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Mouse protoporphyrinogen oxidase

Kinetic parameters and demonstration of inhibition by bilirubin

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The penultimate step of haem biosynthesis, the oxidation of protoporphyrinogen to protoporphyrin, was examined with purified murine hepatic protoporphyrinogen oxidase (EC 1.3.3.4) in detergent solution. The kinetic parameters for the two-substrate (protoporphyrinogen and oxygen) reaction were determined. The limiting K_m for protoporphyrinogen when oxygen is saturating is 6.6 μM, whereas the K_m for oxygen with saturating concentrations of protoporphyrinogen is 125 μM. The $k_{cat.}$ for the overall reaction is 447 h^{-1}. The ratio of $k_{cat.}$ to the K_m for protoporphyrinogen is approx. 20-fold greater than the $k_{cat.}/K_{m,O_2}$ ratio. The ratio of protoporphyrin formed to dioxygen consumed is 1:3. Ubiquinone-6, ubiquinone-10 and dicoumarol stimulate protoporphyrinogen oxidase activity at low concentrations (less than 15 μM), whereas coenzyme Q_0 and menadione show no activation at these concentrations. Above 30 μM, all five quinones inhibit the enzyme activity. FAD does not significantly affect the activity of the enzyme. Bilirubin, a product of haem catabolism, is shown to be a competitive inhibitor of the penultimate enzyme of the haem-biosynthetic pathway, protoporphyrinogen oxidase, with a calculated K_i of 25 μM. The terminal enzyme of haem-biosynthetic pathway, namely ferrochelatase, is not inhibited by bilirubin at concentrations over double the K_i value for the oxidase. In contrast with other enzymic systems, the toxicity of bilirubin is not reversed by binding to albumin.

INTRODUCTION

Protoporphyrinogen oxidase (EC 1.3.3.4), the penultimate enzyme of the haem-biosynthetic pathway, catalyses the removal of six hydrogen atoms from protoporphyrinogen IX to form protoporphyrin IX (Poulson, 1976; Poulson & Polglase, 1975). This enzyme is an integral membrane protein of the mitochondrial inner membrane, but its exact orientation in the bilayer is unknown at the present time (Deybach *et al.*, 1985) and little is known about the enzyme, since it has only recently been purified to apparent homogeneity (Dailey & Karr, 1987).

Although the oxidation of protoporphyrinogen to protoporphyrin *in vitro* can take place non-enzymically, the presence of an enzyme that catalyses this reaction seems to be ubiquitous in cells having aerobic metabolism (Jackson *et al.*, 1974; Poulson & Polglase, 1975; Smith *et al.*, 1976). At present, oxygen is the only known electron acceptor for the protoporphyrinogen oxidase-catalysed reaction in eukaryotic cells (Poulson, 1976). In prokaryotes, Jacobs & Jacobs (1975*a*,*b*) have reported electron acceptors other than oxygen. Nitrate and fumarate could replace oxygen in *Escherichia coli* if cells were grown anaerobically in the presence of the compounds. On the basis of their data, they proposed a coupling between the electron-transport chain and the protoporphyrinogen-oxidizing system.

Protoporphyrinogen oxidase has been implicated in the genetically inherited disease variegate porphyria (Brenner & Bloomer, 1980), although opinions are divided as to whether protoporphyrinogen oxidase alone, or ferrochelatase and the oxidase, are associated with the disease (Becker *et al.*, 1977; Brenner & Bloomer, 1980; Deybach *et al.*, 1981; Viljoen *et al.*, 1983). Originally it was believed that ferrochelatase was the deficient enzyme (Becker *et al.*, 1977), but later Brenner & Bloomer (1980) and Deybach *et al.* (1981) clearly showed that protoporphyrinogen oxidase activity was decreased in variegate-porphyria patients, whereas ferrochelatase activity was normal. Since then, Viljoen *et al.* (1983) confirmed the lower activity of protoporphyrinogen oxidase, but they stressed that a concurrent structural change in ferrochelatase could occur.

More recently it was reported that leucocytes of patients with Gilbert's syndrome (a hereditary chromic hyperbilirubinaemia) exhibit decreased protoporphyrinogen oxidase activity. In Gilbert's syndrome this activity was reported to be decreased to 30% of normal values when measured in peripheral-blood cells (McColl *et al.*, 1985). Whether this decreased activity was attributable to a genetic defect of protoporphyrinogen oxidase, such as is found in variegate porphyria, or resulted from an effect of bilirubin on the normal enzyme, was not determined.

