Characterization of an NADH-dependent haem-degrading system in ox heart mitochondria

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We report the identification of an NADH-dependent haem-degrading system in ox heart mitochondria. The activity was localized to the mitochondrial inner membrane, specifically associated with complex I (NADH:ubiquinone oxidoreductase). The mitochondrial NADH-dependent haem-degradation activity was highly effective and displayed a rate nearly 60% higher than that of the microsomal activity. The following observations suggested the enzymic nature of the activity: (i) haem degradation by complex I did not proceed upon exposure to elevated temperature and extremes of pH; (ii) it displayed substrate specificity; (iii) it was inhibited by a substrate analogue; and (iv) it showed a cofactor requirement. Moreover, the activity was distinctly different from the ascorbate-mediated haem-degradation activity. Also, complex I differed from the microsomal NADPH:cytochrome *c* (*P*-450) reductase inasmuch as the formation of an effective interaction with the microsomal haem oxygenase could not be detected. Addition of purified haem oxygenase to complex I neither influenced the rate of haem degradation nor resulted in the formation of biliverdin IX α . In contrast, addition of haem oxygenase to NADPH:cytochrome *c* (*P*-450) reductase enhanced the rate of haem degradation by nearly 8-fold, and more than 60% of the degraded haem could be accounted for as biliverdin IX α . The haem-degrading activity of complex I appeared to involve the activity of H₂O₂, as the reaction was inhibited by nearly 90% by catalase, and propentdyopents were detected as reaction products. Intact haemoproteins such as cytochrome *c* and myoglobin were not effective substrates. However, the haem undeca peptide of cytochrome *c* was degraded at a rate equal to that observed for haem. Haematohaem was degraded at a rate 50% lower than that observed for haem. It is suggested that the NADH-dependent haem-degradation system may have a biological role in the regulation of the concentration of respiratory haemoproteins and the disposition of the aberrant forms of the mitochondrial haemoproteins.

INTRODUCTION

It is generally accepted that the degradation of the haem molecule (Fe-protoporphyrin, protohaem) can be catalysed by the extramitochondrial cellular components, including both the microsomal and the cytosolic constituents (Maines, 1984). In the microsomal fractions, the isomer-specific degradation of the haem molecule to bile pigments is carried out by the microsomal haem oxygenase system. The system, which consists of one of the two haem oxygenase isoforms (Maines *et al.*, 1986; Trakshel *et al.*, 1986) and NADPH:cytochrome *c* (*P*-450) reductase, catalyses the cleavage of the haem molecule at the α -meso bridge to form biliverdin IX α isomer (Yoshida & Kikuchi, 1978; Kutty & Maines, 1982; Yoshinaga *et al.*, 1982a).

In the microsomal fractions, a second system, the biological importance of which is debatable, has also been characterized. This system involves the destruction of the haem molecule to pyrrolic complexes by H₂O₂ generated by NADPH:cytochrome *c* (*P*-450) reductase (Masters & Schacter, 1976; Guengerich, 1978; Docherty *et al.*, 1984; Yoshinaga *et al.*, 1982b; Schaefer *et al.*, 1985). The nature of degradation product is, however, controversial; the formation of all possible isomers of biliverdin (α , β , γ and δ), as well as the formation of a

mixture of propentdyopents, have been reported. In addition, Cantoni *et al.* (1981) have observed degradation of the haem molecule to non-biliverdin-type compounds by the cytosolic enzyme xanthine oxidase.

In contrast with the rather extensive body of literature gathered on the degradation of haem by the various cytoplasmic components, very little is known of haem-degrading activity of the mitochondrial constituents. The available information consists of only a preliminary report associating a low-level haem-degrading activity with lipoamide dehydrogenase (Matuda & Nakano, 1984). According to that report, lipoamide dehydrogenase, a soluble mitochondrial protein, in the purified state displays low levels of haem-degradation activity.

