Electron-Transport Pathway of the NADH-Dependent Haem Oxygenase System of Rat Liver Microsomal Fraction Induced by Cobalt Chloride

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The hepatic microsomal haem oxygenase activity of rats treated with CoCl_2 was studied kinetically by measuring biliverdin, the immediate product of the reaction. Biliverdin was extracted with diethyl ether/ethanol mixture, and was determined by the difference between A_{690} and A_{800} . The apparent K_m value for NADPH (at $50\text{ }\mu\text{M}$ -haematin) was about $0.2\text{ }\mu\text{M}$ when an NADPH-generating system was used, whereas that for NADH was about $630\text{ }\mu\text{M}$. Essentially the same V_{max} values were obtained for both the NADH- and NADPH-dependent haem oxygenase reactions. No synergism was observed with NADH and NADPH. The NADH-dependent reaction was competitively inhibited by NADP^+ , with a K_i of about $10\text{ }\mu\text{M}$. The inhibition of the NADH-dependent reaction by the antibody against rat liver microsomal NADPH-cytochrome *c* reductase was essentially complete, with a pattern similar to that of the NADPH-dependent reaction. The immunochemical experiment and the comparison of the kinetic values with the reported data on isolated NADH-cytochrome *b_5* reductase and NADPH-cytochrome *c* reductase indicated the involvement of the latter enzyme in NADH-dependent haem oxygenation by microsomal fraction *in situ*.

The administration of cobalt to rats causes a decrease in hepatic haemoproteins such as cytochrome *P*-450 (Tephly & Hibbeln, 1971) and catalase (Yasukochi *et al.*, 1974). At the same time, an acceleration of haem degradation has been observed after the cobalt treatment, which is due to the induction of haem oxygenase (EC 1.14.99.3) present in microsomal fraction (Maines & Kappas, 1974). The oxygenation of haem is supported by either NADPH or NADH as an electron donor (Maines & Kappas, 1977). The involvement of NADPH-cytochrome *c* reductase (EC 1.6.2.4) in the NADPH-dependent haem oxygenase reaction has been demonstrated by immunochemical experiments (Schachter *et al.*, 1972) as well as by reconstitution experiments (Yoshida & Kikuchi, 1977; Maines *et al.*, 1977). Although Maines & Kappas (1977) have concluded from their reconstitution experiment that NADH-cytochrome *b_5* reductase (EC 1.6.2.2) is responsible for the NADH-dependent reaction, the conclusion needs to be re-examined, because the result of a reconstitution experiment does not necessarily imply the involvement of the component in the system *in situ*.

In this paper, we report some kinetic properties of the microsomal haem oxygenase system by directly measuring the formation of biliverdin. We use the term haem oxygenase system to indicate a multi-enzyme complex in microsomal fraction consisting of a reductase and haem oxygenase. Both kinetic and

immunochemical results indicated the participation of NADPH-cytochrome *c* reductase in the NADH-dependent haem oxygenase activity of the microsomal fraction.

Experimental

Treatment of animals and subcellular fractionation

Male Wistar rats (200–300g) were injected subcutaneously with 130 mM-CoCl_2 solution ($250\text{ }\mu\text{mol/kg}$ body wt.) once a day for 2 days in the afternoon. They were starved after the last injection and killed the next morning by decapitation. The livers were quickly excised, perfused thoroughly with 0.25 M -sucrose solution and homogenized with 4 vol. of the sucrose solution in a Potter-Elvehjem homogenizer equipped with a motor-driven Teflon pestle. The homogenate was centrifuged at $9000g$ for 15 min at 4°C (Hitachi 18PR centrifuge, RPR 20-2 rotor, r_{av} 8.25 cm) and the supernatant was centrifuged at $77000g$ for 120 min at 4°C (Hitachi 55P-2 centrifuge, RP 30 rotor, r_{av} 7.7 cm). The cytosol fraction was centrifuged again at $77000g$ for 120 min to remove any contaminating microsomal fraction and was used as an enzyme source of biliverdin reductase (EC 1.3.1.24). The microsomal fraction was suspended in an excess of ice-cold 0.15 M-KCl , recovered by centrifugation at $77000g$ for 120 min and was resuspended in ice-cold water.