In the present paper we examine the effects of oxygen concentration on protoporphyrinogen oxidase activity. The kinetic parameters of this reaction were determined for the two substrates, protoporphyrinogen and oxygen, and the ratio between protoporphyrin produced and

Abbreviations used: CoQ_0, CoQ_6 and CoQ_{10}, coenzymes Q_0, Q_6 and Q_{10}.

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oxygen consumed is presented. In addition, several quinones were investigated as possible electron acceptors.

We also report the effect of unconjugated bilirubin on the activity of purified protoporphyrinogen oxidase. The results clearly demonstrate that bilirubin, either in the presence or absence of a bilirubin-binding protein, is an effective inhibitor of the enzyme.

EXPERIMENTAL PROCEDURES

Materials

All porphyrins and bilirubin were obtained from Porphyrin Products, Logan, UT, U.S.A. 3,3'-Methylene-bis(4-hydroxycoumarin), menadione, CoQ_0, CoQ_6 and CoQ_{10} were purchased from Sigma. All other reagents were of the highest purity available.

Mouse livers were obtained from either Bio Trol, Indianapolis, IN, U.S.A., or Pel Freeze Biologicals, Rogers, AR, U.S.A.

Enzymes

Ferrochelatase was purified from mouse liver mitochondria by the method of Dailey & Fleming (1983) and Dailey *et al.* (1986). Purified protoporphyrinogen oxidase (free of ferrochelatase activity) was eluted from the tandem columns of Reactive Red 120–Sepharose CL-6B and Reactive Blue–Sepharose CL-6B with 1.0% Triton X-100 and 0.5 M-KCl as described previously for the purification of ferrochelatase (Dailey & Fleming, 1983). The purification of protoporphyrinogen oxidase to apparent homogeneity was recently reported by Dailey & Karr (1987).

Enzyme assays

Ferrochelatase was assayed by using the pyridine haemochromogen assay (Dailey *et al.*, 1986). Protoporphyrinogen oxidase activity was measured by a modification of the method of Brenner & Bloomer (1980). All the results presented are means for at least three determinations, with S.D. values less than $\pm 10\%$ of the mean. The assay mixture contained 0.1 M-Tris/acetate, pH 8.1, 0.1% (w/v) Brij 35, protoporphyrinogen and enzyme. The reaction was started by the addition of substrate. Samples (0.1 ml) were withdrawn every 10 min, added to 0.9 ml of 10 mM-Tris/acetate (pH 8.1)/0.1% Brij 35 and the fluorescence determined in a Perkin–Elmer 650-40 spectrofluorometer (excitation wavelength, 405 nm; emission wavelength, 635 nm). The assay was run at 37 °C in the dark, and a blank, containing all reagents but without the enzyme, was run for each set of samples. Protoporphyrinogen IX was prepared just before use by reduction of protoporphyrin IX with sodium amalgam (Jacobs & Jacobs, 1982). The pH was adjusted to approx. 7.0 by titration with a 1.5 M-solution of Mops (free acid).

The different protoporphyrinogen stock solutions used in the kinetic assays were made by the dilution of the protoporphyrinogen solution described above in a buffer containing 0.1 M-Tris, pH 8.0, and 1 mM-EDTA. The actual concentration of protoporphyrinogen was determined spectrophotometrically after photochemical oxidation of the protoporphyrinogen substrate (Jacobs & Jacobs, 1982).

Solutions (1.0 M) of menadione, dicoumarol, CoQ_0, CoQ_6 and CoQ_{10} were prepared by dissolving each compound in ethanol. Different volumes of quinones were added to the enzyme system and the assay reaction was then adjusted to a constant final volume with the assay buffer. The effect of different Q coenzymes on protoporphyrinogen oxidase activity was examined by using enzyme that had been reconstituted into phospholipid vesicles as described by Ferreira & Dailey (1987).

Protoporphyrinogen oxidase activity was measured with various concentrations of oxygen. These assays were run in closed fluorimetric cuvettes, which were filled and stoppered. In these experiments oxygen concentrations were varied by using mixtures of two buffers which had been saturated with either O_2 or N_2 gas. The reaction was started by the injection of substrate through the stopper with an 18-gauge hypodermic needle. The dissolved-oxygen concentrations were determined both by the Winkler titrimetric method as described by Carpenter (1965) and with an oxygen electrode. All results are means for at least three determinations.

Oxygen and protoporphyrinogen consumptions were monitored simultaneously. O_2 uptake was measured at 37 °C under the same conditions as the fluorometric assay, using a Clark-cell oxygen electrode (Yellow Springs Instruments). The continuous readings were recorded on a Perkin–Elmer recorder. Protoporphyrin production was monitored as described above, by withdrawing 0.1 ml samples from the Clark cell every 10 min.