The present investigation was undertaken to explore the presence of a highly active membrane-bound haem-degrading system in the mitochondria. The presence of such a system appeared plausible when considering that the organelle is the locus of the respiratory haemoproteins and that the mitochondrion is the cellular site of haem formation. We selected heart for this investigation, since despite the high tissue concentration of the respiratory haemoproteins, the microsomal fraction of the organ displays an exceedingly low rate of haem oxygenase activity (Maines, 1984).

Abbreviation used: HO-1, haem oxygenase isoenzyme 1.

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EXPERIMENTAL PROCEDURES

Materials

Ox hearts were obtained from the local slaughterhouse, transported on ice to the laboratory and processed immediately. Biochemicals were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Tetrapyrrole complexes were purchased from Porphyrin Products, Logan, UT, U.S.A. Haem undecapeptide of cytochrome *c* was prepared as described previously (Peterson *et al.*, 1980; Kutty & Maines, 1982). All chemicals were of highest purity commercially available.

Fractionation of ox-heart homogenate

All tissue preparations were carried out at 0–4 °C. The mitochondrial fraction was prepared by using a procedure based on that of Sordahl *et al.* (1971). The postmitochondrial supernatant fraction was used for preparation of the cytosol and the microsomal fractions.

For large-scale preparation of mitochondria, the following procedure was utilized. A heavy mitochondrial fraction was isolated from ox heart as described by Azzone *et al.* (1979). The homogenization medium contained 0.25 M-sucrose, 1 mM-EGTA and bovine serum albumin (0.5 mg/ml) in 11 mM-Tris/HCl, pH 7.8. The same medium, without EGTA, was used for the resuspension and washing of the mitochondria.

Preparation of the mitoplast

The mitoplast was prepared by treating the mitochondria with digitonin as described by Schnaitman *et al.* (1967) and modified by Krebs *et al.* (1979).

Preparation of mitochondrial complex I (NADH:ubiquinone oxidoreductase)

The procedure of Hatefi (1978) was used for the isolation of complex I from complex I,III (NADH:cytochrome *c* oxidoreductase). In turn, the latter complex was prepared from frozen mitochondria (Hatefi & Stiggall, 1978).

Other enzyme preparations

NADPH: cytochrome *c* (*P*-450) reductase was purified to homogeneity from rat liver microsomes (Yasukochi & Masters, 1976). The preparations exhibited a specific activity of 30–50 μ mol of cytochrome *c* reduced/min per mg of protein. Haem oxygenase isoenzyme I (HO-1) was purified from the liver microsomal fraction obtained from rats treated with bromobenzene (2 mmol/kg injected subcutaneously) for 24 h to a specific activity of nearly 6000 nmol of bilirubin/h per mg of protein (Maines *et al.*, 1986). Biliverdin reductase was purified to a specific activity of approx. 3000 nmol of bilirubin/min per mg of protein (Kutty & Maines, 1981).

Assay procedures

NADH-dependent haem degradation was determined as follows. A reaction mixture (0.7 ml) containing enzyme preparation (protein concentration varied from 70 μ g/ml for complex I to 0.5 mg/ml for the mitochondrial or the microsomal preparations), cholate (0.4%), haem (17 μ M) and NADH (0.5 mM), was incubated in dark at 37 °C with shaking for a period of 10 min. The blank reaction mixture did not contain NADH. At the end of the incubation period the concentration of haem in the blank and test incubation

mixtures was estimated by the pyridine haemochromogen method (Paul *et al.*, 1953). Each reaction mixture was diluted with water to 1.2 ml, mixed with 0.3 ml of pyridine and 0.15 ml of 1.0 M-NaOH, and divided into two cuvettes. A few grains of Na₂S₂O₄ were added to the sample cuvette and a few crystals of K₃Fe(CN)₆ were added to the reference cuvette and the difference spectrum was recorded, from which the haem concentration in the reaction mixture could be calculated. The differences in haem concentration in the blank and test reaction mixtures was used in calculating the rate of NADH-dependent haem-degrading activity. This was expressed as nmol of haem degraded/min per mg of protein.

