Reduction of Nitro and Azo Compounds by NADPH–Cytochrome P-450 Reductase–Cytochrome c Heme Peptide System

Yasusuke MASUDA* and Masanobu OZAKI

Division of Toxicology, Niigata College of Pharmacy, Niigata 950–21, Japan. Received July 9, 1992

Octa heme peptide, an enzymic digestion product of *Candida Krusei* cytochrome c, was found to catalyze nitro and azo reduction in the presence of NADPH and NADPH–cytochrome P-450 reductase under anerobic conditions. The reduction was dependent on the concentrations of heme peptide and reductase, and inhibited by carbon monoxide and oxygen. Comparison of the activities of the reductase–heme peptide system with liver microsomes revealed that heme peptide was as effective as cytochrome P-450 in nitro reduction, although less effective in azo reduction. Thus, cytochrome c heme peptide may serve as an artificial substitute for cytochrome P-450 at least in the reductive metabolism of xenobiotics.

Keywords cytochrome c heme peptide; anerobic reduction; nitro reduction; azo reduction; NADPH–cytochrome P-450 reductase

Introduction

Heme peptides obtained by enzymic digestion of cytochrome c, unlike native cytochrome c, exhibit peroxidase-like activity.^{1–3)} Végh *et al.*⁴⁾ and Rusvai *et al.*⁵⁾ furthermore reported that heme peptides enhance NADPH oxidation and also catalyze aniline hydroxylation in the presence of NADPH and NADPH–cytochrome P-450 reductase. We have recently showed that heme peptide can catalyze one electron reduction of CCl₄ and CBrCl₃ to trichloromethyl free radicals in the presence of NADPH and NADPH–cytochrome P-450 reductase under anerobic conditions,⁶⁾ such as reported with hepatic microsomal systems.⁷⁾ The similarity of the action between cytochrome heme peptides and cytochrome P-450 hemoproteins may reside on the vacancy of the sixth coordinating position of the porphyrin iron in both molecules,^{8–10)} despite a variety of molecular differences. These studies suggest that cytochrome c heme peptides could be used as a model substance as well as a replacement for cytochrome P-450-mediated reactions. At present, however, no data on xenobiotic metabolizing activity other than aniline hydroxylation and halogenomethane reduction have been reported. The present study shows that heme peptide can also mediate reductive metabolism of nitro and azo compounds.

Materials and Methods

Octa-heme peptide used in the present study was kindly provided by Sankyo Research Laboratory, Japan. This heme peptide was prepared by enzymatic digestion of *Candida Krusei* cytochrome c: initial digestion with pepsin followed by purification by ammonium sulfate fractionation and partition chromatography on Hyflo Supercel, and succeeding digestion of the purified peptic-heme peptide with trypsin followed by chromatographic purification on a DEAE Sephadex A 25 column.^{2,3,9)} The amino acid sequence of this heme peptide, confirmed by protein sequencer, was Cys–Ala–Gln–Cys–His–Thr–Ile–Glu, with the heme bound to the two cysteinyl residues by thio-ether bonding. The actual molecular weight (1748) calculated from the iron content was used in this study.

NADPH–cytochrome P-450 reductase was purified from phenobarbital-pretreated rats by the method of Guengerich and Martin,¹¹⁾ with the specific activity of 54 U/mg protein. Liver microsomes were isolated from normal and phenobarbital-induced rats using 0.15 M KCl–1 mM EDTA–20 mM potassium phosphate buffer, pH 7.4, by the usual differential centrifugation method, washed once, and suspended in 0.1 M potassium phosphate buffer (pH 7.4).

NADPH–cytochrome P-450 reductase activity of the purified enzyme

and microsomal fraction was measured using cytochrome c as an electron acceptor in 0.3 M potassium phosphate buffer (pH 7.7).¹²⁾ One unit was defined as the amount of the enzyme that reduces 1.0 μ mol of cytochrome c per min at 30 °C. Microsomal P-450 content was assayed by the method of Omura and Sato¹³⁾ after solubilizing microsomes in 0.1 M potassium phosphate buffer (pH 7.4) containing 1 mM EDTA, 40% glycerol, 0.2% Emulgen 913, and 0.5% sodium cholate. Protein was determined by the method of Lowry *et al.*¹⁴⁾

