SEQUENCE OF THE REACTION OF HEME CATABOLISM CATALYZED BY THE MICROSOMAL HEME OXYGENASE SYSTEM

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Received 4 October 1974

1. Introduction

The microsomal heme oxygenase is operational in various tissues such as spleen, liver, kidney and macrophages and is thought to play an essential role in the physiological heme catabolism [1–7]. The microsomal heme oxygenase has been assumed to involve a cytochrome P-450 as a terminal oxidase [6], though the conclusive evidence for it has been lacking. However, recently we have obtained evidence that the heme oxygenase of pig spleen microsomes may not involve any type of cytochrome P-450 [8]. As an alternate possibility to account for the heme oxygenase activity in the spleen microsomes, we proposed that the spleen microsomes may contain a protein which readily binds with heme to form a heme–protein complex which behaves as an active enzyme, and then heme on the enzyme protein is decomposed by its own oxidative activity [8]. If this is the case, the sequence of the reaction of heme catabolism catalyzed by the microsomal heme oxygenase system may be quite similar to that for the non-enzymic heme decomposition by the coupled oxidation of myoglobin with ascorbic acid [9–13] which has been assumed to proceed in the following sequence [11,13]: protoheme → hydroxyheme → biliverdin–iron complex or verdoheme → biliverdin).

Considering these circumstances, we attempted an investigation of the reaction sequence of heme catabolism in the reconstituted microsomal heme oxygenase system. The results obtained indicated striking similarities with respect to the sequence of heme decomposition in the heme oxygenase system and in the non-enzymic system with myoglobin and ascorbic acid.

2. Materials and methods

Heme oxygenase and NADPH-cytochrome *c* reductase were partially purified from microsomes of pig spleen and pig liver, respectively, by the procedures described in the previous paper [8]. The heme oxygenase preparation used for the present study could form approx. 70 nmol of bilirubin from hemin per 5 min per mg of protein under te same reaction conditions described in the previous paper [8]. The NADPH-cytochrome *c* reductase preparation used catalyzed the reduction of approx. 2 μmol of cytochrome *c* per min per mg protein at 25°C. Methods of preparation of biliverdin reductase, as well as enzyme activity, used in the present study were the same as those in the preceding paper [13]. Methods of preparation of chlorhemin, biliverdin and of the protein determination were also as described in the preceding paper [13].

Reaction conditions of individual experiments are described in the legends to figures. A Hitachi Model 323 recording spectrophotometer was used for the spectral examination.

3. Results and discussion

The time course of green pigment formation by the heme oxygenase reaction is shown in fig. 1. In this experiment, the reaction was run employing a relatively large amount of heme oxygenase and without addition of biliverdin reductase and also at relatively low temperature (15°C), in the hope that these may help us in detecting some intermediate(s) of the reaction. The reaction was followed by means of difference

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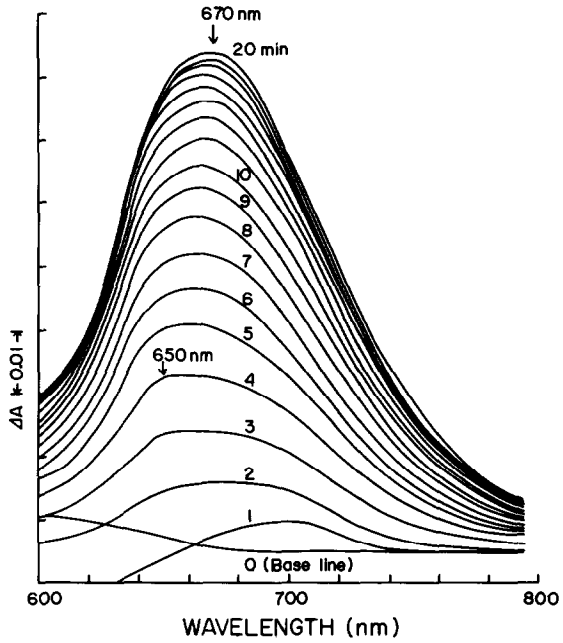

