SPECIFIC REQUIREMENT OF NADPH-CYTOCHROME *c* REDUCTASE FOR THE MICROSOMAL HEME OXYGENASE REACTION YIELDING BILIVERDIN IXα

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1. Introduction

Heme oxygenase is a microsomal protein which catalyzes the degradation of protoheme to yield biliverdin IXα. It was demonstrated [1] that NADPH-cytochrome *c* reductase (EC 1.6.2.3; abbreviated as fp_T here) should be involved in the microsomal heme oxygenase reaction by immunochemical techniques using an antibody to purified fp_T. However, it was reported [2,3] that NADH could also serve as electron donor for heme oxidation and that presence of NADH-cytochrome b_5 reductase (abbreviated as fp_D here) was necessary for this reaction [3]. We report here that the heme oxygenase reaction specifically requires fp_T, and fp_D does not appear to support the heme degradation leading to the formation of biliverdin IXα.

2. Materials and methods

Microsomes were prepared from the liver of rats which had been injected hemin to induce hepatic heme oxygenase [4] and heme oxygenase was purified to apparent homogeneity from the microsomes by the methods [4] similar to those for pig spleen heme oxygenase [5].

fp_T, fp_D and cytochrome b_5 were partially purified from rat liver microsomes by the methods in [5], [6] and [7], respectively. Activities of fp_T and fp_D preparations were determined by reduction of 2,6-dichlorophenolindophenol [8] and 1 unit of fp_T or fp_D was defined as the amount of enzyme catalyzing the reduction of 1 μmol dye/min at 25°C [8]. Both fp_T and fp_D preparations were practically free from cytochrome b_5. The amount of cytochrome b_5 was determined by measuring the reduced minus oxidized difference spectrum of the sample [9].

Antibodies (IgG) against purified fp_T, fp_D and cytochrome b_5 from rat liver microsomes were the generous gifts from Professor T. Omura, Kyushu University.

The formation of bilirubin was used as the measure of heme oxygenase activity on the basis of difference in A_{468} and A_{517} [5,10] or by chloroform extraction method [10]. Biliverdin reductase employed was a highly-purified preparation from pig spleen and the biliverdin reductase preparation was active with either NADPH or NADH (unpublished). Detailed conditions for the assay of heme oxygenase are given in the legends to the respective tables. Protein was determined by the methods in [5].

3. Results

3.1. *Effects of antibodies on the microsomal heme oxygenase reaction*

As can be seen in table 1, both NADPH and NADH could serve as electron donor for heme degradation catalyzed by the microsomal preparation. The yield of bilirubin was highest in the reaction system with NADPH and the yield of bilirubin in the NADH system was increased considerably by increased addition of NADH. However, the heme oxygenase activity with either NADPH or NADH was completely abolished when anti-fp_T IgG was further added to the incubation mixture, whereas the addition of anti-fp_D IgG exhibited practically no effect in either systems. Inhibitory effect of anti-cytochrome

Table 1
Effects of antibodies on the microsomal heme oxygenase reaction with NADPH or NADH as electron donor

Antibody	Relative heme oxygenase activity (%)		
	With 0.5 mM NADPH	With 0.5 mM NADH	With 5 mM NADH
None	100 (1.80 nmol)	100 (0.284 nmol)	100 (1.01 nmol)
Control IgG	76	73	78
Anti-fp_T IgG	0	0	0
Anti-fp_D IgG	100	102	96
Anti-cyt. b_5 IgG	70	75	77

Microsome suspension (0.05 ml, 0.4 mg protein) was mixed with 0.4 mg antibody protein and the mixture incubated for 15 min at 25°C. Then bovine serum albumin (0.5 mg), potassium phosphate buffer (pH 7.4) (100 μmol), hemin (15 nmol) and biliverdin reductase (excess) were added and further incubated for 2 min at 37°C. Reaction was started by the addition of NADPH or NADH and was for 20 min at 37°C. The final volume was 1.0 ml. Bilirubin formed was estimated after extraction 3-times with 3 ml each of chloroform [10]. The values in brackets represent the actual amounts of bilirubin obtained

b_5 IgG was also insignificant as compared with the effect of control IgG. NADH may have served as electron donor apparently by way of fp_T which has been shown to react with NADH, too, although the K_m for NADH is much higher than that for NADPH [11]. Essentially similar results were also obtained in [12].