H_2O_2 was determined fluorometrically by the homovanilic acid/peroxidase coupled assay (Guilbeault, 1967; Green & Wu, 1986); 0.1 mM-NaCN was added to the assay buffer to block any endogenous catalase present as a contaminant.

Protein determination

Protein concentrations were determined with the Pierce BCA protein reagent, with bovine serum albumin as a standard.

RESULTS

Oxidation of protoporphyrinogen by protoporphyrinogen oxidase: role of oxygen

The reaction catalysed by protoporphyrinogen oxidase was examined, assuming protoporphyrinogen to be the primary and oxygen to be the secondary substrate. Oxygen in the concentration range of 204–544 μM stimulated protoporphyrinogen oxidase activity. Assuming rapid-equilibrium binding, the double-reciprocal plot of the oxidase's activity provides the apparent $V_{max.}$ and K_m values for protoporphyrinogen at various concentrations of oxygen. The results obtained from this primary plot were used in secondary plots (Figs. 1 and 2). In Fig. 1(*a*) the ratio of O_2 and the apparent $V_{max.}$ was plotted against the secondary-substrate (O_2) concentration. The slope of this secondary plot gives the reciprocal as $V_{max.}$, and the intercepts on the O_2 axis indicates the (negative) K_m for O_2 (Cornish-Bowden, 1981). In this case the calculated $V_{max.}$ was 6876 nmol of protoporphyrinogen/h per mg of protein and the K_{m,O_2} was 125 μM. This K_m can be defined as the limiting constant for O_2 when protoporphyrinogen is saturating.

The secondary plot in Fig. 1(*b*) illustrates the relationship between the $[O_2]K_m^{app.}/V_{max.}^{app.}$ ratio against the oxygen concentration. The limiting K_m for proto-

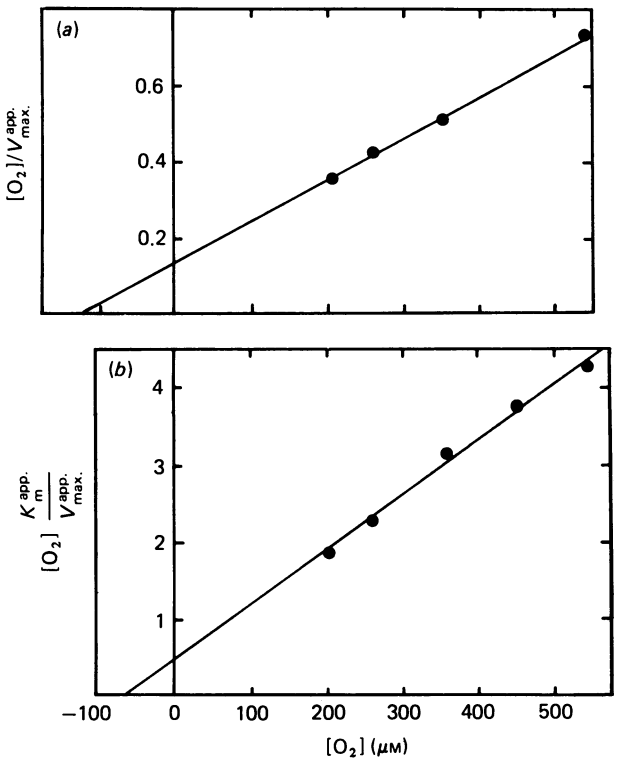

Fig. 1. Secondary plots for the protoporphyrinogen oxidase reaction

(*a*) Determination of K_{m,O_2}. The intercept on the O_2 axis gives the negative value of the constant; K_{m,O_2} is 125 μM. (*b*) Determination of $K_m^{protoporphyrinogen}$ and $K_i^{protoporphyrinogen}$. The slope gives $K_m^{protoporphyrinogen}/V_{max.}$ and the intercept on the O_2 axis gives the negative of $K_i^{protoporphyrinogen}$ $K_{m,O_2}/K_m^{protoporphyrinogen}$ (the velocity is given in arbitrary units).

