A two-molecule mechanism of haem degradation

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Coupled oxidation of octaethylhaemin and phenylhydrazine hydrochloride with $^{16,16}\text{O}_2$ and $^{18,18}\text{O}_2$ produced octaethyl[^{16}O]verdohaemochrome and octaethyl[^{18}O]verdohaemochrome respectively. Reactions of these products with $^{16,16}\text{O}_2$ in the presence of phenylhydrazine hydrochloride yielded octaethyl[$^{16}\text{O},^{16}\text{O}$]biliverdin and octaethyl[$^{18}\text{O},^{16}\text{O}$]biliverdin. The same reactions with $^{18,18}\text{O}_2$ yielded octaethyl[$^{16}\text{O},^{18}\text{O}$]biliverdin and octaethyl[$^{18}\text{O},^{18}\text{O}$]biliverdin. Accordingly, the two oxygen atoms of biliverdin are incorporated from different O_2 molecules in separate reactions, namely the formation of verdohaemochrome and the conversion of verdohaemochrome into biliverdin. These reactions account for a 'two-molecule mechanism' of biliverdin formation from haem with verdohaemochrome participating as an intermediate product.

Biliverdin is the final product in the coupled oxidation of haem and a reducing agent by O_2 . Foulkes *et al.* (1951) postulated the following sequence in the formation of intermediate products in this process: haem→hydroxyhaem→verdohaem→iron biliverdin→biliverdin. A *meso* carbon in the porphyrin ring of haem is hydroxylated to form hydroxyhaem, and this carbon is expelled as CO and replaced by an oxa bridge to form verdohaem. The iron complex of biliverdin is the initial product of verdohaem hydrolysis, and biliverdin results from release of the iron atom by acidification. Biliverdin produced in the enzyme-mediated physiological degradation of haem is rapidly converted into bilirubin in a two-electron reduction in which the terminal oxygen atoms are conserved. Tenhunen *et al.* (1972) found that bilirubin formed enzymically from haem contained two oxygen atoms from O_2 and none from H_2O . Brown & King (1975, 1976, 1978) and King & Brown (1978) confirmed these results, and they showed, moreover, that each of the two oxygen atoms of bilirubin was derived from a different O_2 molecule. Incorporation of the two oxygen atoms of biliverdin from different O_2 molecules was found as well in the coupled oxidation of myoglobin (King & Brown, 1978) and octaethylhaemin (Chaney & Brown, 1978; Jackson *et al.*, 1978).

Biliverdin formed by the hydrolysis of verdohaem would, on the other hand, contain an oxygen atom from H_2O . The absence of such an atom, coupled with the assumption that the conversion of verdohaem into biliverdin could only occur by hydrolysis, resulted in widespread acceptance of the inference that neither in physiological nor in chemical oxidative degradation of haem is verdohaem the precursor of biliverdin (Jackson, 1974; O'Carra, 1975; Clezy, 1978; Schmid & McDonagh, 1979; Brown & Troxler, 1982).

Experimental procedures

Pyridine was distilled over ninhydrin. Tetrahydrofuran was distilled over LiAlH_4 under N_2 . Ethanol was distilled over Na and diethyl phthalate. Other solvents were of analytical grade. $^{18,18}\text{O}_2$ gas (99 atom%) was purchased from Prochem/Isotopes, U.S. Services (Summit, NJ, U.S.A.), and octaethylporphine, purchased from Porphyrin Products (Logan, UT, U.S.A.), was transformed into octaethylhaemin by the method of Bonnett & Dimsdale (1972). Octaethyl[^{16}O]verdohaemochrome was obtained as described previously (Hirota & Itano, 1983). Electron-impact (70 eV) mass spectra were taken with a Shimadzu LKB-9000 spectrometer.

