Autoxidation of Soluble Trypsin-Cleaved Microsomal Ferrocytochrome *b₅* and Formation of Superoxide Radicals

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The rate and mechanism of autoxidation of soluble ferrocytochrome *b₅*, prepared from liver microsomal suspensions, appear to reflect an intrinsic property of membrane-bound cytochrome *b₅*. The first-order rate constant for autoxidation of trypsin-cleaved ferrocytochrome *b₅*, prepared by reduction with dithionite, was $2.00 \times 10^{-3} \pm 0.19 \times 10^{-3} \text{ s}^{-1}$ (mean $\pm$ S.E.M., $n = 8$) when measured at 30°C in 10 mM-phosphate buffer, pH 7.4. At 37°C in aerated 10 mM-phosphate buffer (pH 7.4)/0.15 M-KCl, the rate constant was $5.6 \times 10^{-3} \text{ s}^{-1}$. The autoxidation reaction was faster at lower pH values and at high ionic strengths. Unlike ferromyoglobin, the autoxidation reaction of which is maximal at low O_2 concentrations, autoxidation of ferrocytochrome *b₅* showed a simple O_2 -dependence with an apparent K_m for O_2 of $2.28 \times 10^{-4} \text{ M}$ (approx. 20 kPa or 150 mmHg). During autoxidation, 0.25 mol of O_2 was consumed per mol of cytochrome oxidized. Cyanide, nucleophilic anions, EDTA and catalase each had little or no effect on autoxidation rates. Adrenaline significantly enhanced autoxidation rates, causing a tenfold increase at 0.6 mM. Ferrocytochrome *b₅* reduced an excess of cytochrome *c* in a biphasic manner. An initial rapid phase, independent of O_2 concentration, was unaffected by superoxide dismutase. A subsequent slower phase, which continued for up to 60 min, was retarded at low O_2 concentrations and inhibited by 65% by superoxide dismutase at a concentration of 3 $\mu\text{g/ml}$. It is concluded that autoxidation is responsible for a significant proportion of electron flow between cytochrome *b₅* and O_2 in liver endoplasmic membranes, this reaction being capable of generating superoxide anions. A biological role for the reaction is discussed.

The structural and functional characteristics of cytochrome *b₅*, a major component of microsomal membranes of mammalian liver (Strittmatter & Velick, 1956), invite comparison with other well-characterized protoporphyrin-containing haemoproteins. Structurally, microsomal cytochrome *b₅* comprises an iron protoporphyrin IX prosthetic group embedded in a single polypeptide chain of 150 amino acid residues, which is firmly attached to the membrane by means of a hydrophobic 'tail' segment (Ozols, 1974). The fifth and sixth haem valencies are occupied by imidazole N atoms of histidine-63 and histidine-80 (Ozols & Strittmatter, 1968a; Mathews *et al.*, 1972). There is no spectral evidence for oxyferrocytochrome *b₅*, and neither CO nor CN^- induces binding spectra (Lemberg & Barrett, 1973). The crystal structure of cytochrome *b₅*, isolated from calf liver microsomal suspensions, has been determined to 0.2 nm resolution (Mathews *et al.*, 1972), and that of ferrocytochrome *b₅*, which is isomorphous with the oxidized form, has been established at 0.28 nm resolution (Argos & Mathews, 1975). In contrast with cytochrome *c* (Takano *et al.*, 1973), cytochrome

b₅ does not undergo a conformational change on reduction. Unlike cytochromes *c* (Salemme *et al.*, 1973), cytochrome *b₅* has no system of hydrogen bonds linking iron ligands to the surface of the protein. The haem group lies in a hydrophobic pocket and has one edge exposed to solvent through two propionic acid groups.

Microsomal cytochrome *b₅* is reducible by NADH or NADPH with nucleotide-specific flavoprotein dehydrogenases, and its presence in erythrocytes has been implicated in the catalytic reduction of methaemoglobin (Hultquist & Passon, 1971). As an electron donor it is solely responsible for transmission of electrons to the cyanide-sensitive factor involved in desaturation of fatty acyl-CoA derivatives (Oshino *et al.*, 1971) and it may also be involved in donation of a second electron in mixed-function oxidation reactions catalysed by cytochrome *P-450* (Hildebrandt & Estabrook, 1971).