Reduction of nitro and azo compounds was carried out in a 3-ml vial bottle or a 4-ml cuvette shielded by a Teflon-rubber septum. To ensure that the condition was anerobic, reagents were previously bubbled or flushed with nitrogen as much as possible and the reaction mixture was finally flushed again with nitrogen in ice for more than 10 min. The reaction mixture contained an NADPH-generating system: 10 mM sodium isocitrate, 5 mM MgCl₂ and 0.12 IU/ml of isocitrate dehydrogenase (Oriental Yeast, Japan). For nitro reduction, the reaction was started by the addition of NADPH after a 5-min preincubation at 37 °C, stopped by the addition of trichloroacetic acid (final 5%), and the reduced products of nitro compounds were colorimetrically determined after diazo-coupling with *N*-(1-naphthyl) ethylene diamine.¹⁵⁾ *p*-Aminobenzoic acid, aniline and *p*-aminophenol were used as standards for the reduced products of *p*-nitrobenzoic acid, nitrobenzene and nitrosobenzene, and *p*-nitrophenol, respectively. For the reduced product of chloramphenicol, the optical density (OD) ratio 0.650/1.335 for reduced-chloramphenicol/*p*-aminobenzoic acid at 540 nm¹⁶⁾ was used for calculation. Azo reduction of amaranth and sulfonazo III was regularly assayed spectrophotometrically by recording the OD decrease at 520 and 573 nm, respectively,^{17,18)} using a Hitachi 124 double beam spectrophotometer, with an attached cuvette holder regulated at 37 °C. Experimental details are described in the legends.

Results and Discussion

Nitro Reduction Nitro reduction of *p*-nitrobenzoic acid was first examined under anerobic conditions. As shown in Fig. 1, *p*-aminobenzoic acid formation required both NADPH–cytochrome P-450 reductase and heme peptide in the presence of NADPH (hereafter referred to as reductase–heme peptide system) and NADPH alone had no reductive activity. The reaction was linear with time. With 0.1 U of reductase, the reduction rate was linear up to about 10 μ M heme peptide, above which the rate of *p*-aminobenzoic acid formation on the basis of heme peptide concentrations lowered (Fig. 1A). However, with increasing concentrations of reductase, *p*-aminobenzoic acid formation at 30 μ M heme peptide further increased (Fig. 1B). Thus, the reductive activity of heme peptide seems to be maximally manifested, when the heme peptide (nmol/ml)/reductase (U/ml) ratio is roughly less than 100. The reduction rate was not affected by NADPH concentrations be-

Fig. 1. Nitro Reduction of *p*-Nitrobenzoic Acid by the Reductase-Heme Peptide System

(A) Time-course and heme peptide dependency. (B) NADPH-cytochrome P-450 reductase dependency. In (A), the mixture containing 1 mM *p*-nitrobenzoic acid, 0–100 μM heme peptide (CHP), 0.1 U NADPH-cytochrome P-450 reductase and NADPH-generating system (without NADPH) in 1.0 ml of 0.1 M potassium phosphate buffer (pH 7.4) was prepared in a shielded 3-ml vial bottle under nitrogen gas saturation and preincubated for 5 min at 37°C. The reaction was started by the addition of NADPH (final 200 μM) through the Teflon-rubber septum. At 5, 10 and 20 min, 1.0 ml of 10% trichloroacetic acid solution was added to stop the reaction and the mixture was centrifuged. *p*-Aminobenzoic acid (PABA) in the supernatant was determined by the diazo-coupling method as described in the Method section. In (B), the same procedure as in (A) was used except that the heme peptide concentration was fixed at 30 μM and the amount of reductase was varied. Incubation time was 10 min. Without heme peptide, no reduction of *p*-nitrobenzoic acid occurred even with 800 μM NADPH.

TABLE I. Effects of Carbon Monoxide and Oxygen on Nitro Reduction of *p*-Nitrobenzoic Acid by the Reductase-Heme Peptide System

Incubation atmosphere	<i>p</i> -Aminobenzoic acid formed (nmol)
N ₂	54.7 ± 0.3
N ₂ -CO	1.2 ± 0
Air	0.9 ± 0.3

For N₂ atmosphere, the solution containing 30 μM heme peptide, 0.2 U NADPH-cytochrome P-450 reductase, NADPH-generating system (without NADPH) and 1 mM *p*-nitrobenzoic acid in 1.0 ml of 0.1 M potassium phosphate buffer (pH 7.4) was prepared in a shielded 3-ml vial bottle under nitrogen gas saturation. For N₂-CO atmosphere, carbon monoxide gas was further flushed into the nitrogen-saturated mixture for 5 min. After preincubation of this mixture for 5 min at 37°C, the reaction was started by the addition of 200 μM NADPH through the Teflon-rubber septum and continued for 10 min. For air atmosphere, all these procedures were done under air. The reaction was stopped by the addition of 1 ml of cold 10% trichloroacetic acid, the mixture was centrifuged, and *p*-aminobenzoic acid in the supernatant was determined by the diazo-coupling method. Values are means ± deviations of duplicate experiments.