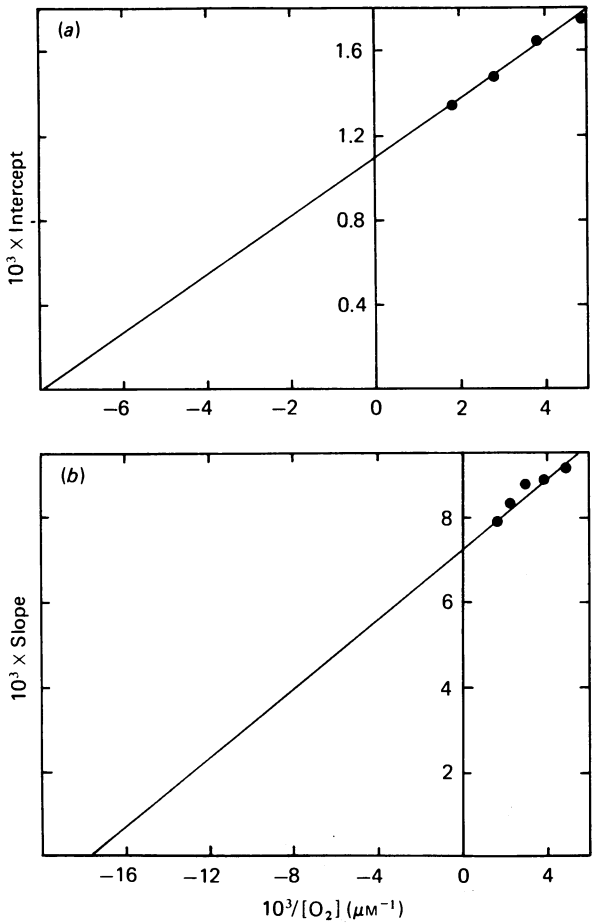

Fig. 2. Dixon & Webb (1979) plots for the protoporphyrinogen oxidase reaction

(*a*) Plot of the intercepts of the $1/v$ axis from the primary plot versus the reciprocal of the oxygen concentration. K_{m,O_2} is 126 μM. (*b*) Plot of the slopes of the primary plot versus the reciprocal of the oxygen concentration. $K_m^{protoporphyrinogen}$ is 6.6 μM (the velocity is given in arbitrary units).

porphyrinogen when O_2 was saturating ($K_m^{protoporphyrinogen}$) was 6.6 μM. This constant is somewhat higher than the value of 5.6 μM reported for protoporphyrinogen oxidase when it was assayed under non-saturating-O_2 conditions (Ferreira & Dailey, 1987). However, the values of the apparent specificity constants ($k_{cat.}^{app.}/K_m^{app.}$) do not vary significantly with increasing concentrations of oxygen (Table 1). The $K_i^{protoporphyrinogen}$, defined as the limiting value of the Michaelis constant for protoporphyrinogen values when the oxygen concentration approaches zero, was 3.5 μM. The data obtained from the primary plot were also plotted as described by Dixon & Webb (1979). The intercepts on $1/v$ axes and the slopes were plotted against the reciprocal of the oxygen concentration (Figs. 2*a* and 2*b*). The K_{m,O_2} (126 μM), the $V_{max.}$ (6840 nmol of protoporphyrinogen/h per mg of protein) and the $K_m^{protoporphyrinogen}$ (6.6 μM) calculated from Figs. 2(*a*) and 2(*b*) were in agreement with the values reported above. The kinetic parameters for the overall reaciton are shown in Table 2.

Table 1. Apparent catalytic and specificity constants of the protoporphyrinogen oxidase catalysed reaction at different concentrations of O_2

$[O_2]$ μM	$k_{cat.}^{app.}$ (h^{-1})	$k_{cat.}^{app.}/K_m^{app.}$ (μM$^{-1}\cdot h^{-1}$)
204	276	52
261	293	54
355	327	54
450	278	58
540	360	61

Stoichiometric ratio of dioxygen consumption to oxidation of protoporphyrinogen

The reaction catalysed by protoporphyrinogen oxidase was monitored by protoporphyrin formation and also by the oxygen consumed. The stoichiometric ratio of O_2 consumed to protoporphyrin produced was 3.0±0.2 (Table 3). This value is independent of variations in oxygen or protoporphyrinogen concentrations. Because of the nature of this value, we examined whether H_2O_2 was a product of the reaction. It was found that H_2O_2 was formed during protoporphyrinogen oxidation (results not shown), but it was not possible to accurately establish a molar ratio between protoporphyrinogen oxidized and H_2O_2 produced.