Cytochrome *b₅* has been described as slightly autoxidizable (Lemberg & Barrett, 1973). However, a significant proportion of total electron flow between cytochrome *b₅* and molecular O_2 in micro-

somal fractions has been observed to take place by autoxidation (Boveris *et al.*, 1972; Berman *et al.*, 1975). The purpose of the present study was to clarify the extent and mechanism of autoxidation of microsomal cytochrome b_5 and to relate these, if possible, to the unique structural properties of this haemo-protein.

We have investigated a soluble preparation of cytochrome b_5 , obtained by tryptic cleavage of hepatic endoplasmic membranes. Tryptic hydrolysis releases, from the *N*-terminal segment of the native cytochrome, a hydrophilic fragment (Kajihara & Hagihara, 1968), which contains the haem and 80–90 amino acid residues (Ozols, 1972). Although smaller in size than the monomer of approx. 17000 daltons released by mild detergents (Ito & Sato, 1968; Spatz & Strittmatter, 1971), the trypsin-cleaved fragment retains the spectral and redox characteristics of microsomal cytochrome b_5 , and the conformation of the polypeptide chain and its haem crevice remains unaltered (Robinson & Tanford, 1975). From our present study it is clear that, like other cytochromes with redox potentials less than or equal to 0.0 V, cytochrome b_5 is readily autoxidizable. The characteristics of autoxidation are consistent with the formation of superoxide radicals as interceding in the autoxidative process.

Materials and Methods

Materials

Horse heart myoglobin and cytochrome *c* (type III) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.; bovine liver catalase was from Worthington Biochemical Corp., Freehold, NJ, U.S.A.; bovine erythrocyte (superoxide dismutase) was from Miles Laboratories, Kankakee, IL, U.S.A.; adrenaline was from BDH Chemicals, Poole, Dorset, U.K.; and O_2 -free N_2 was from Afrox, Epping Industria, Cape Town, South Africa. All other materials were of reagent grade. Aqueous solutions were prepared in water, double-distilled in glass.

Methods

Preparation of soluble ferricytochrome b_5 . Trypsin-cleaved cytochrome b_5 was prepared from the microsomal fraction of calf liver by a modification of the method of Omura & Takasue (1970), in which the microsomal fraction was sedimented from the post-mitochondrial supernatant by low-speed sedimentation after treatment with 8 mM- $CaCl_2$ (Strittmatter *et al.*, 1974). Identical preparations were obtained either with or without the calcium precipitation step. Some studies were also performed on cytochrome b_5 prepared from pig liver. Microsomal suspensions

were freed from excess of Ca^{2+} by dialysis overnight against 0.1 M-Tris/acetate buffer (pH 8.1)/20 mM-EDTA, before tryptic digestion. After purification by column chromatography on columns of Sephadex G-100 and DEAE-cellulose (Omura & Takasue, 1970), stock solutions (approx. 70 μ M) were stored at $-20^\circ C$ in 10 mM-potassium phosphate buffer, pH 7.5, containing 0.2 mM-EDTA. Concentrations of trypsin-cleaved cytochrome b_5 were calculated, in terms of haem, by using $E_{1cm}^{1mM} = 171$ at 423 nm or 25.6 at 556 nm for the fully reduced cytochrome (Strittmatter & Velick, 1956). The cytochrome was homogeneous as determined by polyacrylamide-disc-gel electrophoresis in the presence of sodium dodecyl sulphate (Weber & Osborn, 1969) and by sedimentation analysis. The preparation was not reduced by 1 mM-NADH or -NADPH, indicating the absence of nicotinamide-adenine dinucleotide-cytochrome b_5 reductase activity, nor did CO induce difference spectra when added to oxidized or dithionite-reduced cytochrome, and the preparation was therefore also free from cytochrome *P*-450 and cytochrome *P*-420. The cytochrome thus isolated was fully oxidized, with a maximum absorption at 413 nm in 10 mM-phosphate buffer, pH 7.4. On reduction with dithionite the Soret maximum shifted to 422.5 nm and α and β bands appeared at 556 and 525 nm.