Determination of biliverdin formation

A reaction mixture consisting of 0.1M-potassium phosphate buffer, pH7.4, bovine serum albumin (1mg/ml), 50 μ M-haematin and an appropriate amount of microsomal preparation (less than 1mg of protein) in a final volume of 1.0ml was preincubated for 5min at 37°C. Albumin was added to make the medium similar to those for bilirubin formation. The reaction was started by the addition of electron donor (NADH or NADPH), incubated at 37°C for 3 or 5min and stopped by adding 0.5ml of 20% (w/v) trichloroacetic acid. The reaction was essentially linear with time for at least 5min. Diethyl ether/ethanol (4:1, v/v) (2ml) was added to the acidified sample, agitated vigorously and left for a short time to separate the phases. Peroxide-free diethyl ether was used throughout to avoid the degradation of biliverdin. The upper organic phase was withdrawn and the extraction was repeated three times. The pooled extract was concentrated *in vacuo* at room temperature (20–25°C) and the volume of the concentrated extract was adjusted to 1.5ml with ethanol. Biliverdin was determined from the A_{690} – A_{800} in the dual-wavelength mode by using a Hitachi 556 spectrophotometer. An absorption coefficient of $20.9 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ was used. This value was determined by using a commercial sample of biliverdin. The parallel control experiments were carried out without the addition of electron donors and a difference in the biliverdin formation was taken as the haem oxygenase activity.

Determination of biliverdin reductase activity

A reaction mixture consisting of 0.1M-potassium phosphate buffer, pH7.4, bovine serum albumin (1mg/ml), 12 μ M-biliverdin and an appropriate amount of the postmicrosomal supernatant, in a final volume of 3.0ml, was divided into two equal parts. One of them was used as a reference in spectrophotometry. They were placed in a thermostatically controlled cuvette-holder of a spectrophotometer (Hitachi 556) and preincubated for about 5min at 37°C. The reaction was started by the addition of NADH or NADPH, and the formation of bilirubin was followed at 472nm by using a recorder. The spectrum of bilirubin has an absorption maximum at 472nm under the conditions used. The bilirubin concentration was determined by using an absorption coefficient of $59.1 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$, which was determined by using a commercial bilirubin preparation.

Determination of the overall haem degradation to bilirubin

A reaction mixture consisting of 0.1M-potassium phosphate buffer, pH7.4, bovine serum albumin (1mg/ml), 50 μ M-haematin, microsomal preparation

and an excess amount of biliverdin reductase (post-microsomal supernatant) was used. The procedure was essentially the same as the determination of the biliverdin reductase activity.

Other determinations

Protein concentration was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Succinate-cytochrome *c* reductase activity was assayed by a modification of the method of Stotz (1955) as described previously (Hino *et al.*, 1978). NAD(P)⁺ transhydrogenase (EC 1.6.1.1) activity was assayed by the method of Kaplan (1967).

Immunochemical experiments

The antibody against microsomal NADPH-cytochrome *c* reductase used in the experiments was a highly potent and specific immunoglobulin G purified by affinity chromatography on antigen-conjugated Sepharose, and had no cross-reactivity to cytochrome *b*₅ reductase (Noshiro & Omura, 1978). It was a generous gift from the laboratory of Professor T. Omura.

A mixture consisting of 75mM-potassium phosphate buffer, pH7.4, a fixed amount of microsomal preparation and various amounts of the antibody in a final volume of 0.2ml was incubated for 15min at 37°C. It was diluted with 0.8ml of a mixture of 106mM-potassium phosphate buffer, pH7.4, and 60 μ M-haematin, and incubated for 5min at 37°C. The reaction was started by the addition of either NADH or NADPH, and stopped by adding 0.5ml of 20% (w/v) trichloroacetic acid after 5min incubation. The formation of biliverdin was determined as described above. Control experiments were carried out similarly with immunoglobulin G fraction of non-immunized rabbits purified on a DEAE-cellulose column.