The following activities were used as marker enzymes. Lactate dehydrogenase was assayed as the marker for the cytosol (Kornberg, 1955). Succinate:cytochrome *c* oxidoreductase was assayed as the marker for the mitochondria, as well as for the inner membrane of the mitochondria (Fleischer & Fleischer, 1967). Rotenone-insensitive NADH:cytochrome *c* oxidoreductase was assayed as the marker for the outer membrane of mitochondria as described by Fleischer & Fleischer (1967). Malate dehydrogenase was measured as the marker for the mitochondrial matrix (Ochoa, 1955). KCN-resistant NADH oxidoreductase activity was used as the microsomal marker (Scalera *et al.*, 1980).

The activity of complex I,III was assessed by measuring the NADH-dependent reduction of cytochrome *c* (Hatefi & Stiggall, 1978), and the activity of complex I was determined by estimating the NADH-dependent reduction of K₃Fe(CN)₆ (Hatefi, 1978). NADPH-dependent cytochrome *c* (*P*-450) reductase activity was estimated by monitoring the reduction of cytochrome *c* in the presence of NADPH (Strobel & Dignam, 1978). Biliverdin reductase activity was assayed by monitoring the formation of bilirubin (Kutty & Maines, 1981). Haem oxygenase activity was estimated as described by Maines *et al.* (1986).

Protein was estimated by the biuret method after solubilization of membrane proteins with deoxycholate (Gornall *et al.*, 1949). The method of Lowry *et al.* (1951), with bovine serum albumin as standard, was used for the determination of protein in purified enzyme fractions. The modification introduced by Dullea & Grieve (1975) was utilized for samples containing detergents.

RESULTS

The subcellular distribution of NADH-dependent haem-degradation activity, haem oxygenase activity, and the marker enzymes in ox heart is shown in Table 1. Haem degradation activity was assessed by measuring haem concentration in incubation system in the presence and the absence of NADH, and haem oxygenase activity was determined from the amount of bilirubin formed. As shown, a rather impressive rate of NADH-dependent haem-degradation activity was detected in the mitochondrial fraction. Indeed, the specific activity of the mitochondrial fraction exceeded that of the microsomal fraction by nearly 60%. Only a minute level of activity was detected in the cytosol fraction. On the other hand, essentially all haem oxygenase activity was associated with the microsomal fractions.

The association of NADH-dependent haem-

Table 1. Subcellular distribution of NADH-dependent haem degradation and haem oxygenase activities in ox heart

The subcellular fractions were prepared from ox heart as described in the Experimental procedures section and used for measurements of the above-indicated parameters. The composition of assay systems used for the assessment of enzyme activities are described in detail in the text. Haem concentration was measured by the pyridine haemochromogen method (Paul *et al.*, 1953). Haem oxygenase activity was measured by using NADPH as the cofactor. Total activity was measured in postnuclear supernatant fraction.

Cell fraction	Succinate:cytochrome <i>c</i> oxidoreductase	KCN-resistant NADH oxidoreductase	Lactate dehydrogenase	NADH-dependent haem degradation	Haem oxygenase
Mitochondria					
Specific activity (nmol/min per mg)	640	6.86	70	0.46	0
Activity as % of total	86	33	2	78	*
Microsomes					
Specific activity (nmol/min per mg)	30	18.72	1810	0.29	0.003
Activity as % of total	1	47	9	15	*
Cytosol					
Specific activity (nmol/min per mg)	10	2.63	7220	0.03	0
Activity as % of total	1	14	94	4	*

* Owing to spectral interference, measurement of haem oxygenase activity in the postnuclear fraction was not feasible.

Table 2. Localization of NADH-dependent haem-degradation activity in the mitochondrial fractions

The mitoplasts, the mitochondrial outer membrane and the microsomal fractions were prepared as described in the text. The activity of the marker enzymes and the magnitude of NADH-dependent haem-degradation activity were assessed as described in detail in the Experimental procedures section.