Reduction of ferricytochrome b_5 to ferrocyclochrome b_5 . Stock solutions of ferricytochrome b_5 (1–2 ml) containing approx. 5% (w/v) sucrose were reduced with a slight excess of solid crystalline sodium dithionite and passed, under anaerobic conditions at $4^\circ C$, through a column (1.5 cm $\times$ 26 cm) of Sephadex G-25 which had been equilibrated with de-gassed 10 mM-phosphate buffer, pH 7.5, containing 0.2 mM-EDTA. The buffer reservoir was continuously flushed with a stream of O_2 -free N_2 which had been bubbled through two sodium vanadate trains in series (Meites & Meites, 1948). The reduced cytochrome was eluted with the phosphate buffer from the column, at a rate of approx. 1 ml/min, in a total volume of 3–5 ml, into an all-glass syringe, ahead of dithionite and its oxidation products. The reduced preparation, when eluted from the column, contained approx. 15–20% oxidized cytochrome. The preparation was stored at $0^\circ C$ and all kinetic measurements were performed within 4 h, by which time not more than 55% of the cytochrome was in the oxidized form. Ferromyoglobin was prepared from myoglobin in a manner similar to that described for ferrocyclochrome b_5 . The material eluted from the Sephadex column was more than 95% reduced and was used immediately for O_2 -consumption studies. The concentration of myoglobin was calculated by using $E_{1cm}^{1mM} = 13.8$ at 560 nm for the dithionite-reduced form (Antonini, 1965).

Measurement of first-order rate constants. Rates of autoxidation of ferrocyclochrome b_5 were monitored

spectrophotometrically at either 423 nm, 556 nm or 423 minus 409 nm in an Aminco DW-2 spectrophotometer equipped with 1 cm light-path cells and a thermostatically controlled cell holder. Rate constants of autoxidation faster than $5 \times 10^{-3} \text{ s}^{-1}$ were calculated from first-order plots by allowing the reaction to proceed virtually to completion. Rate constants of slower reactions were calculated by the Guggenheim method (Gutfreund, 1972). Correlation coefficients of first-order plots, fitted to data points by the method of least squares, varied between 0.985 and 0.999. Oxygen concentrations in buffer solutions were varied by mixing air- or O_2 -saturated buffers (20°C and 1 atm) with de-gassed N_2 -equilibrated solutions.

Measurement of O_2 uptake. O_2 uptake or evolution during autoxidation was determined polarographically by means of a Clark-type electrode (Yellow Springs Instruments, Antioch, OH, U.S.A.), connected to a 10 in (25 cm) strip-chart potentiometric recorder. Immediately after it had been eluted from the Sephadex column, 5.0 ml of an anaerobic solution of the dithionite-reduced haemoprotein was introduced, under a blanket of N_2 , into a reaction vessel which was thermostatically maintained at 30°C. After equilibration with stirring, 3.0 ml of aerated buffer was injected to initiate the autoxidation reaction. O_2 consumption by the electrode amounted to 0.33 nmol/min. Uptake or evolution of O_2 was recorded until this background was reached. The concentration of haemoprotein in the reaction mixture and completeness of its conversion into the oxidized form was determined from the absorbance of the Soret peak.

Results

Spectral changes and kinetics of autoxidation of soluble ferrocytochrome b_5

The time-course of decay of a freshly prepared anaerobic solution of ferrocytochrome b_5 to ferricytochrome b_5 after addition to an aerated buffer solution is shown in Fig. 1(a). The initial spectrum and that of the final product are identical respectively with those of the reduced and fully oxidized forms. At wavelengths of 423 nm, 556 nm and 423 minus 409 nm, kinetics are first-order with respect to ferrocytochrome b_5 concentration for 95% of the total reaction (Fig. 1b), with rate constants for the autoxidation reaction, measured at 30°C in 10 mM-phosphate buffer, pH 7.4, calculated from first-order plots, of 2.15×10^{-3} , 2.26×10^{-3} and $2.26 \times 10^{-3} \text{ s}^{-1}$. The measured rate constants were reproducible and varied between $1.6 \times 10^{-3} \text{ s}^{-1}$ and $2.3 \times 10^{-3} \text{ s}^{-1}$ for five different preparations of cytochrome b_5 from calf liver. Cytochrome b_5 prepared from pig liver behaved similarly (Table 1). The susceptibility of ferrocytochrome b_5 to autoxidation was not altered by up to three successive cycles of purification by chromatography on a column of DEAE-cellulose, dithionite reduction and autoxidation. Trace amounts of metal ions catalyse the autoxidation of ferromyoglobin and ferrohaemoglobin (Antonini & Brunori, 1971). EDTA (0.2 mM) added to reaction mixtures had no effect on the autoxidation reaction (Table 1), but nevertheless was included in all buffer solutions used in the present study. The rate constant of autoxidation, under conditions simulating those *in vivo*

Fig. 1. Spectral changes during autoxidation of soluble ferrocytochrome b_5

Dithionite-reduced ferrocytochrome b_5 (2–4 μM) was allowed to autoxidize at 30°C in aerated 10 mM-phosphate buffer, pH 7.2, containing 0.2 mM-EDTA. Spectral scans (a) were performed at 1, 2, 4, 8, 16 and 32 min. First-order plots of ΔE_{423} (●), ΔE_{556} (○) and $\Delta E_{424} - E_{409}$ (▲) are shown in (b). E_t and E_∞ are the measured extinction values at time t and at the virtual completion of the reaction respectively.

