Mechanism of Action of Heme Oxygenase

A STUDY OF HEME DEGRADATION TO BILE PIGMENT BY $^{18O}$ LABELING*

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The formation of bile pigment from heme by a reconstituted heme oxygenase system containing purified bovine spleen heme oxygenase, NADPH-cytochrome P-450 reductase, and biliverdin reductase was studied under an atmosphere containing $^{18O}_2$. The product, bilirubin, was isolated and subjected to mass spectrometry, which revealed incorporation of $^{18O}$ consistent with a two-molecule mechanism, whereby the product bile pigment contains oxygen atoms derived from two different oxygen molecules.

Heme metabolism to bile pigments represents the main pathway for removal of heme during turnover of hemoproteins such as hemoglobin and cytochrome P-450 (1–3). The same process occurs extensively in plant and algal systems, where the reaction is part of the biosynthetic pathway of the functional phycobiliproteins (3). In both systems, the immediate bile pigment product formed is biliverdin IXα (see Fig. 1). In both cases also, further processing of biliverdin requires reduction. In mammalian systems, which have been more extensively studied largely because of their medical significance, the reduction occurs at the central methylene bridge to yield bilirubin, which is excreted in the bile, usually in the conjugated form (Fig. 1).

There is good evidence that endogenous heme clevage is catalyzed by the microsomal enzyme, heme oxygenase (EC 1.14.99.3) (4) which requires molecular oxygen and NADPH. The latter coenzyme supplies electrons via the microsomal electron transport system utilizing the enzyme NADPH-cytochrome P-450 reductase (EC 1.6.4.2) (5). Biliverdin is then converted to bilirubin by the action of the NADPH-requiring enzyme, biliverdin reductase (EC 1.3.1.24) (6). Heme oxygenase activity was initially identified in the microsomal fraction of cells from spleen, liver, and other tissues (4). Using purified heme oxygenase (7) and NADPH-cytochrome P-450 reductase (8), it has been possible to carry out mechanistic studies on the reconstituted enzyme system. Initially, ferric heme binds to heme oxygenase to form a heme:heme oxygenase complex. The ferric heme is then reduced to the ferrous state by NADPH-cytochrome P-450 reductase, and dioxygen is bound (9). Reduction of the oxygenated heme:heme oxygenase complex by NADPH-cytochrome P-450 reductase yields a heme-bound activated oxygen species which initiates heme degradation by hydroxylation of a methene bridge carbon atom to form hydroxyheme. Incorporation of two further atoms of oxygen from molecular oxygen results in ring opening of the hydroxyheme to yield biliverdin and iron with the methene carbon atom being liberated as carbon monoxide (4). In recent studies with the reconstituted heme oxygenase system, less than half of the total heme degraded was converted to bile pigment and carbon monoxide (10). These low yields may be due to the formation of dipyrrolic pentadepsopentapyroles by the action of free hydrogen peroxide which has been formed on and has subsequently dissociated from the heme iron (10). Addition of catalase to this system resulted in near-stoichiometric recovery of biliverdin and carbon monoxide (10).

The role of oxygen in the enzymatic mechanism of heme degradation to bile pigment has been previously studied in a number of systems. Using a rat spleen postmitochondrial preparation, Tenhunen et al. (11) showed that the two lactam oxygen atoms inserted into biliverdin (Fig. 1) were each derived from molecular oxygen. These studies were confirmed by Brown and co-workers (12–14) who developed an approach to determine whether the lactam oxygen atoms incorporated into bile pigment during heme degradation were derived from a single oxygen molecule (one-molecule mechanism) or two oxygen molecules (two-molecule mechanism). This approach has been applied to study bile pigment formation in living rats (12), in a postmitochondrial rat spleen supernatant (13), and in photosynthetic algae (14). In each case, a two-molecule mechanism was observed. However, the mechanism has not yet been studied in this way using the purified and reconstituted enzyme system. We now report such studies both in the presence and absence of catalase confirming the two-molecule mechanism in the reconstituted heme oxygenase system.

EXPERIMENTAL PROCEDURES

Materials—$^{18O}_2$ (99% pure) was obtained from British Oxygen Company. Hemin was purchased from Koch-Light and catalase (33,900 Sigma units/mg protein; stock number C-100, from bovine liver) was from Sigma. All other reagents were from BDH Ltd. or Fisher Scientific and were of the highest grade available.

Enzyme Preparations—NADPH-cytochrome P-450 reductase (EC 1.6.4.2) was purified from pig liver by detergent solubilization, ion-exchange chromatography on DEAE-cellulose, and affinity chromatography on ADP-Sepharose (8). Enzyme activity was assayed by the reduction of cytochrome c in 0.05 M potassium phosphate buffer, pH 7.0.

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7.7, at 25 °C (15). Under these conditions the reductase exhibited a specific activity of 34 μmol of cytochrome c reduced per mg/min.