Table 2. Kinetic parameters for the protoporphyrinogen oxidase catalysed reaction

Parameter	Value
$V_{max.}$	6900 nmol·h^{-1}·mg of protein^{-1}
$K_m^{protoporphyrinogen}$	6.5 μM
K_{m,O_2}	125 μM
$k_{cat.}$	450 h^{-1}
$k_{cat.}/K_m^{protoporphyrinogen}$	68 μM^{-1}·h^{-1}
$k_{cat.}/K_{m,O_2}$	3.6 μM^{-1}·h^{-1}

Table 3. Comparison of molar ratio of O_2 consumed and protoporphyrinogen oxidized in the protoporphyrinogen oxidase-catalysed reaction

(*a*) O_2 consumed (nmol·h^{-1})	(*b*) Protoporphyrinogen oxidized (nmol·h^{-1})	(*a*)/(*b*)
270	102	2.6
280	87	3.2
339	108	3.1
440	142	3.1
594	188	3.2

Inhibition by bilirubin

To determine the effect of bilirubin on protoporphyrinogen oxidase activity, different concentrations of free unconjugated bilirubin were added to the assay mixture and the enzyme activity was measured (Fig. 3). Because, *in vivo*, organic-anion-binding proteins (e.g. albumin, ligandin) can bind to bilirubin, preventing its inhibitory effect on activities of several enzymes (Kamisaka *et al.*, 1975), bovine serum albumin was also included in the assay mixture in some experiments.

At a bilirubin concentration of 68 μM, the oxidase activity was decreased to approx. 30% of the control activity, whether or not albumin was present (Fig. 3). In fact, the inhibition curve of protoporphyrinogen oxidase by bilirubin coincided with that of bound unconjugated bilirubin. This is in contrast with the action of albumin in reversing bilirubin inhibition of mitochondrial enzymes involved in respiration.

In order to investigate the possibility that bilirubin affects not only the penultimate enzyme, but also the terminal enzyme, of the haem-biosynthetic pathway, ferrochelatase activity was determined in the presence of free unconjugated bilirubin. Over the same range of bilirubin concentrations used for protoporphyrinogen oxidase, ferrochelatase activity was not inhibited (Fig. 3). The possibility that either bilirubin or protoporphyrin was adsorbed by other proteins, extraneous lipids or membrane fragments was minimized in our study by the use of purified enzymes.

The kinetics of protoporphyrinogen oxidase inhibition were examined by assaying protoporphyrinogen oxidase activity at different concentrations of free unconjugated bilirubin and substrate. The graphical representation of the data in a double-reciprocal plot, as shown in Fig. 4, indicates that bilirubin is a competitive inhibitor of the oxidase with respect to protoporphyrinogen. From the

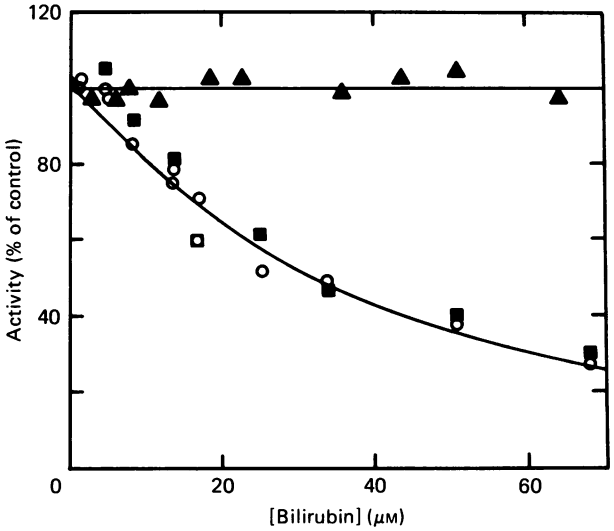

Fig. 3. Effect of bilirubin on the two terminal enzymes of the haem-biosynthetic pathway

The inhibition (percentage of the enzymic activity in the absence of bilirubin) of protoporphyrinogen oxidase in the absence (○) or presence (■) of bovine serum albumin (0.45 g·l^{-1}) and inhibition of ferrochelatase (▲) as a function of bilirubin concentration are shown. Bilirubin stock solution was made by moistening 1 mg of free bilirubin with 50 μl of 2 M-NH_3, adding 500 μl of 20% (v/v) Triton X-100 and adjusting to a final volume of 5 ml with distilled water. Bilirubin was added to the assay mixture before the addition of enzyme and substrate.