Table 1. Rate constants for autoxidation of soluble ferrocytochrome b_5

Autoxidation of freshly prepared ferrocytochrome b_5 (2–4 μM) was measured at 30°C in 10mM-phosphate buffer, pH7.4. Values are presented as means $\pm$ S.E.M. (n) where indicated. EDTA (0.2mM) and superoxide dismutase (10 $\mu\text{g}/\text{ml}$) were added as shown.

Source	Additions	$10^3 \times$ First-order rate constant of autoxidation k (s^{-1})
Calf liver	—	2.05
	EDTA	2.00 ± 0.19 (8)
	EDTA + superoxide dismutase	2.41
Calf liver recycled ($\times 1$)	EDTA	1.71 ± 0.13 (8)
Calf liver recycled ($\times 2$)	EDTA	1.57 ± 0.24 (3)
Pig liver	EDTA	2.29 ± 0.13 (4)

Fig. 2. Effect of pH on autoxidation rate of ferrocytochrome b_5

Determinations were made at 30°C in aerated buffers. Potassium acetate, potassium phosphate and potassium bicarbonate buffers (all 10mM) were used in the pH ranges 3–5, 5–8 and 8–10.8 respectively. KI was added to the buffers to achieve a final $I = 0.07\text{M}$; $\log k$ refers to $\log(10^3 \times k)$ where k is in s^{-1} .

[10mM-phosphate buffer (pH7.4)/150mM-KCl, 37°C] is $5.57 \times 10^{-3} \pm 0.27 \times 10^{-3} \text{s}^{-1}$ (mean $\pm$ S.E.M., $n = 5$).

pH-dependence

The rate constant for autoxidation exhibits a strong dependence on pH, particularly in the range pH4–6 (Fig. 2). At higher pH values the rate constants are much lower. At and below pH3.5, the rates of the reactions are most rapid and no longer first-order with respect to ferrocytochrome b_5 . Below pH4.0, the Soret peak of the spectrum of ferrocytochrome b_5 disappears and other changes indicate a transition from low-spin to high-spin state (Ikeda *et al.*, 1974). The measured ratio of $\Delta \log k / \Delta \text{pH}$ is 0.16 at pH7.0 the same as that reported (0.16) for the autoxidation of ferromyoglobin (Brown & Mebine, 1969).

Fig. 3. Variation with temperature of the autoxidation rates of ferrocytochrome b_5

Determinations were made in 10mM-phosphate buffer (pH7.4)/0.2mM-EDTA. The reaction was initiated by addition of 2–4 μM -ferrocytochrome b_5 and monitored at 423 minus 409 nm. The calculated enthalpy values for the first-order rates of autoxidation in the range 8–34°C and 36–56°C are 45.0 and 83.2 kJ/mol respectively.

Effect of temperature

Autoxidation was enhanced at elevated temperatures. At pH7.0, the Arrhenius plot (Fig. 3) showed a discontinuity at approx. 35°C. Between 5° and 34°C, the activation energy was 45 kJ/mol (10.5 kcal/mol) and was 83.2 kJ/mol (19.9 kcal/mol) between 36° and 56°C. The pH of the phosphate buffer fell by 0.1 unit when the temperature was increased from 5 to 56°C, and rate constants were not corrected. For comparison, George & Stratmann (1952) found that the activation energy for autoxidation of myoglobin was in the range 80–105 kJ/mol (19–25 kcal/mol) when measured between 22° and 38°C. At higher temperatures, deoxyferromyoglobin was rapidly denatured.

Fig. 4. Effect of salts and ionic strength on autoxidation rates of ferrocytochrome b_5

The reaction mixture at 30°C was 10mM-phosphate buffer (pH 7.0)/0.2mM-EDTA, to which KCl (●), KBr (○) and potassium acetate (△) were added. For the purpose of calculation of the final ionic strength, I , the charge on phosphate anions was considered to be 2. Kinetic measurements were as described in Fig. 3.