Fraction	Succinate:cytochrome <i>c</i> oxidoreductase	Rotenone-insensitive NADH:cytochrome <i>c</i> oxidoreductase	NADH-dependent haem degradation	KCN-resistant NADH oxidoreductase
Mitoplast				
Specific activity (nmol/min per mg)	914	18	1.06	2
Activity as % of total mitochondrial activity	96	15	77	11
Outer membrane				
Specific activity (nmol/min per mg)	86	143	0.57	29
Activity as % of total mitochondrial activity	3	88	22	84

degradation activity with the mitochondria was further verified (Table 2). Mitochondrial fraction was treated with digitonin to yield mitoplast and outer-membrane fractions. As suggested by the distribution of marker enzymes, the contamination of the mitoplast with the outer membrane and microsomes was minimal. Moreover, the data suggest that, for the most part, NADH-dependent haem-degradation activity of the mitochondrial fraction is associated with the mitoplast.

As is shown, more than 75% of the haem-degrading activity was present in the fraction which contained the bulk of succinate:cytochrome *c* oxidoreductase activity.

Subsequently, the enzymic nature of the reaction was investigated and the role of ascorbate was examined. It is known that, under oxidizing conditions, ascorbate catalyses the degradation of haem (O'Carra & Colleran, 1969). As shown in Table 3, ascorbate effectively supported the degradation of haem. Indeed, the rate of

Table 3. Evidence for the enzymic nature of the mitoplast NADH-dependent haem-degradation reaction

The mitoplast preparation was obtained from the heart mitochondrial fraction as described in the Experimental procedures section. Degradation of haem was assessed from difference in haem concentration at the end of the incubation period (10 min, 37 °C) in the presence and the absence of reducing agents or cofactors. The control assay system contained mitoplasts, 0.5 mg of protein/ml, NADH (0.5 mM), cholate (0.4%) and haem (17 μ M), in phosphate buffer (0.1 M, pH 6.9). The concentration of ascorbate was 10 mM, and those of Fe^{2+} and EDTA were 1 mM.

Assay system	Haem degraded (nmol/min per mg)
Mitoplast + NADH + haem	1.06
Mitoplast + ascorbate + haem	1.48
Heat-treated mitoplast + NADH + haem	0.04
Heat-treated mitoplast + ascorbate + haem	1.31
Mitoplast treated at pH 2.0 + NADH + haem	0.04
Mitoplast treated at pH 11.5 + NADH + haem	0.04
Mitoplast + NADH + haem + Zn-protoporphyrin (40 μ M)	0.55
Mitoplast + haem + NADPH (0.5 mM)	0.17
Mitoplast + NADH + pyridine haemochrome	0.00
Mitoplast + haem + Fe^{2+} *	0.13
Mitoplast + haem + EDTA + Fe^{2+}	0.13

* 0.1 M-Hepes buffer, pH 6.9, instead of phosphate buffer was used when Fe^{2+} was present.

Fig. 1. Time course of haem degradation by mitoplasts

A reaction mixture containing mitoplasts (0.5 mg of protein/ml), sodium cholate (0.4%) and haem (17 μ M) in 0.1 M-potassium phosphate buffer, pH 6.9, was incubated in the presence (●) or absence (○) of NADH (0.5 mM) at 37 °C in the dark with shaking. At indicated time intervals, portions (0.7 ml) were removed for the determination of haem concentration. The amount of haem degraded was calculated by subtracting the amount present at specified time point from that present at zero time.

haem degradation in the presence of ascorbate exceeded the rate supported by NADH. However, the NADH-dependent activity was heat-labile, and a 95% loss in activity was noted when the mitoplast preparation was heated for 15 min at 90 °C. In contrast, the ascorbate-dependent activity was not susceptible to heat inactivation. Also, the NADH-dependent activity was nearly abolished when the mitoplast preparation was exposed to extremes of pH, i.e. pH 2 and pH 11.5. Moreover, the addition to the assay system of Zn-protoporphyrin, a structural analogue of the substrate, haem, resulted in a 50% decrease in activity. NADPH was not an effective substitute for NADH as the cofactor; NADPH at the concentration of 0.5 mM was only 16% as effective as NADH (0.5 mM). In addition, pyridine haemochrome was not degraded by mitoplasts in the presence of NADH. Fe^{2+} , either alone or with EDTA, was not effective in replacing NADH.