Heme oxygenase was purified from bovine spleen by detergent solubilization, ammonium sulfate precipitation, ion-exchange chromatography, and gel filtration essentially as described by Yoshida and Kikuchi (7). Enzyme activity was assayed at 37 °C in the presence of NADPH-cytochrome P-450 reductase in 0.1 M potassium phosphate buffer, pH 7.4, by following the increase in absorbance at 458 nm due to formation of bilirubin, in the presence of biliverdin reductase. The reaction was initiated by addition of NADPH to a final concentration of 1 mM. The heme oxygenase preparation exhibited a specific activity of 10 nmol of bilirubin formed per mg/min.

Biliverdin reductase (EC 1.3.1.24) was partially purified to step III from bovine kidney as described by Tenhunen et al. (6).

Enzymic Heme Degradation in a \(^{18} \text{O}\) Atmosphere—The incubation mixture in a total volume of 20 ml of 0.1 M potassium phosphate, pH 7.4, consisted of 0.55 mg of purified heme oxygenase, 0.3 mg of purified NADPH-cytochrome P-450 reductase, 6.0 mg of partially purified biliverdin reductase, and 34 μM methemalbumin. Where applicable catalase was added to a final concentration of 1.6 × 10⁻³ units/ml. Incubation mixtures were placed in a sealed flask which was evacuated to minimize feasible pressure, taking care to avoid excessive foaming of the enzyme mixture. This precided rigorous degassing normally employed in \(^{18} \text{O}\) experiments. Following introduction of an artificial air mixture containing a known enrichment of \(^{18} \text{O}\), the reaction was initiated by addition of NADPH (to a final concentration of 1 mM) from a side arm. Incubations proceeded for 30 min at 37 °C with gentle shaking in the dark.

Extraction and TLC of Bilirubin—Product bilirubin was extracted by a modification of the method of Blanckaert et al. (18). After acidifying to pH 2.5 by addition of 15 ml of glycine HCl buffer, pH 1.8, and addition of 8 g of NaCl, bilirubin was extracted into chloroform:ethanol (1:1, 2 × 20 ml), and solvent was removed under a stream of nitrogen. Bilirubin was purified by thin-layer chromatography on plates coated with Silica Gel GF₃₄ which fluoresces under UV light and developed in 1% acetic acid in chloroform (17).

Mass Spectrometry—Samples of the gas phase removed before and after incubation were analyzed on an AE1 MS 10 mass spectrometer. Samples of bilirubin were analyzed underivatized by mass spectrometry on an AE1 MS 9 mass spectrometer. All results were obtained by averaging at least five repetitive scans.

**RESULTS**

**Extraction and Purification of Bilirubin IXα for Mass Spectrometry**—Yields of bilirubin IXα of the order 100–200 μg were achieved by incubation of heme oxygenase with the reconstituted heme oxygenase system both in the presence and absence of catalase. The procedure used for extraction of the bilirubin achieved virtually quantitative recovery of the bile pigment as assessed by the complete decoloration of the aqueous phase after the second chloroform/ethanol extraction. Thin-layer chromatography purification of the bilirubin IXα was, however, necessary before mass spectroscopic analysis due to the presence of impurities, largely unreacted heme in the final organic extract. It was also essential to demonstrate the absence of the IIIα and XIIIα isomers which could, in principle, be formed by acid-mediated dipyrrole exchange reactions (17) during the extraction procedure. Such exchange would have made discrimination between a one-molecule and two-molecule mechanism impossible. Heme remained very close to the origin in the TLC system used and was easily removed from the bilirubin, and the IIIα and XIIIα isomers of bilirubin were present only as very minor traces. TLC of the extract obtained from incubation in the absence of catalase also revealed the presence of a broad colorless band (R₂ 0.06–0.18) visible on the fluorescent plate under UV light. This may be related to recent observations that the yield of bile pigment and carbon monoxide obtained from degradation of heme by the reconstituted heme oxygenase system is significantly less than the amount of heme degraded (10), possibly due to formation of dipyrroles by the action of free peroxide (18, 19). These conclusions are supported by the results of Experiment 2, in which case a greatly reduced amount of the colorless product was observed when catalase was added to the incubation.

**\(^{18} \text{O}\) Enrichment in Gas Phase of Incubation**—The enrichment of \(^{18} \text{O}\) in the gas phase for incubation was determined approximately by calculation from the volumes of \(^{18} \text{O}_₂\) and \(^{16} \text{O}_₂\) used to obtain the desired gas mixture and also by mass spectrometric analysis on samples of the gas phase obtained before and after incubation. The calculated \(^{18} \text{O}_₂\) enrichment in the gas phase for experiment 1 was 50%. The mass spectrometric analyses of the pre- and postincubation gas phases both yielded \(^{18} \text{O}_₂\) enrichment of 52%. Similarly for incubation in the presence of catalase (Experiment 2), the calculated enrichment was 33% with an observed value of 33% for mass spectrometric analysis of both the pre- and postincubation gas phases. The agreement between the values measured before and after reaction demonstrates that no leakage had occurred during the incubation period. The possibility of scrambling in \(^{18} \text{O}_₂\) and \(^{16} \text{O}_₂\) to produce \(^{18} \text{O}_₂\) was precluded, since no peak at m/z 34 was observed either before or after the reaction.