Reagents

NAD⁺, NADH, NADP⁺, NADPH and glucose 6-phosphate dehydrogenase were purchased from Oriental Yeast Co., Tokyo, Japan, and haemin (bovine, type I), biliverdin dihydrochloride (grade II) and bilirubin (bovine gall-stone, Sigma grade) were from Sigma Chemical Co., St. Louis, MO, U.S.A. Horse heart cytochrome *c* and acetylpyridine-adenine dinucleotide were purchased from Boehringer und Söhne, Mannheim, Germany, and bovine serum albumin (fraction V) was from Armour Pharmaceutical Co., Kankakee, IL, U.S.A. Cholic acid, obtained from Wako Pure Chemical Industries, Osaka, Japan, was used after recrystallization from hot ethanol solution. Diethyl ether was used after treatment with an aqueous solution of FeSO₄. All other reagents used were of analytical grade.

Results

Kinetic parameters of the microsomal haem oxygenase system and other related enzyme activities

Kinetic information about the microsomal NADH-dependent haem oxygenase system is difficult to obtain by the method used by previous investigators (Tenhunen *et al.*, 1968; Yoshida & Kikuchi, 1977; Maines *et al.*, 1977). They measured bilirubin formation by a coupled reaction with biliverdin reductase, a cytosol enzyme. Low affinity of the enzyme for NADH tends to limit the reaction. Thus, to measure kinetic parameters of the NADH-dependent haem oxygenase system, it is necessary to use a method to measure the formation of biliverdin, the immediate product of the reaction. The method is described in the Experimental section. Biliverdin was extracted from the acidified reaction mixture with diethyl ether containing ethanol, and the recovery was essentially complete when diethyl ether/ethanol (4:1, v/v) was used. Biliverdin was measured as the absorption difference at a wavelength pair of 690 and 800 nm (Fig. 1).

Kinetic parameters of the microsomal haem oxygenase activity obtained by this method, as well as those of other related enzyme activities, are shown in Table 1. The apparent K_m values (at 50 μM -haematin) of the haem oxygenase system for NADPH were 0.16 μM with an NADPH-generating system and 59 μM with an NADPH preparation. The discrepancy between the K_m values for NADPH with and without an NADPH-generating system could not be ascribed to the inhibition of the reaction by NADP^+ , because we used freshly dissolved NADPH and measured the initial velocity. The K_m value for NADH was found to be 630 μM , but the V_{max} values of the reaction were essentially the same irrespective of electron donors used: NADH supports the microsomal haem oxygenase activity with the same V_{max} value as for NADPH, though the K_m value for the former was more than 1000 times that for the latter.

The K_m value for NADH of the overall haem catabolism to form bilirubin was as high as 7 mM, which may reflect the high K_m value (about 8 mM) of biliverdin reductase for NADH. O'Carra & Colleran (1971) gave the K_m value of partially purified biliverdin reductase for NADH as 200–500 μM .

Haem oxygenase activity in the presence of both NADH and NADPH

The effect of NADH on the NADPH-dependent haem oxygenase activity was studied to see whether

Fig. 1. *Absorption spectra of the extracts*
Microsomal preparation was incubated in the reaction mixture of haem oxygenase assay for the time-intervals given on the traces (0, 2, 7, 16, 32 min) at 37°C. Biliverdin was extracted and condensed to 1.5 ml. Under the conditions of this experiment, the absorption spectrum of an authentic biliverdin showed an absorption maximum at 690 nm, which corresponds to the shoulder part around 700 nm of the spectra of the extracts. The insert shows the time course of the reaction as measured with the wavelength pair of 690 nm and 800 nm.

Table 1. *Kinetic parameters of microsomal haem oxygenase, biliverdin reductase and overall reactions*
Activities of the haem oxygenase system, biliverdin reductase and the overall reaction to form bilirubin were determined as described in the Experimental section. The values are given as means $\pm$ s.d. for the numbers of independent experiments in parentheses.