Further data supporting the enzymic nature of the NADH-dependent haem oxidation activity are provided in Fig. 1. In this study the time course of haem degradation by mitoplasts was studied in a large reaction mixture, and the concentration of haem in portions of the mixture was determined at specified time intervals. As shown, the linearity of the reaction rate was lost after 10 min of incubation. Moreover, although a measurable amount of haem was degraded in the absence of NADH, omission of NADH from the system resulted in considerable loss of haem-degrading activity. It should be noted that all data presented here have been corrected for activity in the absence of NADH by including a blank reaction mixture devoid of a cofactor in all experiments.

The distribution of haem-degradation activity in the mitoplast was investigated. The inner-membrane and the matrix fractions were separated by extensive sonication of the mitoplast preparation followed by differential centrifugation. More than 90% of haem-degradation activity and succinate:cytochrome *c* oxidoreductase activity (marker enzyme for inner membrane) were associated with the pellet (inner membrane), whereas over 70% of malate dehydrogenase (marker for matrix) was associated with the supernatant (matrix). Therefore the inner membrane appeared to be the site of haem-degrading activity. Within the mitochondrial inner membrane, the complex I portion of complex I,III of the electron-transport chain is the initial acceptor of electrons from NADH. Hence the possibility was examined that the NADH-dependent haem-degradation activity is associated with complex I. In this experiment the relative distribution of activity in various fractions obtained during the purification of complex I was compared with the accompanying NADH-dependent ferricyanide-reduction rate displayed by the fractions (Table 4); the latter parameter was used as a marker for complex I (Hatefi, 1978). As is shown, haem oxidation was associated with complex I insofar that the activity co-purified along with the complex.

The haem-degradation activity of complex I was sensitive to cholate concentration in the reaction mixture. The activity was accelerated with increasing cholate concentrations, with the maximum activity being attained at a concentration near 0.4%. At this concentration the rate of haem degradation exceeded that observed in the absence of the detergent by nearly 5-fold. The higher concentrations of cholate proved inhibitory to haem oxidation. The observed detergent requirement

Table 4. Distribution pattern of NADH-dependent haem-degradation activity in mitochondrial inner-membrane fractions

The S1 and complex I,III were prepared from mitochondria as described by Hatefi & Stiggall (1978). Complex I was prepared from complex I,III as described by Hatefi (1978). The preparations were used for measurements of NADH-dependent haem-degradation activity and the rate of ferricyanide reduction as described in the Experimental procedures section.

Fraction	Total protein (mg)	NADH-dependent haem degradation (nmol/min per mg)	Ferricyanide reduction (μ mol/min per mg)
Mitochondria	898.0	0.53	4.57
S1	175.0	2.11	8.96
Complex I,III	23.0	5.04	30.65
Complex I	10.5	8.64	41.87

Fig. 2. Formation of propentdyopents as products of haem oxidation mediated by complex I, NADPH:cytochrome *c* (*P*-450) reductase or H_2O_2