Effects of salts and ionic strength

The effect of ionic strength on the rate constant, k , for autoxidation was investigated by increasing the ionic strength of the buffered reaction medium with potassium salts up to a maximum of 0.5M. The results (Fig. 4) show a general increase in the rate constant with ionic strength (I). With KCl, an approximately linear relationship between $\log k$ and the square root of ionic strength was obtained in the range $I = 0.025$ – 0.4 mol/l. At the latter concentration k reached a maximum, with a value of $6.2 \times 10^{-3} \text{ s}^{-1}$. Higher ionic strengths decreased the rate constant. No maximum was observed for stimulation by potassium acetate or KBr. Nucleophiles had little specific effect on the autoxidation reaction. Rate constants at pH 7.0 and 30°C in the presence of 75mM- $\text{Na}_2\text{S}_2\text{O}_3$, sodium thiosulfate or NaF were 2.88×10^{-3} , 4.20×10^{-3} and $2.94 \times 10^{-3} \text{ s}^{-1}$ respectively. At pH 5.2 the rate constant was $6.7 \times 10^{-3} \text{ s}^{-1}$ in the presence of 75mM- $\text{Na}_2\text{S}_2\text{O}_3$ and $5.5 \times 10^{-3} \text{ s}^{-1}$ in its absence.

Effect of O_2 concentration

The autoxidation rates of ferrohaemoglobin and ferromyoglobin are known to be enhanced at low O_2 partial pressure, being first-order with respect to deoxyferrohaemoprotein concentration and maximal for myoglobin at 0.16kPa (1.2mmHg) which is identical with the O_2 concentration at half-saturation for the oxygenation reaction (George & Stratmann, 1952). The autoxidation of ferrocytochrome b_5 increases with increasing concentrations of O_2 in the medium (Fig. 5). A double-reciprocal plot of O_2

Fig. 5. Effect of O_2 concentration on rates of autoxidation of ferrocytochrome b_5

Autoxidation of ferrocytochrome b_5 was determined as described for Fig. 4 in mixtures of deaerated anaerobic and aerated or O_2 -equilibrated buffer. k is in s^{-1} . A double-reciprocal plot of the data is shown in the inset. The calculated K_m for O_2 is $2.28 \times 10^{-4} \text{ M}$.

concentration versus rate constant shows that in the range 20–950 μM , the reaction is O_2 -dependent and the kinetics are compatible with a mechanism in which molecular O_2 is a substrate in the reaction. The calculated apparent K_m for O_2 of $2.3 \times 10^{-4} \text{ M}$ (approx. 20kPa or 150mmHg) indicates low affinity of the cytochrome for O_2 .

O_2 uptake during the autoxidation reaction

O_2 uptake was followed polarographically during autoxidation of ferrocytochrome b_5 . The reaction was initiated by addition of aerated buffer to an anaerobic solution of dithionite-reduced ferrocytochrome b_5 . The saturation of O_2 in solution (41%) immediately after mixing 3 vol. of aerated and 5 vol. of deaerated buffer was slightly greater than that expected (38%) and indicates that no significant binding of O_2 to the reduced cytochrome had occurred during this time. O_2 uptake was monophasic and first-order for at least 90% of the reaction with respect to that fraction of O_2 which remained to be taken up during the reaction (Fig. 6a). The rate constant, k , of $1.84 \times 10^{-3} \text{ s}^{-1}$, is close to that obtained by spectrophotometric measurements under the same conditions of pH, temperature, ionic strength and O_2 concentration ($k = 2.00 \times 10^{-3} \text{ s}^{-1}$). During autoxidation 0.251 mol of O_2 was absorbed per mol of cytochrome, which is the expected result if a single electron was involved during univalent electron transfer between ferrocytochrome b_5 and molecular O_2 . The presence of catalase (10units/ml) in the reaction mixture had no effect on the amount of O_2 consumed during autoxidation. Previous claims that autoxidation of both oxyferrohaemoglobin and oxyferromyoglobin consume 0.5

Fig. 6. O_2 uptake or evolution during autoxidation of ferrocyanochrome b_5 and oxyferromyoglobin

A portion (3ml) of freshly prepared $161\mu\text{M}$ -ferrocyanochrome b_5 (a) or $227\mu\text{M}$ -deoxyferromyoglobin (b) was added to 5.0ml of aerated 10mM-phosphate buffer (pH 7.0)/0.2M-EDTA, containing 10 units of catalase. O_2 uptake or evolution was measured polarographically as described under 'Methods'. First-order rate constants, calculated from semi-log plots, shown in insets, were $1.84 \times 10^{-3}\text{s}^{-1}$ and 0.31h^{-1} for ferrocyanochrome b_5 and ferromyoglobin respectively; $\ln[O_2]$ is $\log_{10}$ (mol of O_2 remaining to be taken up or evolved per mol of haem).