Electron donor	Haem oxygenase system		Biliverdin reductase K_m (μM)	Overall reaction K_m (μM)
	K_m (μM)	V_{max} (nmol of biliverdin formed/min per mg of microsomal protein)		
NADPH*	0.16 $\pm$ 0.10 (4)	0.74 $\pm$ 0.26 (4)	—	—
NADPH	59.2 $\pm$ 0.4 (3)	0.76 $\pm$ 0.16 (3)	9	20
NADH	630 $\pm$ 190 (4)	0.78 $\pm$ 0.15 (4)	8000	7000

* With an NADPH-generating system consisting of 5 mM-glucose 6-phosphate, 10 mM-nicotinamide, 0.5 mM-MgCl₂ and 0.5 unit of glucose 6-phosphate dehydrogenase.

Fig. 2. Effect of NAD^+ or $NADP^+$ on the microsomal $NADH$ -dependent haem oxygenase activity. Haem oxygenase activity was determined by measuring biliverdin with 0.5 mM - $NADH$ as an electron donor, with various concentrations of NAD^+ (●) or $NADP^+$ (○) as indicated on the abscissa.

Table 2. Haem oxygenase activity with $NADH$ or $NADPH$, or both

The activity was determined by measuring biliverdin as described in the Experimental section.

	Electron donors (mM)		Haem oxygenase activity (nmol of biliverdin formed/min per mg of microsomal protein)
	$NADPH$	$NADH$	
Expt. 1	0.75	—	0.76
	—	1.0	0.51
	—	2.0	0.67
	0.75	1.0	0.87
	0.75	2.0	0.81
Expt. 2	0.75	—	0.59
	—	4.0	0.61
	0.75	4.0	0.56

there is any synergistic phenomenon between $NADH$ and $NADPH$. If there are independent $NADH$ - and $NADPH$ -dependent haem oxygenase systems, we may expect an additive effect with $NADH$ and $NADPH$. The microsomal haem oxygenase activity was determined by measuring biliverdin at a given concentration of $NADPH$ (0.75 mM) and various concentrations of $NADH$. As shown in Table 2, the haem oxygenase activity was essentially the same when $NADH$ or $NADPH$ was added alone or the both electron donors were added together. This clearly

Fig. 3. Lineweaver-Burk plots showing the reaction rates of microsomal haem oxygenase system as a function of $NADH$ with or without $NADP^+$

Haem oxygenase activity was determined by measuring biliverdin as described in the Experimental section, with $NADP^+$ (○, $10\text{ }\mu\text{M}$; △, 1 mM) or without $NADP^+$ (●).

shown in Expt. 2 of Table 2, in which the concentrations of electron donors were sufficiently high. The absence of synergism between NADH and NADPH suggests that a single haem oxygenase system is involved for both electron donors.

Effects of NAD⁺ and NADP⁺ on NADH-dependent haem oxygenase activity

Fig. 2 shows the effect of the increasing concentrations of oxidized nicotinamide coenzymes on the microsomal NADH-dependent haem oxygenase activity. NAD⁺ inhibited the reaction competitively with NADH, with a K_i value of about 16 mM. In contrast, the effect of NADP⁺ on the NADH-dependent reaction was rather complex. NADP⁺ inhibited the reaction at low concentration (about 10 μ M), whereas it activated the reaction at intermediate concentrations of about 1 mM. The reaction was inhibited again when NADP⁺ concentration exceeded 2 mM.

Lineweaver-Burk plots of the NADH-dependent activity against NADH concentration in the presence of 10 μ M- and 1 mM-NADP⁺ are shown in Fig. 3. With 10 μ M-NADP⁺ the inhibition pattern was biphasic, and at a concentration of NADH higher than about 0.8 mM it gave competitive-type inhibition with a K_i value of about 9.2 μ M. At low concentrations of NADH (less than about 0.8 mM) the reaction was activated by the addition of 10 μ M-NADP⁺. Such activation at low concentrations of NADH was more pronounced with 1 mM-NADP⁺. With 1 mM-NADP⁺ the plot gave a straight line, with decreased K_m value (48 μ M). The value is very much smaller than that determined in the absence of NADP⁺ (630 μ M) and is strikingly similar to that for NADPH (59.2 μ M) given in Table 1. The V_{max} value was also decreased by the addition of 1 mM-NADP⁺, but the decrease of the K_m value caused the activation of the NADH-dependent reaction at 0.5 mM-NADH and 1 mM-NADP⁺ shown in Fig. 2.