3.2. *Reactions with the heme·heme oxygenase complex*

A complex of heme and heme oxygenase was prepared in a similar way to that in [5] and a series of experiments shown in table 2 were performed. In these experiments, reactions were carried out in a cuvette placed in a recording spectrophotometer and decrease A_{405} due to degradation of protoheme was followed (405 nm represents an A_{max} of the ferric form of heme·heme oxygenase complex [5]). In the reaction system with fp_T plus NADPH, the A_{405} decreased linearly for initial 2–3 min, then declined and reached plateau. At 25 min after the start of the reaction, biliverdin reductase was further added to the incubation mixture and immediately the spectrum was recorded. In this reaction system the hemin that disappeared had been converted quantitatively to biliverdin as judged from the amount of bilirubin obtained. The results obtained in the reaction system with fp_T plus NADH were essentially similar to those in the fp_T plus NADPH system except that the rate of the reaction was considerably lower in the fp_T plus NADH system.

In the fp_D plus NADH system, however, no measurable amount of bilirubin was obtained after

Table 2
Degradation of heme bound to heme oxygenase with fp_T or fp_D as an electron-donating system

Electron donor	Time of reaction (min)	Hemin disappeared (nmol)[a]	Bilirubin formed (nmol)
fp_T + NADPH	25	0.56	0.57
fp_T + NADH	60	0.60	0.46
fp_D + NADH	100	0.53	0.00

[a] Calculated on the assumption that mM extinction coefficient of the ferric heme·heme oxygenase complex at 405 nm is 140 [5]

Reaction mixture contained, in 2 ml final vol., 40 μg protein of heme oxygenase saturated with hemin by about 70%, 200 μg bovine serum albumin, 0.05 unit of fp_T or fp_D, 200 μmol potassium phosphate buffer (pH 7.4) and 0.1 μmol NADPH or NADH. Reaction was started by the addition of NADPH or NADH and was at 25°C. Excess amount of biliverdin reductase (0.05 ml) was further added at indicated time of reaction

the addition of biliverdin reductase, although a significant amount of heme disappeared during the 100 min incubation period though at a very low rate.

3.3. *Reduction of heme bound to heme oxygenase by various reducing systems*

It has been assumed that reduction of heme is a prerequisite for the oxidative degradation of heme in the heme oxygenase reaction [13]. Therefore, rates of reduction of the heme-heme oxygenase complex were compared under various conditions. As shown in fig.1, the fp_T plus NADPH system was the most effective in reducing the heme bound to heme oxygenase, and the rate of reduction with the fp_T plus NADH system was nearly half of that with the fp_T plus NADPH system. This is in good accord with the observation that the rate of heme degradation in the fp_T plus NADH system was about half of that in the fp_T plus NADPH system as judged by the decrease in A_{405} (cf. section 3.2; data not shown). On the other hand, heme reduction observed in the fp_D plus NADH system was extremely slow, indicating that fp_D can hardly donate electron to the heme which is bound to heme oxygenase. However, heme reduction in this reaction system was significantly enhanced when a small amount of cytochrome b_5 (final, 0.5 μM) was further added to the incubation mixture. This may be analogous to the well known observation that reduction of cytochrome c by the fp_D plus NADH system is greatly enhanced by the presence of cytochrome b_5 [14,15].

