INABILITY OF THE NADH-CYTOCHROME b_5 REDUCTASE SYSTEM TO INITIATE HEME DEGRADATION YIELDING BILIVERDIN IXα FROM THE OXYGENATED FORM OF HEME · HEME OXYGENASE COMPLEX

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1. Introduction

The microsomal heme oxygenase system, which catalyzes the oxidative degradation of protoheme to biliverdin IXα, consists of heme oxygenase (EC 1.14.99.3) and NADPH-cytochrome *c* reductase (EC 1.6.2.4; fp_T). With microsomes, however, the heme oxygenase reaction is also supported by NADH [1]. In this case, however, NADH may serve as electron donor by way of fp_T [2,3]. In fact, we demonstrated that the NADH-cytochrome b_5 reductase system, which was reconstituted from NADH-cytochrome b_5 reductase (EC 1.6.2.2, fp_D and cytochrome b_5 partially purified from rat liver microsomes, failed to support the heme degradation to form biliverdin IXα from the heme · heme oxygenase complex, although the reconstituted fp_D system could effectively reduce the heme bound to the heme oxygenase, using NADH as electron donor [3]. The reason why the fp_D system could not support the biliverdin formation has remained unsolved.

On the other hand, recently we obtained an oxygenated form of the heme · heme oxygenase complex and revealed that a reducing equivalent was indispensable for the onset of heme degradation from the oxygenated form of the complex [4]. Therefore, the possibility arises that the fp_D system may hardly be able to donate electron to the oxygenated form of the heme · heme oxygenase complex to initiate the heme degradation leading to the formation of biliverdin IXα. The present study has revealed that this is really the case.

2. Materials and methods

Heme oxygenase was purified from pig spleen microsomes [5] and the complex of ferric heme and heme oxygenase was prepared as in [5]. fp_T [5], fp_D [6], and cytochrome b_5 [7] were partially purified from pig liver microsomes by the method referred. The fp_T preparation was free from either of cytochrome b_5 and fp_D, and the fp_D preparation was free from cytochrome b_5 and fp_T. Activity of fp_T was determined by reduction of horse heart cytochrome *c* (purchased from Sigma) in 2 ml system [8], and 1 unit of fp_T was defined as the amount of enzyme catalyzing the reduction of 1 μmol of cytochrome *c* (cyt. *c*-unit)/min at 25°C. Activity of fp_D was determined by reduction of 2,6-dichlorophenolindophenol and 1 dye-unit was defined as the amount of enzyme catalyzing the reduction of 1 μmol dye/min at 25°C [8]. Activity of the fp_D system was determined by reduction of cytochrome *c* in the presense of cytochrome b_5 (cyt. *c*-unit); 0.02 cyt. *c*-unit of the fp_D system consisted of ~0.14 dye-unit of fp_D and 0.25 nmol cytochrome b_5 under our experimental conditions. The amount of cytochrome b_5 was determined by measuring the α-band of reduced form of cytochrome b_5 [9]. Highly purified biliverdin reductase was obtained from the soluble fraction of pig spleen [10]. Solution A was a mixture of 1 mM EDTA, 0.1% Triton X-100, and 0.1% sodium cholate. Chemicals were obtained from commercial sources described in [5,11].

3. Results

3.1. *Comparison of rates of reduction of the heme bound to heme oxygenase by the fp_T system, the fp_D system and fp_D*

The reaction was carried out in a Thunberg-type cuvette filled with CO gas, employing ~0.02 cyt.

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c-unit of respective reductases and 5.5 nmol heme of the ferric heme · heme oxygenase complex, in 2 ml final vol. Reaction was started by introducing NADPH or NADH, and the increase A_{421} was recorded. With the fp_T system, the half-reduction time of ferric heme · heme oxygenase complex was ~150 s, while it was ~435 s with the fp_D system. When cytochrome b_5 was omitted from the fp_D system, the half-reduction time was >1500 s. These results are consistent with our observations with preparations from rat liver microsomes.

3.2. *Heme oxygenase reaction with the fp_D system*

We reported that the oxygenated heme bound to heme oxygenase was readily and quantitatively converted to biliverdin IXα when the oxygenated heme · heme oxygenase complex was incubated with the fp_T system [4]. However, when the fp_D system was used in place of the fp_T system, the absorption of the oxygenated form of the complex (curve I in fig.1) only decreased slowly, maintaining a spectrum characteristic of the oxygenated complex. Curve III represents an absorption spectrum recorded ~1 h after the start of the reaction. Also no bilirubin was formed when biliverdin reductase and NADPH were added to the incubation mixture giving curve III; further addition of desferrioxamine, a ferric iron chelator, exerted no effect (data not shown; cf. [11]). These observations indicate that neither biliverdin IXα nor biliverdin–iron chelate was formed by incubation with the fp_D system and some nonspecific heme degradation occurred in this reaction system.