The similarity of the K_m value for NADH in the presence of 1 mM-NADP⁺ to that for NADPH suggests the possibility that an NAD(P)⁺ transhydrogenase activity may catalyse the transfer of electrons from NADH to NADP⁺, and NADPH thus formed is used as an electron donor for the haem oxygenation. When NAD(P)⁺ transhydrogenase activity of microsomal fraction was determined with acetylpyridine-adenine dinucleotide as a hydrogen acceptor, the specific activity was about 25% of that in the mitochondrial fraction. To examine the involvement of the transhydrogenase activity in the NADH-dependent haem oxygenation, we studied the effect of cholate on the reaction, because the microsomal transhydrogenase activity was inhibited completely by 0.1% cholate, as was reported for the mitochondrial transhydrogenase activity (Rydström *et al.*,

Fig. 4. Effect of cholate on microsomal haem oxygenase activity in the presence of NADP⁺

Haem oxygenase activity was determined with 1 mM-NADP⁺ and various concentrations of NADH given on the abscissa, and with (●) or without (○) 0.1% cholate.

Fig. 5. Effects of antibody against microsomal NADPH-cytochrome *c* reductase on the NADH- and NADPH-dependent haem oxygenase activities

A mixture containing 0.5 mg of microsomal preparation and various amounts of antibody against microsomal NADPH-cytochrome *c* reductase given on the abscissa was incubated and remaining activities of the NADH-dependent (●—●) and NADPH-dependent (○—○) haem-oxygenation reactions were determined. Control experiments were carried out with control immunoglobulin G fraction, for the NADH-dependent (●—●) and NADPH-dependent (○—○) reactions. The concentrations of NADH and NADPH were 1.0 mM and 0.5 mM respectively.

1976). Fig. 4 shows the double-reciprocal plot of the NADH-dependent haem oxygenase activity against NADH, in the presence of 1 mM-NADP⁺ and with or without 0.1% cholate. Cholate not only increased the K_m value for NADH from 58.8 μ M to 480 μ M, but also largely removed the inhibition caused by NADP⁺ on the V_{max} value. The K_m value in the presence of cholate is similar to that determined in the absence of NADP⁺ and cholate, given in Table 1.

Inhibition of NADH- and NADPH-dependent haem oxygenase activities by antibody against microsomal NADPH-cytochrome c reductase

All the kinetic results described above suggest that NADPH-cytochrome *c* reductase is involved in the NADH-dependent haem oxygenase activity. To examine this possibility further, we studied the effect of the antibody against microsomal NADPH-cytochrome *c* reductase on the NADH- and NADPH-dependent haem oxygenase activities. As shown in Fig. 5, both activities were inhibited in an essentially identical manner by increasing the ratio of the antibody to microsomal protein, and the maximal inhibition observed was more than 90%. Immunoglobulin G fraction of non-immunized rabbits had no effect on the activities under the conditions of this experiment. When similar immunochemical experiments were carried out in the presence of 0.2% cholate, essentially the same results were obtained (results not shown).