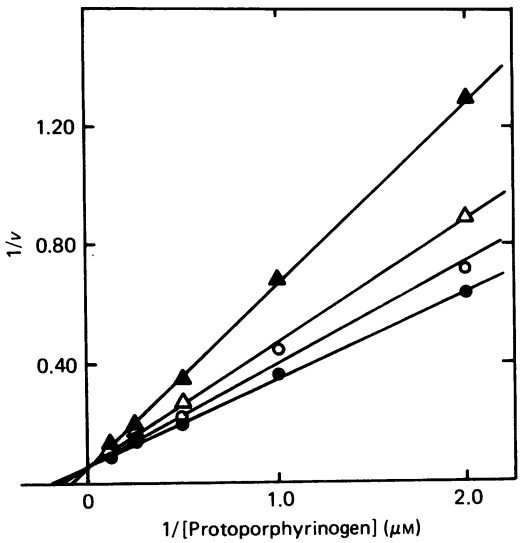

Fig. 4. Competitive inhibition of protoporphyrinogen oxidase by bilirubin

Lineweaver–Burk plots of the initial velocity of protoporphyrinogen oxidase in the presence of (●) 0 μM-, (○) 8.5 μM-, (△) 17 μM- and (▲) 34 μM-bilirubin are shown.

Dixon plot ($1/v$ against [I]) an inhibition constant (K_i) of 25 μM was obtained (Fig. 5). This pattern of inhibition suggests that bilirubin and protoporphyrinogen compete for the (same) catalytic site on the protoporphyrinogen oxidase.

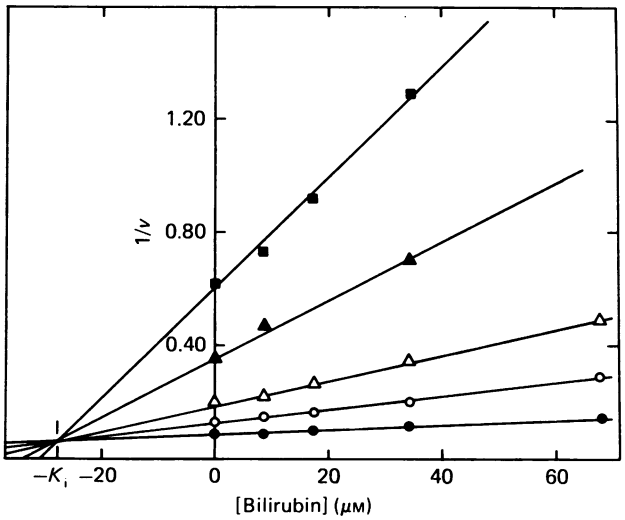

Fig. 5. Reciprocal of protoporphyrinogen oxidase velocity plotted against the inhibitor concentration (Dixon plot)

The intercept gives the negative of the K_i; $K_i = 25\ \mu M$. Protoporphyrinogen concentrations: ■, 0.5 μM; ▲, 1.0 μM; △, 2.0 μM; ○, 4.0 μM; ●, 8 μM.

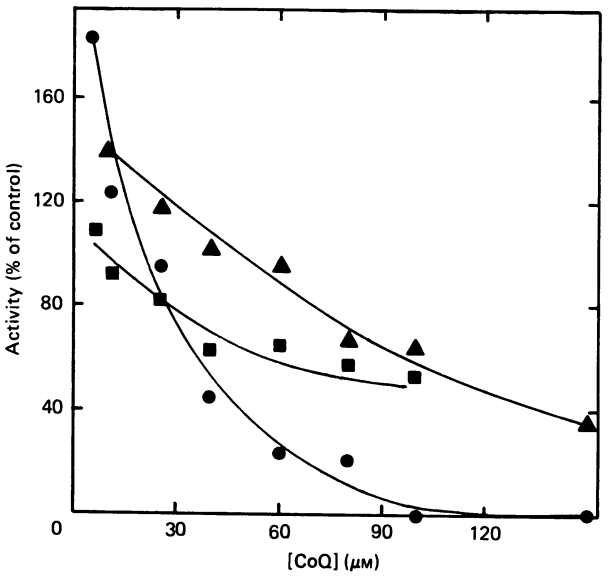

Fig. 6. Effect of CoQ_{10} (●), CoQ_6 (▲) and CoQ_0 (■) on protoporphyrinogen oxidase activity

Data points are averages of three determinations. Experimental details are given in the Materials and methods section.

Effect of potential electron acceptors on protoporphyrinogen oxidase activity

To determine whether protoporphyrinogen oxidase may use other electron acceptors rather than O_2, a series of compounds (CoQ_0, CoQ_6, CoQ_{10}, quinones and flavins) was examined. Because of the low partition of CoQs in aqueous solutions, the studies with CoQs were done with protoporphyrinogen oxidase reconstituted into phospholipid vesicles. Different concentrations of CoQ were added to the assay mixture, and the oxidase activity was measured (Fig. 6). When the concentration of CoQ_0 was increased up to 100 μM, the enzymic activity decreased to approx. 50% of the control value. Concentrations of CoQ_6 up to 40 μM slightly stimulated the enzyme activity, but above this concentration the coenzyme became inhibitory. For concentrations greater than 30 μM, CoQ_{10} rapidly inactivated the reconstituted protoporphyrinogen oxidase. At low concentrations (5–10 μM) the coenzyme had a slight stimulatory effect.