tween 100 to 800 μM, but gradually increased by increasing the concentration of *p*-nitrobenzoic acid (0.02 to 4 mM) (data not shown). The nitro reduction by the reductase-heme peptide system, like that by the microsomal system,¹⁹ was inhibited by 98–99% in the air or in the presence of carbon monoxide (Table I). Unlike the case of P-450, the reduced heme peptide-carbon monoxide complex had a sharp Soret peak at 411 nm and a trough at 425 nm in the carbon monoxide difference spectrum of reduced heme peptide (Fig. 2).

Other nitro compounds such as chloramphenicol, nitrobenzene, and *p*-nitrophenol were also reducible (Table II). The high reduction rate of nitrosobenzene to aniline suggests that reduction of nitro to nitroso group may be a rate limiting step, as reported by the liver microsomal system.¹⁹

Azo Reduction Reduction of amaranth was also depen-

Fig. 2. Carbon Monoxide Difference Spectrum of Reduced Heme Peptide in the Presence of NADPH and NADPH-Cytochrome P-450 Reductase under Anaerobic Conditions

The nitrogen saturated solution containing 20 μM heme peptide and 0.5 mM NADPH in 3.0 ml of 0.1 M phosphate buffer (pH 7.4) was prepared in duplicate in a shielded 3-ml cuvette under nitrogen atmosphere. NADPH-cytochrome P-450 reductase (0.2 U) was added to each cuvette through the Teflon shield. After complete reduction of heme peptide, which was checked by an increase in OD at 550 nm (absorption peak of α -band of reduced heme peptide), carbon monoxide gas was bubbled into one of the cuvettes for 5 min and the difference spectrum was recorded.

TABLE II. Reduction of Some Nitro and Nitroso Compounds by the Reductase-Heme Peptide System

Substrates	Amines formed (nmol)
<i>p</i> -Nitrobenzoic acid	46.0 ± 4.9
Chloramphenicol	27.9 ± 0.1
Nitrobenzene	46.7 ± 1.0
Nitrosobenzene	553 ± 33
<i>p</i> -Nitrophenol	23.9 ± 1.1

Reaction mixtures containing 1 mM of each substrate, 10 μM heme peptide, 0.1 U NADPH-cytochrome P-450 reductase and 200 μM NADPH with the generating system in 1.0 ml of 0.1 M phosphate buffer (pH 7.4) were incubated for 20 min under nitrogen atmosphere (details of the procedure were described in the legend of Fig. 1). The reaction was stopped by the addition of 1.0 ml of 10% trichloroacetic acid solution, the mixture was centrifuged, and amines in the supernatant were determined as described in the Method section.

dent on both reductase and heme peptide concentrations (Fig. 3). Since non-enzymatic reduction of amaranth occurred with increasing concentrations of NADPH, 100 μM NADPH was used in this experiment. As with nitro reduction, amaranth azo reduction was inhibited in the presence of carbon monoxide or in the air by 89% and 92%, respectively. A similar inhibition was reported with liver microsomes.¹⁷ Sulfonazo III was reduced in the presence of NADPH and reductase, with no enhancement by heme peptide (data not shown), which is in agreement with the report that its microsomal reduction was not inhibited by carbon monoxide.¹⁸

Comparison with Microsomal Reductive System Relative reductive potencies of heme peptide to P-450 were next examined using microsomes isolated from normal and phenobarbital-pretreated rat livers. As shown in Table III, the ratio of P-450 (nmol)/reductase(U) = 2.3–3.7 in microsomes was too deviated from that of the reductase-heme peptide system used so far, i.e. large excess of reductase in microsomes, to extrapolate this ratio to the data on the reductase-heme peptide system. Therefore, the activities in both systems were compared under equimolar concentra-

TABLE III. Comparison of Nitro and Azo Reductive Activities between Liver Microsomal and Reductase-Heme Peptide Systems