A reaction mixture (0.7 ml) containing complex I (70 μ g of protein/ml), cholate (0.4%), haem (4 μ M) and NADH (0.5 mM) in 0.1 M-potassium phosphate buffer, pH 6.9, was incubated in a shaking incubator at 37 °C for 30 min. Thereafter, an equal volume of 2 M-NaOH was added and the reaction mixture was divided into reference and test reaction mixtures. A small amount of $Na_2S_2O_4$ was added to the test system, and both the test and the blank mixtures were kept at 100 °C for 2 min. After cooling to room temperature, the spectrum of test mixture against the reference mixture was recorded. Also, a reaction mixture (0.7 ml) containing NADPH:cytochrome *c* (*P*-450) reductase (6.5 μ g of protein/ml), haem (4 μ M) and NADPH (0.5 mM) in 0.3 M-potassium phosphate buffer, pH 7.4, was processed. Propentdyopents produced by heating a solution of haem (1%) at pH 8.0 with H_2O_2 (5%) were converted into pentdyopents by the addition of NaOH and $Na_2S_2O_4$ and used for the reference spectrum. —, H_2O_2 ; ····, NADPH:cytochrome *c* (*P*-450) reductase; ----, complex I.

may be related to the ability of the detergent to facilitate a productive interaction between complex I, which is an integral part of mitochondrial membrane, and haem, a lipophilic substrate.

In the next experiment we investigated the nature of oxygen radical involved in complex-I-catalysed haem degradation by evaluating the effects of catalase and

superoxide dismutase on the magnitude of the activity of the complex. The addition of catalase, at a final concentration of 100 units/ml, to the assay system inhibited degradation of haem by approx. 80%. In contrast, an appreciable inhibition of activity was not detected when superoxide dismutase was added to the assay system (100 units/ml). On the basis of these findings it appeared that the catalytic activity of complex I was mediated through H_2O_2 generated by the complex.

The results of the following experiments suggest the formation of propentdyopents among the products of oxidation of haem by complex I (Fig. 2). For these experiments reaction mixtures, either containing complex I, NADH and haem, or NADPH, haem and NADPH:cytochrome *c* (*P*-450) reductase, were incubated at 37 °C, in the dark, until at least 95% of the haem was degraded. The positive control consisted of a system containing H_2O_2 and haem. The propentdyopents formed during incubation period were converted into pentdyopents, by heating the mixtures with $Na_2S_2O_4$ in the presence of 1 M-NaOH (Stokvis reaction) (Von-Dobeneck, 1979). Pentdyopents are characterized by an absorption maximum at ~ 525 nm. As shown in Fig. 2, the product of degradation of haem by complex I was readily converted into pentdyopents. Also, the product of oxidation of haem catalysed by NADPH:cytochrome *c* (*P*-450) reductase, and H_2O_2 , underwent the Stokvis reaction, as previously reported by Guengerich (1978).

In subsequent experiments the possibility of the formation of biliverdin IX α as the end product of haem degradation by complex I was investigated. Moreover, the possibility of interaction between complex I and haem oxygenase and the formation of the bile pigment was examined. In those experiments, the formation of biliverdin was measured in the presence of exogenously added biliverdin reductase, which converts biliverdin IX α to bilirubin IX α . Also, a purified preparation of liver HO-1 was utilized. The reference assay system consisted of an incubation mixture containing purified NADPH:cytochrome *c* (*P*-450) reductase and HO-1. In all systems, NADH was used as the cofactor. This selection did not pose a problem, since NADH can effectively serve as the cofactor for degradation of haem by haem oxygenase (Maines *et al.*, 1986). Moreover, insofar as cholate is inhibitory to HO-1 (Maines *et al.*, 1986), the detergent was not added to the systems which contained the enzyme; on the other hand, the activity of purified haem oxygenase is routinely detected in the presence of Triton X-100 (Trakshel *et al.*, 1986). Therefore this

Table 5. Interaction of complex I and NADPH:cytochrome *c* (*P*-450) reductase with haem oxygenase

The enzymes and complex I were obtained as described in the Experimental procedures section. Degradation of haem and the formation of bilirubin were determined in assay systems (0.7 ml) containing the enzyme sources indicated in the Table: haem (4 μ M), NADH (0.5 mM), biliverdin reductase (0.5 μ g/ml) and detergent. When the assay system contained complex I, cholate was used as the detergent (0.4%), otherwise Triton X-100 (0.04%) was added. The concentrations of complex I, NADPH:cytochrome *c* (*P*-450) reductase and haem oxygenase used in the assay systems were 70 μ g of protein/ml, 6.5 μ g of protein/ml and 4 μ g of protein/ml respectively. The amount of haem degraded was determined as described in the Experimental procedures section.