Preparation of octaethyl[^{18}O]verdohaemochrome

Into a solution of 278 mg (0.446 mmol) of octaethylhaemin in 500 ml of pyridine/water (4:1, v/v) in a 500 ml three-necked flask, 129 mg

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(0.892 mmol) of solid phenylhydrazine hydrochloride wrapped in paper was dropped at once under N_2 . After closure of the flask with rubber stoppers and stoppage of N_2 flow, the flask was evacuated with a vacuum pump. After closure of the vacuum cock, a glass container with 100 ml (4.46 mmol) of $^{18,18}O_2$ was attached to the flask. The seal to the $^{18,18}O_2$ container was broken, and the vacuum cock was opened to admit $^{18,18}O_2$ into the evacuated flask, which was then filled with N_2 from a balloon. The reaction mixture was stirred for 1 day, during which time the flask was occasionally inverted to transfer the solution into the $^{18,18}O_2$ container. The resulting dark-green solution was evaporated at 40–50°C under vacuum. The residue was fractionated by t.l.c., the major green zone was scraped off and eluted, and the eluate was purified by column chromatography under N_2 as described previously (Hirota & Itano, 1983). A better incorporation yield of ^{18}O was obtained in a second preparation with minor modifications in mixing procedures. Phenylhydrazine hydrochloride, dissolved in 10 ml of water, was injected into the octaethylhaemin solution under a stream of N_2 through a needle inserted through the rubber stopper of the reaction flask. The latter was not evacuated as in the first preparation, but contained N_2 at atmospheric pressure, when it and the container of $^{18,18}O_2$ were connected.

Reaction of octaethylverdohaemochrome with O_2 and phenylhydrazine

To a solution of 15.7 mg (0.02 mmol) of octaethylverdohaemochrome in 50 ml of tetrahydrofuran in a 100 ml flask, 28.9 mg (0.2 mmol) of phenylhydrazine hydrochloride in ethanol was injected under a stream of N_2 through a rubber stopper. After stoppage of the flow of N_2 , 100 ml (4.46 mmol) of O_2 was added, and the solution was stirred for 1 day as its colour gradually changed from green to blue. The solution was then evaporated under vacuum, and octaethylbiliverdin was obtained from the residue as described previously (Hirota & Itano, 1983). The ratio of intensities of molecular-ion peaks was determined from the mass spectrum of the product.

Reaction of octaethylverdohaemochrome with H_2O_2 and phenylhydrazine

Octaethylverdohaemochrome (15.7 mg, 0.02 mmol) was dissolved in 50 ml of tetrahydrofuran under argon. The colour of the solution changed from green to dark brown as soon as H_2O_2 (0.03 mmol as aqueous 30% solution) was injected through the rubber stopper sealing the reaction vessel. When phenylhydrazine hydrochloride (4.4 mg, 0.03 mmol), dissolved in 2 ml of ethanol,

was added 10 min later, the colour of the solution changed rapidly to dark blue. After 10 more min, the solution was evaporated to dryness under vacuum, a process that required about 20 min because of considerable foaming. Octaethylbiliverdin was obtained from the residue as described previously (Hirota & Itano, 1983).

Reaction of octaethylverdohaemochrome with H_2O in tetrahydrofuran

Octaethylverdohaemochrome (15.7 mg, 0.02 mmol) and H_2O (3.6 mg, 0.2 mmol) in 50 ml of tetrahydrofuran were stirred under argon for 1 day. The solution remained dark green, and some octaethylverdohaemochrome was observed by t.l.c. at the end of this period. The solution was evaporated, and octaethylbiliverdin was obtained as described previously (Hirota & Itano, 1983). The experiment was repeated without the addition of H_2O to the tetrahydrofuran. Change in colour did not occur, and some octaethylverdohaemochrome was seen by t.l.c. after 1 day.

Results

Octaethyl[^{18}O]verdohaemochrome

The first preparation yielded 58 mg (16.6%) of product: m/z (rel. intensity) 595 (12.5), 594 (56.9), 593 (M^+ , 100), 592 (16.7), 591 (25); 86.9 atom% incorporation of ^{18}O . The second preparation yielded 51 mg (14.6%) of product: m/z (rel. intensity) 595 (16.9), 594 (58.9), 593 (M^+ , 100), 592 (11.0), 591 (15.3); 95.3 atom% incorporation of ^{18}O (Found: C, 68.4; H, 6.8; N, 10.7; calc. for $C_{45}H_{53}N_6^{17,91}OFeCl$: C, 68.7; H, 6.7; N, 10.7).