Discussion

All the results presented in this paper demonstrate that NADPH-cytochrome *c* reductase is a component of the microsomal NADH-dependent haem oxygenase system, namely (a) there was no synergism between NADH and NADPH, (b) the K_m value for NADH (630 μ M) is similar to the K_m for NADH of NADPH-cytochrome *c* reductase (1300 μ M; Ichikawa & Yamano, 1969) and is too high to be attributed to NADH-cytochrome *b*₅ reductase, which has a K_m

value of 2.7 μ M (Strittmatter & Velick, 1957), (c) the NADH-dependent haem oxygenase activity was competitively inhibited by NADP⁺ with a K_i value of about 10 μ M, which is similar to that of isolated NADPH-cytochrome *c* reductase for NADPH (2.1 μ M; Noshiro & Omura, 1978), and (d) the NADH-dependent haem oxygenase activity was strongly inhibited by the antibody against NADPH-cytochrome *c* reductase in a manner identical with that of the NADPH-dependent haem oxygenase activity.

The electron-transport pathway of the microsomal NADH-dependent haem oxygenase system can therefore be formulated as shown in Fig. 6. Both the NADPH-dependent and NADH-dependent activities are mediated by the same flavoprotein, NADPH-cytochrome *c* reductase. The situation is quite similar to that of the NADH-dependent microsomal drug metabolism shown by Noshiro & Omura (1978). They gave the K_m value of the microsomal NADH-dependent aniline hydroxylation reaction for NADH as 670 μ M, which is similar to the K_m value of the NADH-dependent haem oxygenase activity given in the present paper. Our conclusion does not agree with that of Maines & Kappas (1977), who showed by a reconstitution experiment that NADH-cytochrome *b*₅ reductase is responsible for the NADH-dependent haem oxygenase reaction. Our immunochemical experiment clearly showed that the electrons from NADH were supplied to haem oxygenase almost exclusively via NADPH-cytochrome *c* reductase and that the participation of NADH-cytochrome *b*₅ reductase is negligible. The discrepancy between the results obtained from a reconstituted system and for intact microsomal preparation is of interest when considered in relation to the mechanism by which the interaction of haem oxygenase with NADPH-cytochrome *c* reductase or NADH-cytochrome *b*₅ reductase is controlled. Our results indicate that haem oxygenase can interact only with NADPH-cytochrome *c* reductase in the microsomal membranes, but not with NADH-cytochrome *b*₅ reductase,

Fig. 6. Schematic representation of microsomal haem oxygenase system

whereas in a reconstituted system as described by Maines & Kappas (1977) both flavoproteins interact with haem oxygenase. The control mechanism might not require any integrity of membrane organization, because it took place even in the presence of cholate, which destroys the membrane structure.

We have shown the complex effect of NADP^+ on the haem oxygenase reaction with NADH as an electron donor: NADP^+ at low concentrations (about $10\mu\text{M}$) inhibited the reaction, whereas it accelerated the reaction at intermediate concentrations (about 1 mM). When NADP^+ concentrations exceeded 2 mM , the reaction was again inhibited. The first inhibition phase at low concentrations of NADP^+ may be explained by the competition between NADH and NADP^+ for NADPH-cytochrome *c* reductase. The acceleration of the reaction by NADP^+ at intermediate concentrations may be ascribed to the formation of NADPH by NAD(P)^+ transhydrogenase activity present in the microsomal preparation. Similarly, the inhibition of the reaction by NADP^+ at high concentration could be explained by the competition between NADPH formed by the transhydrogenase activity and NADP^+ , because the kinetic pattern modified by NADP^+ was returned to the original one by the addition of cholate, an inhibitor of the transhydrogenase activity. Although the equilibrium of the transhydrogenase reaction is favourable to the formation of NADH, the low concentration of NADPH formed by the reverse reaction may be sufficient to support the haem oxygenase reaction via NADPH-cytochrome *c* reductase, the K_m of which for NADPH is very low. The transhydrogenase activity found in the microsomal fraction could not be ascribed to contamination by mitochondria, because the contamination by mitochondria in the microsomal fraction was less than 3%, based on protein, when succinate-cytochrome *c* reductase was used as a marker enzyme of mitochondria (de Duve *et al.*, 1955). Slight activation by sonication of the microsomal preparation (about 10%) compared with the activation of the mitochon-

drial activity (about 100%) also indicates that the microsomal transhydrogenase activity is indigenous to microsomal membranes. The nature of the microsomal transhydrogenase activity is not clear.

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