (1). Oxidation states Fe(II) and Fe(III) have been suggested for the iron atom of **III** (19, 20). We have assigned Fe(II) to **III** to avoid the complication of an odd-electron transfer in the formation of **IV**, a ferrous compound; reaction **3** requires only O_2 (1) and is not known to produce a free radical. Quantitative formation of biliverdin (**IX**) by successive treatment of verdohemochrome (**IV**) with alkali and then acid (Fig. 4, reactions **4** and **5**) has been reported (13). Since O_2 by itself did not react with **IV**, a two-electron transfer to a dioxygen ligand, as in reaction **1**, does not occur in the formation of **VI** or **VII**. Ascorbate and O_2 or phenylhydrazine and O_2 must first produce H_2O_2, which then reacts with **IV**. Initial addition of H_2O_2 to the Fe(II) of **IV** to produce **VI** is suggested because this reaction was blocked by $TosCH_2NC$. The similarity between the spectra that resulted from the addition of H_2O_2 (Fig. 3) and hydroxide (Fig. 1) to verdohemochrome suggests that **VI** becomes **VII**, a structural homologue of **V**.

Addition of aqueous HCl to the product of the reaction between verdohemochrome and H_2O_2 led to the slow formation of biliverdin. Thus, hydrolysis of **VII** to H_2O_2 and biliverdin is slow. In contrast, addition of phenylhydrazine or ascorbate to **VII** resulted in the rapid formation of iron biliverdin (**VIII**), from which acidification rapidly released biliverdin. The very similar spectra that resulted from hydroxylation of verdohemochrome (Fig. 1) and reduction of the product of the reaction between verdohemochrome and H_2O_2 (Fig. 3) suggest that the products may be the same equilibrium mixture of iron biliverdin (**VIII**) and its hemiketal (**V**).

One oxygen atom derived from O_2 forms the oxygen bridge of verdohemochrome, and a second is incorporated when verdohemochrome is cleaved to biliverdin in reactions **6–9**. This mechanism thus accounts for the presence in biliverdin of two oxygen atoms from different molecules of O_2. *β-meso*-Phenylbiliverdin, a product of the aerobic reaction of phenylhydrazine with oxyhemoproteins (21), was not a product of the aerobic reaction of phenylhydrazine with verdohemochrome.

In the nonhydrolytic mechanism that we postulate for the cleavage of verdohemochrome, reaction with H_2O_2, an oxidation, is followed by reaction with phenylhydrazine or ascorbate, a reduction. Because the net change from verdohemochrome to biliverdin is neither an oxidation nor a reduction, we propose oxidoreductive cleavage as a descriptive name for the process. The overall conversion from ferrous protoporphyrin to ferrous biliverdin in this model of coupled oxidation requires three moles of O_2 and three pairs of reducing equivalents, stoichiometry in reasonable agreement with that of the microsomal heme oxygenase system (22, 23).

Although the amount of water added to the reaction mixture for oxidoreductive cleavage was the minimum needed to solubilize sodium ascorbate, more than half the resulting biliverdin contained oxygen from water. Nearly half of these oxygen atoms came from the ascorbate solution; the rest must have come from water in 2-picoline. The high rate of hydrolysis of verdohemochrome in the absence of strong ligands may account for the finding that biliverdin from the coupled oxidation of hemoglobin and ascorbate contained only one atom of oxygen from O_2 (3). Hydrolysis would have been the predominant mode of cleavage of verdohemochrome in the aqueous medium in which the reaction was carried out. In contrast, hydrolysis was not observed when octaethylchlorohemin and *meso*-hydroxyoctaethylchlorohemin were converted to biliverdin with ascorbate and O_2 in pyridine/water (10:1 or 97:3) (10). These results are in accord with our observation that verdohemochrome resisted hydrolysis in pyridine.

We thank Dr. Martin Tientze for determining the ^1H NMR spectra at 360 MHz. This work was supported by National Institutes of Health Grant AM 14982.

1. Lemberg, R. (1956) *Rev. Pure Appl. Chem.* **6**, 1–23.
2. Tenhunen, R., Marver, H., Pimstone, N. R., Trager, W. F., Cooper, D. Y. & Schmid, R. (1972) *Biochemistry* **11**, 1716–1720.
3. Anan, F. K. & Mason, H. S. (1961) *J. Biochem. (Tokyo)* **49**, 765–767.
4. Brown, S. B. & King, R. F. G. J. (1975) *Biochem. J.* **150**, 565–567.
5. Brown, S. B. & King, R. F. G. J. (1978) *Biochem. J.* **170**, 297–311.
6. King, R. F. G. J. & Brown, S. B. (1978) *Biochem. J.* **174**, 103–109.
7. Jackson, A. H., Lee, M. G., Jenkins, R. T., Brown, S. B. & Chaney, B. D. (1978) *Tetrahedron Lett.*, 5135–5138.
8. Bonnett, R. & McDonagh, A. F. (1973) *J. Chem. Soc. Perkin Trans. 1*, 881–888.
9. Levin, E. Y. (1966) *Biochemistry* **5**, 2845–2852.
10. O'Carra, P. & Colleran, E. (1969) *FEBS Lett.* **5**, 295–298.
11. Traylor, T. G. & Stynes, D. V. (1980) *J. Am. Chem. Soc.* **102**, 5938–5939.
12. Yoshida, T. & Kikuchi, G. (1977) *J. Biol. Chem.* **253**, 4230–4236.
13. Foulkes, E. C., Lemberg, R. & Purdom, P. (1951) *Proc. R. Soc. London Ser. B.* **138**, 386–402.
14. Lemberg, R. & Legge, J. W. (1949) *Hematin Compounds and Bile Pigments* (Interscience, New York), pp. 184–191, 214–222.
15. Jackson, A. H., Kenner, G. W. & Smith, K. M. (1968) *J. Chem. Soc. C*, 302–310.
16. Fuhrhop, J.-H., Besecke, S., Subramanian, J., Mengersen, C. & Riesner, D. (1975) *J. Am. Chem. Soc.* **97**, 7141–7152.
17. Clifcorn, L. E., Meloche, V. W. & Elvehjem, C. A. (1935) *J. Biol. Chem.* **111**, 399–409.
18. Wang, C. M. & Brinigar, W. S. (1979) *Biochemistry* **18**, 4960–4977.
19. Bonnett, R. & Dimsdale, M. J. (1972) *J. Chem. Soc. Perkin Trans. 1*, 2540–2548.
20. Dolphin, D., Muljiani, Z., Rousseau, K., Borg, D. C., Fajer, J. & Felton, R. H. (1973) *Ann. N.Y. Acad. Sci.* **206**, 177–198.
21. Saito, S. & Itano, H. A. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 5508–5512.
22. Tenhunen, R., Marver, H. S. & Schmid, R. (1968) *Proc. Natl. Acad. Sci. USA* **61**, 748–755.
23. Tenhunen, R., Marver, H. S. & Schmid, R. (1969) *J. Biol. Chem.* **244**, 6388–6394.