Fig. 1. Green pigment formation by the heme oxygenase reaction. The reaction was carried out at 15°C in a cuvette with 1 cm light path containing in a final volume of 1 ml, 100 μmol of potassium phosphate buffer (pH 7.5), 15 nmol of hemin, 250 μg as protein of partially purified heme oxygenase, 125 μg as protein of partially purified NADPH-cytochrome *c* reductase and 0.4 μmol of NADPH. The reference cuvette contained the components listed above except that NADPH was replaced by water. The reaction was started by the addition of NADPH and the difference spectra were recorded successively at 1-min intervals. The numbers in the figure indicate the time lapse of incubation. Some spectra recorded in the later stage were not shown in the figure.

spectra. In the early stage of the reaction the absorption at about 650 nm increased first, then the absorption peak gradually shifted to longer wavelength side, and in the later stage of the reaction, only the absorption showing its maximum at 670 nm was apparent; the absorption peak at 670 nm corresponds to the absorption maximum of biliverdin at neutral pH [14]. The origin of the absorption with an apparent peak at 650 nm is unclear at present, though hydroxyheme seems to be a likely candidate. On the other hand, when the reaction was carried out in the presence of biliverdin reductase added, the absorption in the longer wavelength side was only trace and instead, a large absorption showing its peak at 465 nm appeared indicating the rapid conversion of biliverdin to bilirubin during the course of the reaction (fig.2). These data indicate that the major final product of the heme oxygenase reaction is biliverdin. The data in fig.2 also indicate that biliverdin formed in the heme oxygenase reaction may be almost exclusively the α-isomer in view of the fact that biliverdin reductase is highly specific for the α-isomer of biliverdin [15].

Fig.3 represents the results of another series of time study of the heme catabolism in the absence of biliverdin reductase. The reaction mixtures were treated with NaOH, $Na_2S_2O_4$ and CO at indicated times of incubation. Protoheme diminished along with the duration of incubation and in turn, there arose two absorption peaks in the spectrum, the one at 640–650 nm and the other at 760–800 nm. As discussed in the preceding paper [13], the absorption peak around 640–650 nm may be ascribed to hydroxyheme (choleheme) and the absorption showing its maximum at 760–800 nm would correspond to biliverdin.

However, verdoheme has also been shown to be converted to biliverdin when reduced by $Na_2S_2O_4$ in alkaline solution [13]. Therefore, to see whether the reaction mixture in the heme oxygenase system contained verdoheme or some compound of the same oxidation level (a biliverdin–iron chelate) as an intermediate for the formation of biliverdin, we extracted the pigments with pyridine from the reaction mixture in the middle of incubation. The reaction conditions in this experiment were same as for fig.1, except that biliverdin reductase was also added to the reaction mixture to minimize the accumulation of biliverdin. As shown in fig.4, the pyridine extract exhibited an absorption peak at 670 nm which corresponds to the absorption maximum of authentic biliverdin in pyridine. However, when $Na_2S_2O_4$ was added to the pyridine extract, there appeared a distinct absorption peak at about 656 nm which corresponds to the absorption peak of pyridine verdohemochrome, then the absorption diminished gradually, in harmony with the known characteristics of pyridine verdohemochrome [16,17]. We have observed that the biliverdin solution in 50% pyridine–water loses its characteristic absorption promptly when added with $Na_2S_2O_4$. We also ascertained in an independent experiment that no verdoheme was formed when hemin was incubated with the heme oxygenase system in 50% pyridine. Therefore it is