mol of O_2 per mol of haem iron oxidized were shown to be due to traces of dithionite reductant contaminating the reaction mixture (Brown & Mebine, 1969) and when care was taken to remove excess of reductant and its oxidation products by a mixed-bed ion-exchange procedure, net evolution of 0.6–0.7 mol of O_2 per mol of oxyferromyoglobin autoxidized was observed. As a check on the experimental procedure, especially with regard to complete removal of dithionite and its oxidation products during Sephadex column chromatography, the experiment was repeated with ferromyoglobin. Net evolution of 0.66 mol of O_2 per mol of oxyferromyoglobin autoxidized occurred (Fig. 6b). The rate constant, k , for autoxidation of oxyferromyoglobin, calculated from the curve for evolution of O_2 , was 0.31h^{-1} . George & Stratmann (1952) obtained a k value of 0.30h^{-1} from spectrophotometric measurements when the reaction was conducted at 30°C in 0.6M-phosphate buffer, pH 5.69.

Fig. 7. Effect of adrenaline on first-order rate constant of autoxidation of ferrocyanochrome b_5

Experimental conditions and kinetic measurements were as described for Fig. 3. The reaction was performed at 30°C .

Univalent reduction of O_2 during autoxidation of ferrocyanochrome b_5

Adrenaline has been used to demonstrate univalent reduction of oxygen (McCord & Fridovich, 1969). The superoxide anion, $O_2^{\cdot-}$, causes oxidation of adrenaline to adrenochrome, and the formation of adrenochrome, monitored spectrophotometrically at 485 nm, has been used to demonstrate the production of $O_2^{\cdot-}$ during autoxidation of ferredoxins (Misra & Fridovich, 1971) and in the course of univalent reduction of O_2 by reduced flavins and quinones (Misra & Fridovich, 1972). Attempts to demonstrate adrenochrome formation during autoxidation of ferrocyanochrome b_5 in the presence of adrenaline were unsuccessful, owing to significant changes in the absorbance spectrum of the haemoprotein at 485 nm as a result of conversion into the ferrihaem form. However, when adrenaline was added to reaction mixtures it significantly enhanced rates of autoxidation of ferrocyanochrome b_5 . The effect was linear in the concentration range studied, which was up to 1 mM-adrenaline (Fig. 7). At 0.6 mM-adrenaline, autoxidation rates were enhanced 10-fold when compared with controls without adrenaline. By comparison, maximal and half-maximal rates of adrenochrome formation during oxidation of FMNH₂ occurred at 0.5 and 0.2 mM-adrenaline respectively (Misra & Fridovich, 1972), and half-maximal rates of adrenochrome formation accompanying autoxidation of ferredoxin occurred with 0.125 mM-adrenaline (Misra & Fridovich, 1971).

Fig. 8. Reduction of cytochrome *c* by ferrocytochrome b_5

Cytochrome *c* ($3.96 \mu\text{mol}$) was incubated at 30°C in aerated 10 mM-phosphate buffer, pH 7.4, containing 0.2 mM-EDTA. In (a) spectral scans were performed immediately before (–2) and 0.5, 2, 4, 7, 30 and 60 min after the reaction had been initiated by addition of a total of $1.90 \mu\text{mol}$ of a dithionite-reduced preparation of cytochrome b_5 of which $1.0 \mu\text{mol}$ was in the reduced state. In (b) the reaction was monitored at 550 nm in aerated buffer (●) and anaerobically (○). The sensitivity of slow aerobic reduction of cytochrome *c* to inhibition by superoxide dismutase is shown in (c).

Generation of $\text{O}_2^{\cdot-}$ during autoxidation of ferrocytochrome b_5 could also be demonstrated when it acted as a reductant of cytochrome *c*. Spectral scans taken immediately after mixing ferrocytochrome b_5 with an excess of ferricytochrome *c* showed a peak absorption at 550 nm, but none at 556 nm, indicating that the equilibrium mixture contained predominantly ferricytochrome b_5 and ferrocytochrome *c* (Fig. 8a). On further incubation there was continuing reduction of cytochrome *c* at a much lower rate without change in the redox status of cytochrome b_5 , since successive spectral scans showed no relative change in E_{556} . The slow phase of reduction was dependent on O_2 concentration (Fig. 8b) and was inhibited by low concentrations of superoxide dismutase (Fig. 8c). The rapid phase of reduction of cytochrome *c* was unaffected by superoxide dismutase ($10 \mu\text{g/ml}$).