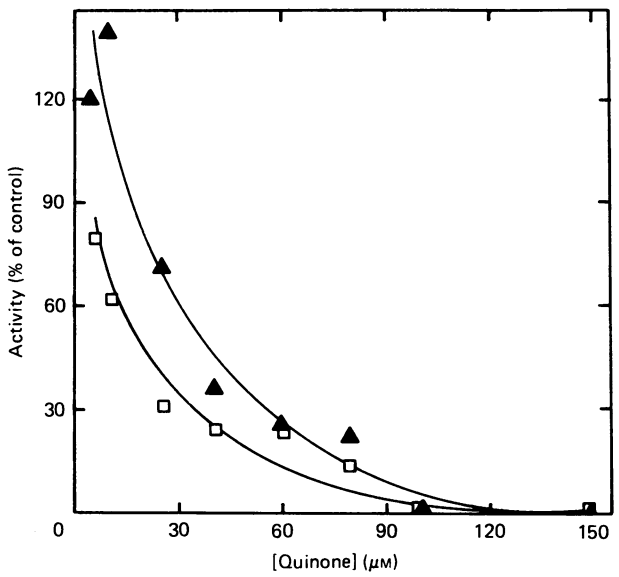

Fig. 7. Effect of menadione (□) and dicoumarol (▲) on the oxidation of protoporphyrinogen by protoporphyrinogen oxidase

Procedures used were the same as those in Fig. 6.

Dicoumarol and menadione (vitamin K_3) were found to be strong inhibitors of protoporphyrinogen oxidase. However, the oxidase activity increased slightly when dicoumarol was present at concentrations below 10 μM (Fig. 7). FAD was also tested as an alternative electron acceptor, and no significant activating or inhibitory effect was observed over the range of 0–60 μM.

DISCUSSION

Protoporphyrinogen oxidase as an enzyme that catalyses a six-electron transfer is rare, but not unique. Xanthine oxidase is also an example of an enzyme capable of accepting six electrons (Hille & Massey, 1981; Malmstrom, 1982), two each at the Mo and flavin sites and one each at the two Fe–S centres. The first two steps correspond to two-electron oxidations, producing H_2O_2, whereas the last two steps are one-electron processes, yielding superoxide ($O_2^{-\cdot}$). Other oxidases (i.e. flavoproteins, haemoproteins or metalloflavoproteins) have also been shown to produce H_2O_2 as a first step in oxygen reduction (Malmstrom, 1982). Because protoporphyrinogen oxidase has only recently been purified (Dailey & Karr, 1987), its structure and metal content remains unknown, but there is no evidence for a chromophoric cofactor associated with the purified enzyme, so its catalytic mechanism may be quite different from these other enzymes.

Coproporphyrinogen oxidase, the antepenultimate enzyme of the haem-biosynthetic pathway, catalyses the oxidative decarboxylation of two propionate side chains to yield the two vinyl groups at positions 2 and 4 on the porphyrin macrocycle (Yoshinaga & Sano, 1980*a*,*b*). This enzyme appears unique in that it does not contain or utilize chromophoric cofactors but appears to make use of protein tyrosine residues as electron acceptors (Yoshinaga & Sano, 1980*b*). It will be of interest to determine if protoporphyrinogen oxidase possesses a similar catalytic mechanism to deal with electron transfer.

Protoporphyrinogen and oxygen are considered as primary and secondary substrates respectively for protoporphyrinogen. The stoichiometric ratio of O_2 consumed to protoporphyrin produced in the '*in vitro*' reaction catalysed by purified oxidase was 1 mol of protoporphyrin produced to 3 mol of O_2 consumed (Table 3). This suggests that, in stoichiometric balance, H_2O may not be the product formed, but rather H_2O_2. Unfortunately, owing to the low activity of protoporphyrinogen oxidase and the sensitivity of the substrate and product to light and oxidation, it was not possible to accurately quantify H_2O_2 at the levels produced in our assays to establish a molar ratio between H_2O_2 formed to protoporphyrinogen oxidized.