	Cyt. P-450 (nmol/mg prot.)	NADPH-cyt. P-450 reductase (U/mg prot.)	Nitro and azo reduction (nmol substrate reduced)		
			<i>p</i> -Nitrobenzoic acid	Chloramphenicol	Amaranth
Microsomes					
Normal	1.08 ± 0.02	0.469 ± 0.009	6.6 ± 0.5	13.6 ± 0.2	102 ± 4
Phenobarbital-induced	2.18 ± 0.02	0.595 ± 0.018	18.5 ± 0.2	18.1 ± 0.3	176 ± 2
Reductase-heme peptide system					
Eq. to normal microsomes			10.5 ± 0.4	12.6 ± 0.2	7.9 ± 0.9
Eq. to phenobarbital-induced microsomes			26.9 ± 0.1	26.9 ± 0.1	25.5 ± 1.4

The reductase-heme peptide system contained equivalent amounts of NADPH-cytochrome P-450 reductase and heme peptide to microsomal NADPH-cytochrome P-450 reductase and cytochrome P-450 content, respectively. For *p*-nitrobenzoic acid and chloramphenicol reduction, the reaction mixture containing 1 mg microsomal protein or its equivalent reductase-heme peptide system, 200 μ M NADPH with the generating system and 1 mM of each substrate in 1.0 ml of 0.1 M potassium phosphate buffer (pH 7.4) was incubated at 37°C for 20 min under anaerobic conditions (details of the procedure were described in the legends of Fig. 1 and Table II). For amaranth reduction, in which 300 μ M amaranth was used, the reaction was stopped by adding 30 μ l of 10% Triton X-100 to solubilize microsomes and 2 ml of cold water, kept in ice and the remaining amaranth content was immediately determined colorimetrically at 520 nm after appropriate dilutions. Non-enzymatic reduction of amaranth by NADPH was subtracted. Values are means of duplicate experiments with their ranges.

Fig. 3. Azo Reduction of Amaranth by the Reductase-Heme Peptide System

(A) Heme peptide dependency. (B) NADPH-cytochrome P-450 reductase dependency. In (A), the solution containing 80 μ M amaranth, NADPH-generating system (without NADPH) and 0.2 U/ml NADPH-cytochrome P-450 reductase in 3.0 ml of 0.1 M phosphate buffer (pH 7.4) was prepared in a shielded 3-ml cuvette under nitrogen atmosphere and equilibrated at 37°C. NADPH (100 μ M) was initially added to record a slight non-enzymatic reduction of amaranth and then various concentrations of heme peptide (CHP) were added cumulatively. The decrease in OD change at 520 nm was recorded for 4 min after each addition. The amount of amaranth reduced was calculated from the OD of the amaranth standard solution at 520 nm, since amaranth was completely decolorized by reduction. The non-enzymatic reduction of amaranth was subtracted. In (B), the same procedure as in (A) was applied except that heme peptide was included in the preincubation mixture at a fixed concentration of 15 μ M and increasing amounts of reductase were added cumulatively after the addition of NADPH.

tions of reductase and heme. It is evident that nitro reduction occurred to a roughly comparable degree in both systems. The rate of amaranth azo reduction by the reductase-heme peptide system was comparable with that of nitro reduction. However, microsomal amaranth reduction was much greater than that by the reductase-heme peptide system, which may be due to a difference in substrate-binding characteristics between both systems. Further experiments using purified P-450 are necessary for exact comparisons.

Thus, under anaerobic conditions, the reductase-heme peptide system was found to catalyze the reductive metabolism of nitro and azo compounds to amines. Many similarities were observed between reductase-heme peptide and microsomal systems: First, the reduction by heme peptide requires the presence of both NADPH-cytochrome P-450 reductase and NADPH, which indicates that, like a microsomal system, electrons are successively transferred from NADPH to reductase, heme peptide and then to

substrates. Second, both reductive systems operate only under anaerobic conditions and are inhibited by CO and in the presence of oxygen. Third, heme peptide showed the same order of reductive activity as observed with microsomal cytochrome P-450 at least in the case of nitro reduction.

In the presence of oxygen, this heme peptide is very auto-oxidizable, giving no reduced spectrum in the presence of NADPH and reductase. Under such conditions, in particular, heme peptide markedly enhances NADPH oxidation⁴⁾ accompanied by self-destruction of the heme portion,⁶⁾ which may be caused by formation of active oxygen species such as H₂O₂, since the addition of a high concentration of catalase, but not superoxide dismutase, considerably delayed the heme destruction (unpublished observations). Formation of O₂⁻ has also been suggested.²⁰⁾ It remains to be examined if modifications of heme peptide molecules or improvements of incubation conditions could manifest such various types of monooxygenase reactions as exhibited by microsomes.

In conclusion, cytochrome c heme peptide may substitute for P-450 at least in the reductive metabolism of nitro and azo compounds as well as of halogenomethanes. Heme peptide could be useful as a model substance for P-450.

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