**Mass Spectrometry of Bilirubin IXα**—Good mass spectra were obtained for both bilirubin IXα samples which corresponded well with the mass spectrum obtained for authentic unlabeled bilirubin IXα (Fig. 2), characterized by a strong molecular ion at m/z 584. The spectra of the bilirubin from Experiments 1 and 2 which show strong incorporation of \(^{18} \text{O}\) to yield substantial peaks at m/z 586 and m/z 588 is qualitative evidence in favor of the two-molecule mechanism, since the one-molecule mechanism would predict incorporation only at m/z 588 (see below).

The data may be analyzed quantitatively by measurement of peak heights and correction for naturally abundant isotopes as previously described (12). Following these procedures, the values obtained for the relative abundance of species at m/z 584, m/z 586, and m/z 588 are shown in Table 1. For each experiment, the total observed incorporation of label is readily evaluated (derived as half the per cent abundance of species at m/z 586 plus the per cent abundance of species at m/z 588 (20)). For Experiments 1 and 2, the observed incorporations were 45.2 and 28.1%, respectively. These values are close to but significantly less than the values of enrichment in the gas phase obtained by direct calculation and by mass spectrometric analysis (see above). It seems possible that these small differences are due to traces of residual \(^{16} \text{O}_₂\) in the incubation mixture, due to the inadvisability of carrying out exhaustive degassing because of the danger of enzyme denaturation.

Predicted incorporation for both the one-molecule mechanism and the two-molecule mechanism for 45.2 and 28.1% enrichment is shown in Table 1 (for details of derivation see Ref. 12). This shows that there is good quantitative agreement between the observed results and those predicted for the two-molecule mechanism for both Experiments 1 and 2.
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For each experiment, the observed incorporations were determined by measurement of the appropriate peaks in the mass spectrum and correction for naturally abundant isotopes as previously described (Ref. 12). The values for Experiments 1 and 2 represent the mean of 6 and 8 mass spectral measurements, respectively. The total incorporation was derived as the sum of the per cent incorporation at m/z 588 and half the per cent incorporation of m/z 586 (Ref. 20). The predictions for the two mechanisms are based on the total incorporation.

<table>
<thead>
<tr>
<th>Mechanism</th>
<th>m/z 586</th>
<th>m/z 588</th>
<th>Total Incorporation</th>
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<tbody>
<tr>
<td>Observed</td>
<td>30.7</td>
<td>48.4</td>
<td>21.0 45.2</td>
</tr>
<tr>
<td>Predicted, one-molecule</td>
<td>54.8</td>
<td>0</td>
<td>45.2</td>
</tr>
<tr>
<td>Predicted, two-molecule</td>
<td>50.0</td>
<td>49.5</td>
<td>20.4</td>
</tr>
</tbody>
</table>

TABLE I

$^{18}$O incorporation of bilirubin formed by heme oxygenase under mixtures of $^{18}$O$_{2}$ and $^{16}$O$_{2}$.

DISCUSSION

From these experiments it is clear that heme degradation by the reconstituted heme oxygenase system proceeds by a two-molecule mechanism in both the presence and absence of catalase. It has recently been shown that catalase must be added to the reconstituted heme oxygenase system to achieve stoichiometric recovery of bile pigment and carbon monoxide from degraded heme (10). In the absence of catalase or an intact membrane structure as found in microsomal preparations, it appears that an uncoupling of the reaction occurs such that hydrogen peroxide, formed on the heme by the action of NADPH-cytochrome P-450 reductase, can dissociate from the heme (10). The free hydrogen peroxide may then degrade heme by a pathway not involving formation of bile pigment and carbon monoxide in a similar manner to that recently observed for heme degradation catalyzed by purified NADPH-cytochrome P-450 reductase alone (21). The present results clearly show that the formation of hydrogen peroxide in the reconstituted enzyme system does not influence the mechanism of oxygen insertion in bile pigment formation by heme oxygenase. These results are consistent with previous studies using living rats (12) and rat spleen microsome-rich supernatant (13) and lend further support to the likelihood that heme oxygenase is the enzyme primarily responsible for bile pigment formation in animals.

The precise molecular pathway by which a two-molecule mechanism may be achieved is not yet clear. Recently, however, evidence has been presented for the existence of a contact point absorbing at 688 nm, which may be intermediate between heme and biliverdin (22). Formation of this compound apparently involves elimination of carbon monoxide and insertion of only one oxygen atom from molecular oxygen. Further reaction of this compound with molecular oxygen results in biliverdin formation. Such a scheme would clearly be consistent with the two-molecule mechanism. A similar scheme involving verdoheme has recently been postulated (23). However, it also remains possible that heme degradation could proceed via three independent steps involving attack by molecular oxygen, followed by elimination of carbon monoxide and formation of iron-biliverdin (3).

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REFERENCES

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