Since protoporphyrinogen oxidase catalyses a six-electron reaction, electron acceptors other than, or in addition to, O_2 seemed a possibility. Because of its location on the mitochondrial inner membrane, obvious candidates were quinones, and so five representatives of these compounds were examined, namely vitamin K_3, dicoumarol, CoQ_0, CoQ_6 and CoQ_{10}. Their reactions, as shown above, were complex and not what one would anticipate from a putative electron acceptor. At relatively low concentrations (e.g. 10 μM), most of them showed a stimulatory effect, whereas slightly higher concentrations (e.g. 50–100 μM) caused strong inhibition of oxidase activity. Interestingly, CoQ_{10}, which is present in the inner mitochondrial membrane of most mammals, showed the greatest activation of the enzyme activity at low concentrations, although its maximal effect was less than 100% stimulation. However, the fact that dicoumarol, which is a known inhibitor of quinone-utilizing systems, exhibits the same stimulatory activity at low concentrations as is found with CoQ_{10}, suggests that the stimulatory activity seen at low concentrations may be an artefact of the system and not due to the quinones acting as electron acceptors. Indeed, the data suggest that none of the potential electron acceptors evaluated serve as physiological acceptors. FAD, another possible electron acceptor, had neither a strong inhibitory or stimulatory effect on protoporphyrinogen oxidase activity.

The observed '*in vitro*' inhibition of protoporphyrinogen oxidase but not of ferrochelatase by bilirubin is an interesting and perhaps biomedically significant observation in the light of the report that individuals suffering from Gilbert's syndrome have decreased protoporphyrinogen oxidase activity (McColl *et al.*, 1985). Gilbert's syndrome has been associated with a diminished capacity to conjugate bilirubin and with an impaired hepatic uptake of bilirubin and other cholephilic anions (Berthelot & Dhumeaux, 1978; Berthelot *et al.*, 1982). This syndrome affects approx. 5% of the population; curiously, jaundice generally does not appear before 15 to 20 years of age. Although bilirubin concentrations in plasma of Gilbert's-syndrome patients (up to about 50 μM) are lower than in other hyperbilirubinaemias (e.g., Crigler–Najjar Types I and II) (Wolkoff *et al.*, 1983), the unconjugated bilirubin that accumulates intracellularly may be sufficient to inhibit the penultimate enzyme of the haem-biosynthetic pathway. This would explain the lower values of protoporphyrinogen oxidase activity reported for peripheral-blood cells (McColl *et al.*, 1985). Considering that the concentration of serum bilirubin in Gilbert's-syndrome patients may be 50 μM (normal human blood bilirubin concentrations vary between 3.5 and 24 μM) and that unconjugated bilirubin has a K_i of 25 μM with respect to protoporphyrinogen oxidase, bilirubin concentrations characteristic of Gilbert's syndrome could lead to abnormal haem biosynthesis.

The kinetic data obtained for the isolated purified enzyme are interesting and of value in attempting to determine the reaction mechanisms, but it is difficult to extrapolate the physiological meaning of these '*in vitro*'-determined catalytic parameters for an enzyme that is situated in the mitochondrial inner membrane juxtaposed to the terminal and antepenultimate enzymes of the pathway. It is known that the enzymes of the haem-biosynthetic pathway are present in excess of what is normally needed and that 5-aminolaevulinate synthase, the first and rate-limiting enzyme of the pathway, controls the flow of intermediates in normal tissues (Kappas *et al.*, 1983). Only in the porphyrias does one find that a later enzyme can become rate-limiting. Thus it is that substrate flow to protoporphyrinogen oxidase will normally be at a relatively low level, with substrate concentrations well below its calculated K_m. Indeed, one would not expect to find micromolar concentrations of free porphyrinogens and porphyrins, since they would prove to be quite harmful to a cell. It may be that the terminal membrane-associated enzymes are present in a binary or ternary complex with substrate/product, moving rapidly from one enzyme to another without intermediate build-up. In such a situation, enzymes which alone *in vitro* appear to have low catalytic efficiency actually function with high efficiency *in vivo*. Such properties of multienzyme complexes have been reviewed recently by Srivastava & Bernhard (1986). So it may be that the relatively low $k_{cat.}$ values reported above for the isolated enzyme may not be an accurate reflection of the enzyme's efficiency *in situ*. Preliminary studies in this laboratory aimed at examining possible interactions between the terminal two enzymes of this pathway have yielded evidence for such protein–protein interactions. Further experimentation will be required to unequivocally show the formation of such a complex and then thoroughly examine its catalytic interactions and kinetic parameters.

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