Discussion

The autoxidizability of myoglobin has been ascribed to changes in properties of the haemoprotein during isolation procedures (Antonini & Brunori, 1971). We have considered whether the relatively

rapid rates of autoxidation of trypsin-cleaved ferrocytochrome b_5 are characteristic of native membranous cytochrome, since the first-order rate constant for the autoxidation reaction is 60-fold greater than the reaction of mammalian myoglobin. The method of solubilization by tryptic hydrolysis, as used in the present study, does not appear to alter the structure and redox properties of the hydrophilic haem-containing domain (Spatz & Strittmatter, 1971; Robinson & Tanford, 1975).

That autoxidizability is a significant and inherent property of cytochrome b_5 , not altered during extraction and purification procedures, is suggested by a number of observations. First-order rate constants, determined for autoxidation of several preparations from two mammalian species, have been found to be reproducible, unaltered by successive cycles of dithionite reduction, autoxidation and recovery by column chromatography, and approximate in value to those for NADH-reduced microsomal ferrocytochrome b_5 when measured in the absence of substrates for cytochrome *P*-450 and fatty acid desaturase pathways (Berman *et al.*, 1975).

Mechanisms of autoxidation of the haemoproteins, haemoglobin, myoglobin and horseradish peroxidase remain obscure. All of these protoporphyrin haemoproteins have weak sixth ligands and are able to bind O_2 reversibly to form stable oxyferroderivatives. For haemoglobin (Hb), which has been extensively studied, HbO_2 has been considered to be a likely intermediate during the autoxidation reaction (Wang *et al.*, 1958). Autoxidation of haemoglobin generates superoxide radicals (Wever *et al.*, 1973), suggesting that $O_2^{\cdot-}$ dissociates from HbO_2 to leave a ferrihaem complex. However, i.r. and other evidence indicates strong covalent bonding between haem iron and O_2 (Barlow *et al.*, 1973), contrary to previous descriptions (Weiss, 1964; Peisach *et al.*, 1968; Yamamoto *et al.*, 1973) of haem- O_2 bonding in terms of an $Fe^{3+}-O_2^-$ ion pair.

Ferrocycytochrome b_5 does not form a stable complex with molecular O_2 . In the polarographic study reported here (Fig. 6), anaerobic solutions of ferrocycytochrome b_5 did not bind O_2 when added to an oxygenated medium, nor was O_2 evolved during autoxidation. The relatively high apparent K_m for O_2 during autoxidation, indicating low O_2 affinity, is also relevant and unlike an FeO_2 interaction. Several sequences, especially of those amino acids involved in binding of proximal and distal histidine residues, show striking similarities between cytochrome b_5 , haemoglobin and myoglobin (Ozols & Strittmatter, 1968b). A comparison of the haem-binding pocket in globins and cytochrome b_5 has shown a remarkable structural and topological equivalence (Rossman & Argos, 1975). The problems posed by autoxidizability of O_2 -binding haemoproteins on the one hand and of the non- O_2 -binding cytochromes (b -type and c) on the other may be resolved if this property is inherent in their protein structure and/or haem type rather than their ability to bind O_2 reversibly. Autoxidation of cytochrome b_5 , like that of haemoglobin and myoglobin, is sensitive to pH, salt concentration and temperature. Thus a value for $\Delta \log k / \Delta pH$ of 0.16 suggests an effect on ionizable groups adjacent to the O_2 -binding site rather than a mechanism in which H^+ ions participate directly in the reaction. Salt effects do not indicate whether dissociation of $O_2^{\cdot-}$ is from the Fe^{3+} or another nucleophilic centre. These considerations suggest the possibility that an $Fe-O_2$ bond is neither a prerequisite nor an intermediate during autoxidation of protohaemoproteins, analogous to autoxidation reactions of most non-biological ferrous complexes in which electron transfer occurs by an outer sphere pathway (Bennett, 1973).

It has been suggested that the environment of the haem, especially the hydrophobicity of the haem pocket, stabilizes the oxyferrohaem state and that autoxidation proceeds on unliganded ferrohaem (Wang, 1962). Thus autoxidation of myoglobin is maximal at low pO_2 values and first-order with

respect to deoxyferromyoglobin over a wide range of O_2 concentrations (George & Stratmann, 1952). Phelps *et al.* (1974) showed that oxyferroperoxidase is relatively stable, but decays rapidly to ferriperoxidase in the presence of ferropoxidase by a mechanism which they suggest involves intermolecular electron transfer. This latter reaction could provide a model for terminal oxidase systems.