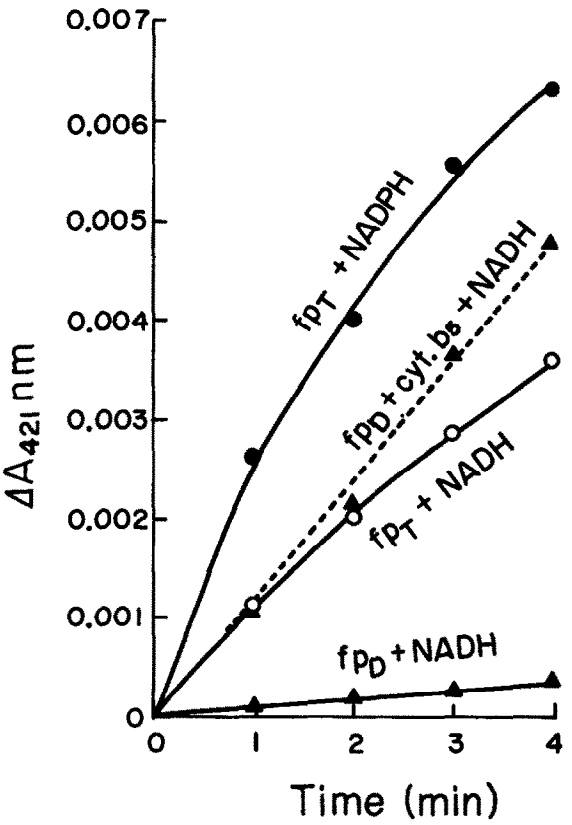

Fig.1. Reduction of heme bound to heme oxygenase. Reaction was in a Thunberg-type cuvette filled with CO gas at 25°C. Reaction mixture contained, in 2 ml final vol., 40 μg protein of heme oxygenase saturated with hemin by about 70%, 0.02 unit fp_T or fp_D, 0.1 μmol NADPH or NADH, 1 nmol cytochrome b_5 when added, and other additions similar to those for table 2. Reference cuvette contained the reducing system alone in the same buffer and ΔA_{421} was measured.

Then, considering the possibility that cytochrome b_5 might enhance heme degradation in the reaction system with fp_D plus NADH, the effect of addition of cytochrome b_5 on the bilirubin formation was examined in the reconstituted heme oxygenase system with 15 μM hemin as substrate. As shown in table 3, however, practically no bilirubin was obtained even when as much as 7 nmol cytochrome b_5 (final, 3.5. μM) was added to the reaction system with fp_D plus NADH.

Table 3
Effect of addition of cytochrome b_5 on the formation of bilirubin in the reaction system with fp_D and NADH

Electron donor	Bilirubin formed (nmol)
fp_T + NADPH (0.5 mM)	5.78
fp_T + NADH (5 mM)	3.91
fp_D + cyt. b_5 + NADPH (0.5 mM)	0.00
fp_D + cyt. b_5 + NADH (5 mM)	0.00

Reaction mixture contained, in 2 ml final vol., partially-purified heme oxygenase (14 units [5], 0.2 mg protein), 0.4 mg bovine serum albumin, 200 μmol potassium phosphate buffer (pH 7.4) 30 nmol hemin, 1 unit fp_T or fp_D, an excess amount of biliverdin reductase, 7 nmol cytochrome b_5 when added, and indicated concentration of NADPH or NADH. Reaction was for 10 min at 37°C

4. Discussion

The present study demonstrated that the microsomal heme oxygenase reaction specifically requires fp_T. fp_D could hardly serve as the electron donor system and could not support the formation of biliverdin IXα or bilirubin although some non-specific heme degradation possibly resulted by formation of H_2O_2 [16,17] occured during incubation of the heme·heme oxygenase complex with fp_D plus NADH. fp_D could effectively reduce the heme on the heme oxygenase when cytochrome b_5 was also present. However, the presence of cytochrome b_5 did not help the formation of biliverdin IXα from protoheme as judged from the observation shown in table 3. This would mean that reduction of heme may not be the sufficient condition for heme degradation leading to the formation of biliverdin IXα. The reason for fp_D apparently abolishing the stereospecificity of heme oxidation is unclear at present; one possibility is that fp_D might cause some conformational change of heme oxygenase. In other words, conformation of the heme oxygenase protein may be an important factor in bringing about the α-methene-specific oxidation of protoheme, and fp_T may well maintain an appropriate configuration of the heme oxygenase molecule, essential for the stereospecificity of the microsomal heme oxygenase reaction. We have shown [13] that when the heme·heme oxygenase complex alone was incubated with ascorbic acid in air, the heme bound to heme oxygenase was also rapidly oxidized, specifically at the α-position.

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