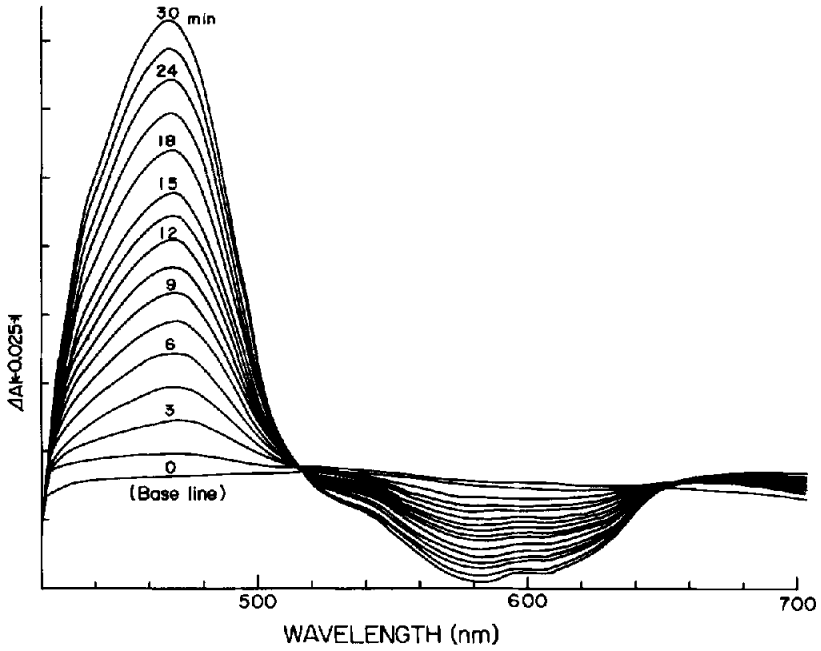

Fig. 2. Bilirubin formation by the heme oxygenase reaction in the presence of biliverdin reductase added. The complete reaction mixture contained, in a final volume of 2 ml, 200 μmol of potassium phosphate buffer (pH 7.5), 30 nmol of hemin, 60 μg as protein of partially purified heme oxygenase, 250 μg as protein of partially purified NADPH-cytochrome *c* reductase, 200 μg as protein of partially purified biliverdin reductase, and 0.8 μmol of NADPH. NADPH was not added to the reaction mixture in the reference cuvette. Other experimental conditions were same as those in fig.1, except that incubation was carried out at 25°C. The spectra were recorded successively at 1.5-min intervals. The numbers in the figure indicate the time lapse of incubation. Some spectra recorded in the later stage were not shown in the figure.

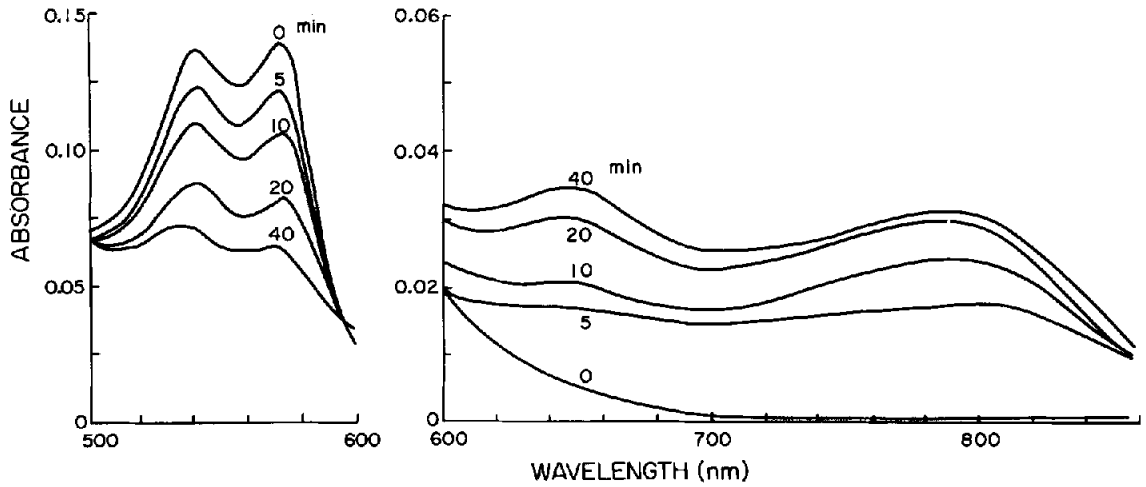

Fig. 3. Absorption spectra of the reaction mixtures of the heme oxygenase reaction which were treated with NaOH, $Na_2S_2O_4$ and CO at various times of incubation. The composition of the reaction mixture was similar to that for fig.1, except that 40 μg as protein of heme oxygenase and 100 μg as protein of NADPH-cytochrome *c* reductase were employed. Reactions were carried out at 25°C in test tubes. At indicated times of incubation the reaction mixtures were added with 0.3 ml of 1.5 N NaOH solution, a small amount of powdered $Na_2S_2O_4$, then bubbled with CO.