Enzyme system	Haem degraded (nmol/h)	Bilirubin formed (nmol/h)
NADPH:cytochrome <i>c</i> (<i>P</i> -450) reductase + haem oxygenase	5.52	3.48
Complex I + haem oxygenase	6.12	0
Complex I	5.82	0
NADPH:cytochrome <i>c</i> (<i>P</i> -450) reductase	0.66	0

Table 6. Degradation of intact haemoproteins and haem derivatives by complex I

The complex I of the mitochondrial inner membrane was prepared as described by Hatefi (1978). The control system for measuring the NADH-dependent haem degradation consisted of complex I (70 μ g of protein/ml), cholate (0.4%), substrate (17 μ M), and NADH (0.5 mM), in 0.1 M-potassium phosphate buffer, pH 6.9. The extent of haem-degrading activity was assessed as described in the Experimental procedures section.

Substrate	Haem degraded (nmol/min/mg)
Haem	8.8
Haematohaem	4.2
Cytochrome <i>c</i>	0
Myoglobin	0
Haem undecapeptide of cytochrome <i>c</i>	9.2

detergent was present in the haem oxygenase assay system. As shown in Table 5, the NADPH:cytochrome *c* (*P*-450) reductase and haem-oxygenase-containing system, as expected, effectively catalysed the degradation of haem, and over 60% of the haem degraded could be accounted for by the amount of bilirubin formed. However, the formation of biliverdin IX α could not be detected in the system containing haem oxygenase and complex I. Also, this addition did not enhance the rate of haem degradation by the complex. In the systems containing complex I or NADPH:cytochrome *c* reductase alone, the formation of bilirubin could not be detected.

The substrate specificity of complex I was examined by measuring the degradation of certain intact haemoproteins, including cytochrome *c* and myoglobin, certain haem derivatives, including haematohaem, and haem undecapeptide of cytochrome *c* (Table 6). As is shown, intact haemoproteins were not degraded to any measurable extent by complex I. In contrast, haem undecapeptide was degraded essentially at the same rate as was haem. The rate of haematohaem degradation was 50% of that of haem. The observed substrate specificity of complex I is consistent with an enzymic mode of haem degradation activity and suggests that a specific orientation of the haem molecule and its binding to the complex are required for catalytic action.

DISCUSSION

The present study reports the identification and the characterization of a haem-degrading system in ox heart mitochondria. Our findings show that the haem-degrading activity is predominantly associated with complex I of the inner membrane of the organelle and suggest that the activity is enzymic in nature. Complex I has been extensively characterized and has been shown to catalyse the oxidation of NADH, with ubiquinone as the electron acceptor. Complex I is an FMN-containing protein complex, and the flavoprotein portion is implicated as the primary oxidant of NADH (Hatefi, 1985). Also, this portion of the complex has been implicated as a site of superoxide anion and H₂O₂ formation in the mitochondria (Turrens & Boveris, 1980). In this respect complex I resembles the microsomal enzyme NADPH:cytochrome *c* (*P*-450) reductase, also a flavoprotein and noted for its ability to degrade haem by generating H₂O₂ (Masters & Schacter, 1976; Guengerich, 1978).