Conversion of octaethylverdohaemochrome into octaethylbiliverdin with O_2 and phenylhydrazine

Octaethyl[^{16}O]verdohaemochrome and $^{16,16}O_2$ yielded 4.1 mg (37%) of octaethyl[$^{16}O,^{16}O$]biliverdin: m/z (rel. intensity) 556 (10.1), 555 (42.4), 554 (M^+ , 100), 553 (5.8), 552 (1.3); 100 atom% incorporation of ^{16}O . Octaethyl[^{18}O]verdohaemochrome (86.9 atom% ^{18}O) and $^{16,16}O_2$ yielded 43 mg (39%) of product: m/z (rel. intensity) 558 (14.0), 557 (43.0), 556 (M^+ , 100), 555 (9.8), 554 (11.6); 100 atom% incorporation of ^{16}O . Octaethyl[^{18}O]verdohaemochrome (95.3 atom% ^{18}O) and $^{18,18}O_2$ (99%) yielded 3.5 mg (32%) of product: m/z (rel. intensity) 560 (10.0), 559 (43.3), 558 (M^+ , 100), 557 (17.7), 556 (20); 89.0 atom% incorporation of ^{18}O . Previous result: octaethyl[^{16}O]verdohaemochrome and $^{18,18}O_2$ yielded 2.3 mg (41%) of octaethyl[$^{16}O,^{18}O$]biliverdin with 81.7% incorporation of ^{18}O (Hirota & Itano, 1983).

Reaction of octaethylverdohaemochrome with H₂O₂ and phenylhydrazine

Octaethylbiliverdin (3.9 mg, 35%) was obtained.

Hydrolysis of octaethylverdohaemochrome

In 50 ml of tetrahydrofuran with 3.6 mg (0.2 mmol) of water, 1.7 mg (15%) of octaethylbiliverdin was obtained. In 50 ml of tetrahydrofuran without added water, 1.5 mg (13.5%) of octaethylbiliverdin was obtained.

Discussion

The process in which an oxygen atom from each of two O₂ molecules is incorporated into a molecule of biliverdin is known as the 'two-molecule mechanism' (Brown & King, 1975; Jackson *et al.*, 1978). Structures have been proposed for hypothetical molecules as possible intermediates that would circumvent the participation of verdohaem as well as accommodate the two-molecule mechanism in biliverdin formation (Clezy, 1978; Rajananda & Brown, 1983). Such compounds, which contain the elements of two O₂ molecules and whose conversion into biliverdin would result in the simultaneous incorporation of an oxygen atom from each of the O₂ molecules, have not been observed. Since verdohaem is invariably produced in the coupled oxidation of haem and haemoproteins, ring closure of the iron complex of biliverdin has been postulated to explain its formation (Rajananda & Brown, 1983).

In previous studies, we prepared verdohaemochrome IX α by the coupled oxidation of myoglobin and ascorbate (Saito & Itano, 1982) and octaethylverdohaemochrome by the coupled oxidation of octaethylhaemin and phenylhydrazine (Hirota & Itano, 1983). Using ¹⁸O₂, we converted these verdohaemochromes into biliverdins that contained an atom of ¹⁸O. We showed by spectrophotometry that H₂O₂ reacts rapidly with verdohaem to form an intermediate that is reduced to biliverdin by ascorbate or phenylhydrazine, and we proposed that incorporation of an oxygen atom from O₂ into biliverdin takes place via H₂O₂ formed by the reaction of O₂ and a reductant.