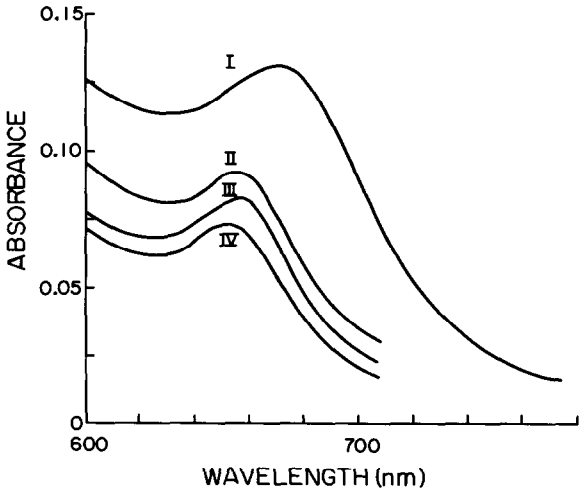

Fig. 4. Absorption spectrum of the pyridine extract of the reaction mixture which had been incubated in the presence of biliverdin reductase. The reaction mixture with the components as those for fig.1 and containing additionally 100 μg as protein of biliverdin reductase was incubated in a test tube for 15 min at 15°C. Then an equal volume of pyridine was added to the reaction mixture and the clear pyridine extract was obtained by centrifugation. Curve I represents the absorption spectrum of the pyridine extract as obtained. Curves II, III and IV represent the absorption spectra measured at 4, 6, and 10 min after the addition of $Na_2S_2O_4$, respectively.

apparent that a small amount of verdoheme (or biliverdin–iron chelate) was present in the reaction mixture of the heme oxygenase system.

With respect to the chemical nature of the immediate precursor of biliverdin in the reaction of the heme oxygenase system, it is worth-noting that the biliverdin formed by the heme oxygenase reaction has been shown to contain two lactam oxygen atoms derived from molecular oxygen and not from water [6] and this was taken as indicating that verdoheme may not be an obligatory intermediate in the heme oxygenase reaction. If verdoheme is an obligatory intermediate for the formation of biliverdin, one oxygen atom of water must be introduced to biliverdin during hydrolysis of verdoheme; this appeared to be the case with the biliverdin obtained by the acid treatment of the intermediate(s) of the coupled oxidation of hemoglobin according to Anan and Mason [18], although it has not been clarified whether the protein-bound precursor of biliverdin in this reaction system is verdoheme or the biliverdin–iron complex. In our reconstituted heme oxygenase system, the amount of heme oxygenase used for the reaction was very small as compared with the amount of hemin added (in terms of molar ratio, assuming the apparent molecular size of the heme oxygenase preparation as 200 000 [8]), and the reaction yielded at a high rate and almost exclusively biliverdin as the final reaction product. These situations seem to support the view that the immediate precursor of biliverdin in the heme oxygenase reaction is probably the biliverdin–iron chelate rather than verdoheme. Elucidation of the exact chemical nature of the reaction products of heme catabolism awaits further investigations.

Based on the experimental findings so far available, heme catabolism in the heme oxygenase system seems to proceed as depicted in fig.5. Namely, protoheme binds to the heme oxygenase protein to form a transient hemeprotein, then the ferric heme is reduced to ferrous heme by the function of NADPH-cytochrome *c* reductase. Ferrous heme readily reacts with the molecular oxygen and the activated oxygen species would attack the α-methene bridge to form hydroxyheme. Hydroxyheme would be further oxidized to yield biliverdin–iron chelate, being accompanied by the concomitant CO evolution [6,19]. The biliverdin–iron chelate would be easily converted to biliverdin by releasing iron, though the mechanism of iron release is not clear as yet.