The enzymic nature of NADH-dependent haem-degradation activity of complex I is suggested by several lines of evidence, and the activity is clearly distinct from the previously described system, namely the 'haem α -methenyl oxygenase' of Nakajima *et al.* (1963). The haem-degrading activity of the latter system, which was originally believed to be enzyme-mediated, was later shown to be attributable to ascorbate endogenous to the liver homogenate (Levin, 1967). As noted in the present study (Table 3), although both the ascorbate-dependent and the NADH-dependent activities were associated with the complex I preparation, only the NADH-dependent activity was sensitive to heat denaturation. In addition, unlike the ascorbate-dependent activity, which is reported insensitive to extremes of the pH (Levin, 1967), the NADH-dependent haem-degradation activity displayed a high degree of sensitivity to changes in pH. Moreover, the NADH-dependent activity exhibited the following properties: substrate specificity (Tables 3 and 6), inhibition by substrate analogue (Table 3) and requirement for a specific cofactor (Table 3), such properties fulfilling the criteria commonly assumed for enzymic reactions.

The ability to degrade the haem molecule to pyrroles other than the bile pigments is not common to all flavoproteins. In the microsomal fraction, apparently only NADPH:cytochrome *c* (*P*-450) reductase can catalyse such degradation activity (Guengerich, 1978). However, although in this respect the reductase may differ from the other microsomal flavoproteins, the

results of the present study show that it is not a unique cellular flavoprotein in its ability to degrade haem. It is noteworthy that the presently described NADH-dependent haem-degrading activity of complex I constitutes the first such activity ascribed to mitochondrial membranes. Furthermore, as shown here, this activity proceeds at a rather high rate.

Considering the high levels of haem oxygenase activity which are associated with the microsomal fraction of various organs, haem degradation by the microsomal NADPH:cytochrome *c* (*P*-450) reductase appears to be, at most, an activity auxiliary to that of the haem oxygenase. As shown in Table 5, in the absence of haem oxygenase, degradation of haem catalysed by the reductase alone amounted to only a fraction of that observed in the presence of haem oxygenase in the system. Moreover, as judged by the absence of detectable amounts of bilirubin, this reaction did not lead to the formation of biliverdin IX α isomer. Accordingly, this finding suggests the possibility that the primary function of the reductase in a system which contains haem oxygenase is to transport reducing equivalents for the isomer-specific oxidation of haem by haem oxygenase. On the other hand, the NADH-dependent haem-degradation activity of complex I may constitute a functional haem-degrading system in mitochondria. This suggestion is plausible in the light of the observation that a measurable rate of haem oxygenase activity could not be detected in the mitochondria (Table 1).

In contrast with the reductase, the haem-degrading activity of complex I was not facilitated in the presence of haem oxygenase, suggesting the inability of the haem-haem-oxygenase complex to accept electrons from the flavoprotein. The fact that complex I utilizes NADH as the cofactor does not appear to account for inability of the complex to interact effectively with haem oxygenase. Haem oxygenase activity can utilize NADH as the cofactor (Maines *et al.*, 1986), and NADPH:cytochrome *c* (*P*-450) reductase can transport electrons from NADH to haem bound to haem oxygenase (Noguchi *et al.*, 1979). Nonetheless, the mechanisms of haem degradation by complex I and NADPH:cytochrome *c* (*P*-450) reductase share certain properties: both systems appear to involve the activity of H₂O₂ produced in the system (Fig. 2) and exhibit a requirement for the presence of a detergent for optimum activity (Table 5; Guengerich, 1978).

The mitochondria are the site of haem biosynthesis and the location of the respiratory haemoproteins. It follows, unless the degradation of haem and its complexes solely takes place extramitochondrially, the presence of a haem-degrading system in the mitochondria may well serve a useful function in the control of the molecular integrity of respiratory haemoproteins, and the regulation of the total cellular haem level. This concept is consistent with the finding that complex I exhibited a rather elevated rate of activity when haem undecapeptide of cytochrome *c*, a proteolytic fragment of cytochrome *c*, was used as the substrate (Table 6). The presence of a system with reactivity toward peptide-bound haem complexes perceivably could prevent accumulation of aberrant haemoproteins and their denatured derivatives. This would also pre-empt the necessity for the transport of such complexes from the mitochondria to the cytoplasm. This postulate is supported by the report that aberrant forms of

respiratory haemoproteins are degraded within the mitochondria (Luzikov, 1985).

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