In an experiment the ferric heme · heme oxygenase complex was incubated with the fp_D system in air. As shown in fig.2, the absorption in the Soret region of the ferric form of the complex decreased, and the peak shifted to longer wavelength side and finally settled at 412 nm. In the visible region two absorption peaks around 540 and 575 nm became distinct with the time of incubation. The absorption spectrum depicted by curve II in fig.2 closely resembles that of curve I in fig.1 which represents an oxygenated form of the heme · heme oxygenase complex (cf. [4]). These observations indicate that the ferric heme bound to heme oxygenase was first reduced by the fp_D system to the ferrous state, followed by binding with molecular oxygen to form the oxygenated heme · heme oxygenase complex. With the fp_T system, we failed to observe an oxygenated form of the complex during the reaction [11] probably

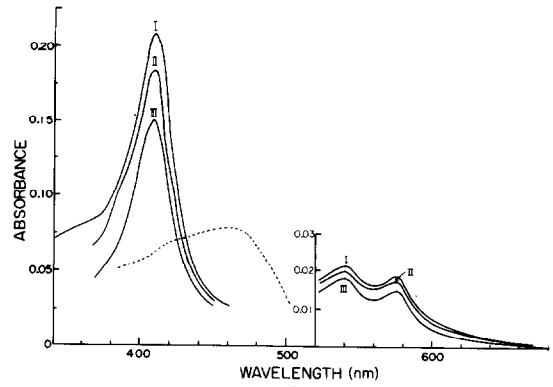

Fig.1. Reaction with the oxygenated heme bound to heme oxygenase and the fp_D system. Sample cuvette contained, in 2 ml final vol., ~1.6 μM heme of the oxygenated form of heme · heme oxygenase complex and 0.2 mg bovine serum albumin in 110 mM potassium phosphate buffer (pH 7.4) containing solution A. The complex was omitted in the reference cuvette. Then, 0.02 cyt. c-unit of the fp_D system (0.05 ml) and 0.05 ml 2 mM NADH were added to both cuvettes and the spectral change was followed at 25°C. Curve I, the oxygenated form of the complex; curve II, 10 min after the start of the reaction; curve III, 1 h after the start of the reaction. For comparison, in an independent experiment, 0.02 cyt. c-unit of fp_T (0.025 ml), excess of highly purified biliverdin reductase (0.025 ml) and 0.05 ml 2 mM NADPH were added to a mixture showing curve I, and the absorption spectrum of the final product (bilirubin) is depicted by dotted line.

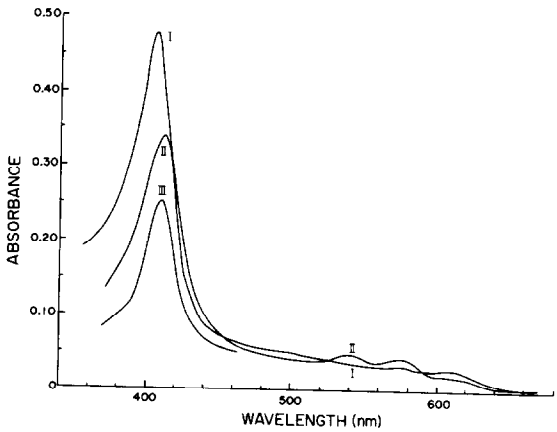

Fig.2. Reaction with the ferric heme bound to heme oxygenase and the fp_D system in air. Sample cuvette contained, in 2 ml final vol., ~3.4 μM heme of the ferric form of the complex in 110 mM potassium phosphate buffer (pH 7.4) containing solution A. The complex was omitted in the reference cuvette. Temp., 25°C. Curve I, the ferric form of the complex; curve II, 30 min after the addition of 0.02 cyt. c-unit of the fp_D system (0.05 ml) and 0.05 ml 2 mM NADH to both cuvettes; curve III, 90 min after the start of the reaction.

because the heme, once oxygenated, is rapidly decomposed to biliverdin in this reaction system.

To account for the observation that the fp_D system could not serve as electron donor system in the heme oxygenase reaction, we had suspected the possibility that the fp_D system might cause some conformational change of the heme oxygenase protein so as to lose the stereospecificity of heme oxidation. To test this possibility, we examined the effects of additions of fp_D (0.14 dye-unit), fp_D plus cytochrome b_5 (0.25 nmol), and fp_D plus cytochrome b_5 plus NADH (100 nmol), respectively, on the rate of heme degradation in the reaction system containing 1.6 μM heme of the ferric heme · heme oxygenase complex, fp_T (0.02 cyt. *c*-unit), and NADPH (100 nmol), in 2 ml final vol. In all systems tested, however, the time required for degradation of half the amount of heme used, as measured by decrease in A_{405} at 25°C, was 240 s, indicating that the fp_T-supported heme degradation was not affected at all by the simultaneous presence of any component of the fp_D system.

4. Discussion

It is interesting that the fp_D system could donate few electrons to the oxygenated form of heme · heme oxygenase complex to initiate heme degradation leading to the formation of biliverdin IXα, while it could effectively reduce the ferric form of the complex. With the fp_D system, the oxygenated heme slowly underwent nonspecific degradation to some unknown product(s), possibly by the action of H_2O_2 formed during the incubation with the fp_D system [12,13]. Also the fp_D system did not affect at all the fp_T-supported heme oxygenase reaction. We may conclude that the microsomal heme oxygenase is strictly linked with the fp_T system in the physiological heme degradation. It is also worth noting that the oxygenated form of heme · heme oxygenase complex was formed when the ferric heme · heme oxygenase complex was incubated with the fp_D system. These observations support findings [4] that heme degradation starts from the oxygenated form of the complex and that a reducing equivalent is indispensable for the onset of heme degradation.

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