According to this scheme, it is predictable that heme

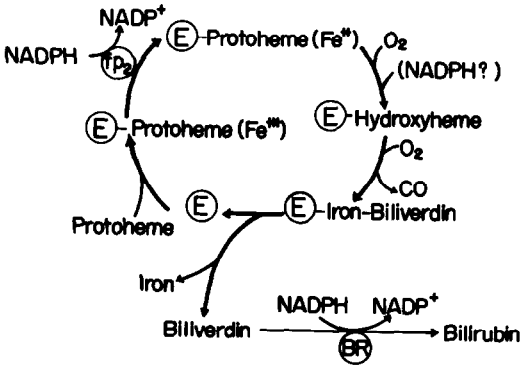

Fig. 5. Possible reaction sequence of heme catabolism in the heme oxygenase reaction. 'E', 'fp_2' and 'BR' represent heme oxygenase, NADPH-cytochrome *c* reductase and biliverdin reductase, respectively.

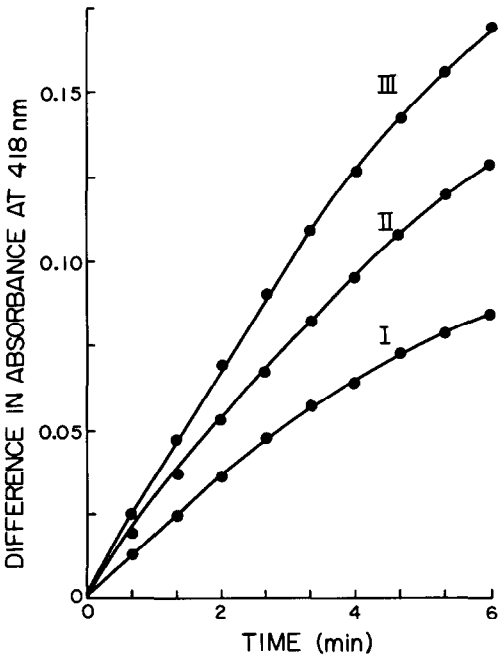

Fig. 6. Enhancement by heme oxygenase of the heme reduction by NADPH-cytochrome *c* reductase. Reactions were carried out in a Thunberg type cuvette filled with CO gas at 25°C. The complete reaction mixture contained, in a final volume of 2 ml, 200 μmol of potassium phosphate buffer (pH 7.5), 30 nmol of hemin, varied amounts of heme oxygenase (0, 80 and 160 μg as protein for Curves I, II and III, respectively), 160 μg as protein of NADPH-cytochrome *c* reductase, and 0.8 μmol of NADPH. NADPH was not added to the reaction mixture in the refference cuvette. Reactions were started by the addition of NADPH and the difference spectra were recorded successively at 20-sec intervals. In the figure the differences in absorption at 418 nm were plotted.

oxygenase might help the reduction of heme by NADPH-cytochrome *c* reductase. In fact, heme oxygenase was found to significantly enhance the reduction of heme by NADPH-cytochrome *c* reductase as shown in fig.6. On the other hand, although hemin can be reduced at a considerably high rate by incubation with NADPH-cytochrome *c* reductase alone, degradation of heme does not occur unless heme oxygenase is further added to the reaction mixture [8]. Thus, it is clear that heme oxygenase positively participates in the reaction of heme degradation.

In conclusion, heme oxygenase is a microsomal protein which binds with heme and provides a suitable environment for the autocatalytic oxidation of heme moiety, so that the heme catabolism could proceed at a much higher rate and in much a specific way than in other artificial non-enzymic reactions. According to our hypothesis, the iron of heme must be indispensable for the reaction. In consistent with this view, Schacter and Waterman [20] recently reported that the microsomal heme oxygenase activity was specific for iron–porphyrin among various metalloporphyrins tested.

Acknowledgement

We thank Miss S. Takahashi for her cooperation in some of the experiments. This work was aided in parts by Grants from the Ministry of Education of Japan (No. 978040 and 948024).

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