In the reaction of phenylhydrazine with O₂, the formation of H₂O₂ proceeds much more rapidly than the reduction of H₂O₂ to H₂O (Stroh & Ebert, 1964); nevertheless hydrolysis of octaethylverdohaemochrome by H₂¹⁸O produced in the auto-oxidation of phenylhydrazine must be considered as a possible alternative mechanism for the incorporation of an oxygen atom from ¹⁸O₂ into octaethylbiliverdin. Octaethylverdohaemochrome was dissolved in tetrahydrofuran to which the maximum amount of water that could have been

produced in our coupled oxidation experiments, a 10-fold excess, had been added. Some octaethylverdohaemochrome remained after 1 day of stirring under argon, and the yield of octaethylbiliverdin was less than half that from coupled oxidation. In contrast, the successive treatment for only 10 min each with H₂O₂ and phenylhydrazine hydrochloride in 50% excess resulted in about the same yield as did coupled oxidation. The reaction of H₂O₂ with octaethylverdohaemochrome to form a brown intermediate was extremely rapid. Thus, not only is the rate of formation of H₂O₂ from O₂ and phenylhydrazine greater than that of H₂O, but also the rate of reaction of H₂O₂ with octaethylverdohaemochrome is greater than that of H₂O. These experiments confirmed that H₂O₂ must be the primary intermediate through which an oxygen atom from O₂ is incorporated into biliverdin in coupled oxidation, though hydrolysis can occur as a side reaction.

The yield of octaethylbiliverdin when octaethylverdohaemochrome was stirred in tetrahydrofuran without water added was nearly as great as that with water added. This result indicated that water must have re-entered our redistilled solvent from the atmosphere and possibly also from the apparatus in the course of transfers and manipulations. Hydrolysis by extraneous H₂¹⁶O therefore accounts at least in part for the less than quantitative incorporation of ¹⁸O into octaethyl[¹⁶O, ¹⁸O]biliverdin (Hirota & Itano, 1983) and octaethyl[¹⁸O, ¹⁸O]biliverdin. In addition, some ¹⁶O₂ could have entered the reaction system from the atmosphere during the setting up and the 1-day span of the experiments. The incorporation yield of ¹⁸O into octaethyl[¹⁸O]verdohaemochrome was about 90 atom% in two preparations. Here the only possible source of ¹⁶O was leakage of atmospheric O₂, because the oxygen atom of verdohaemochrome can only be derived from O₂ (Foulkes *et al.*, 1951).

A reductant such as ascorbate or phenylhydrazine thus takes part in two reactions in the conversion of verdohaem into biliverdin by coupled oxidation, the reduction of O₂ to H₂O₂ and the reduction of the verdohaem-H₂O₂ intermediate to biliverdin. Formation and reduction of this intermediate is a non-hydrolytic process for the conversion of verdohaem into biliverdin. The existence of this route invalidates the premise on which the exclusion of verdohaem as an intermediate in haem degradation has been based, that this conversion can only take place by hydrolysis. The hydrolytic and non-hydrolytic pathways are compared in Scheme 1.

The present work has demonstrated the successive incorporation of the two oxygen atoms of biliverdin in separate steps, namely the formation

Scheme 1. Hydrolytic and non-hydrolytic pathways in the formation of biliverdin from verdohaem

Octaethylverdohaem from the coupled oxidation with O_2 of octaethylhaem and phenylhydrazine contained an oxygen atom from O_2 . Octaethylbiliverdin formed by the hydrolysis of octaethylverdohaem contains an oxygen atom each from O_2 and H_2O . On the other hand, octaethylbiliverdin produced by reacting octaethylverdohaem with phenylhydrazine and O_2 incorporated a second oxygen atom from O_2 . This atom is postulated to be added to octaethylverdohaem via H_2O_2 formed by the reduction of O_2 by phenylhydrazine. Reduction of the verdohaem- H_2O_2 intermediate by phenylhydrazine results in the product of a two-molecule mechanism, namely a biliverdin molecule in which the two oxygen atoms are derived from different O_2 molecules.

of verdohaemochrome and the conversion of verdohaemochrome into biliverdin. This process constitutes a two-molecule mechanism of coupled oxidation of haem in which verdohaem is an intermediate and biliverdin is the end product. The mechanism is consistent with the observation (Foulkes *et al.*, 1951) that verdohaem formation precedes biliverdin formation. Formation of octaethylverdohaem from octaethylbiliverdin by ring closure, a dehydration, is unlikely to occur in the solvent, pyridine/water, in which we prepared octaethylverdohaemochrome.

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