The acceleration of autoxidation caused by adrenaline is compatible with its high affinity for superoxide anions and suggests that displacement of $O_2^{\cdot-}$ from cytochrome b_5 is rate-limiting for autoxidation. Experiments with cytochrome c provide direct evidence that autoxidation of cytochrome b_5 generates superoxide anions. The interaction of ferrocycytochrome b_5 with ferricytochrome c is biphasic. Stopped-flow experiments indicate that the initial rapid phase of reduction of cytochrome c has a second-order rate constant of between 10^6 and $10^7 M^{-1} \cdot s^{-1}$, which is compatible with direct haem-haem interaction and unaffected by superoxide dismutase (M. C. Berman & C. M. Adnams, unpublished work). Comparison of the E_0' value of cytochrome b_5 of +20 mV (Velick & Strittmatter, 1956) with the E_0' value for cytochrome c of +225 mV (Henderson & Rawlinson, 1956) predicts an equilibrium in favour of ferrocycytochrome c . The slow phase of reduction of cytochrome c is compatible with a mechanism in which $O_2^{\cdot-}$ is generated by autoxidation of the small amount of ferrocycytochrome b_5 present in the equilibrium mixture, since it is dependent on the presence of O_2 and inhibited by superoxide dismutase.

The reversibility of the reaction:

has been considered. Under physiological conditions the measured pseudo-first-order rate constant for the autoxidation of ferrocycytochrome b_5 of $5.6 \times 10^{-3} s^{-1}$ permits calculation of a second-order rate constant k_1 ($[O_2] = 240 \mu M$) of $23 M^{-1} \cdot s^{-1}$. An equilibrium constant $K = 10^{-6}$ can be calculated from E_0' ($cyt\ b_{5(ox)}/cyt\ b_{5(red)} = +0.02 V$ (Velick & Strittmatter, 1956) and E_0' ($O_2/O_2^{\cdot-} = -0.33 V$). From k_1 and K a rate constant for the reverse reaction k_{-1} of $2.3 \times 10^7 M^{-1} \cdot s^{-1}$ can be calculated. A second-order rate constant of this magnitude indicates significant reversal of the forward reaction at a rate appreciably less than the diffusion-controlled limit and is consistent with the reaction shown in eqn. (1). The acceleration of autoxidation of ferrocycytochrome b_5 by adrenaline can be explained by removal of $O_2^{\cdot-}$ and a consequent lowered rate of the reverse reaction. A similar conclusion was reached for the autoxidation mechanism of cytochrome c when superoxide dismutase was used to scavenge $O_2^{\cdot-}$ (Cassell & Fridovich, 1975).

McCord & Fridovich (1969) first proposed that the biological role of superoxide dismutase activity was to protect cellular activities against the deleterious effects of superoxide anions. Several sources of $O_2^{\cdot-}$ have been described, including haemoproteins (Wever *et al.*, 1973), flavins (Fridovich & Handler, 1961), ferredoxin (Misra & Fridovich, 1971) and tetrahydropteridines (Nishikimi, 1975). Fried *et al.* (1973) have, however, proposed a biological role for superoxide generators as a source of 'activated' O_2 . Superoxide anions appear to be involved during hydroxylation reactions catalysed by liver microsomal cytochrome $P-450$ (Strobel & Coon, 1971).

According to the scheme proposed by Hildebrandt & Estabrook (1971), cytochrome b_5 can function as a component of the mixed-function oxidase system and transfer a second electron from NADH or NADPH to activate molecular O_2 bound to cytochrome $P-450$. Microsomal suspensions generate $O_2^{\cdot-}$ and although the generator has been reported to be co-purified with NADH-cytochrome c reductase (Aust *et al.*, 1972), we wonder whether cytochrome b_5 , in view of its autoxidizability, might function as an $O_2^{\cdot-}$ donor during microsomal hydroxylation reactions. The present observations may also be relevant to the problem of the formation of H_2O_2 during mitochondrial electron transport, which is compatible with an origin at the cytochrome b level (Boveris & Chance, 1973).

Whatever the role of cytochrome b_5 in microsomal metabolism, the seemingly disproportionate NADH-cytochrome b_5 reductase activity in microsomal membranes appears to be necessary to maintain the cytochrome in the fully reduced state. This function is thus analogous with that of NADH-methaemoglobin reductase activity in erythrocytes, in which cytochrome b_5 has been implicated as an obligatory electron-transfer factor between the reductase and methaemoglobin (Hultquist & Passon, 1971).

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