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REACTIONS OF MERCAPTANS WITH CYTOCHROME *c* OXIDASE AND CYTOCHROME *c*

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Summary

1. The steady-state oxidation of ferrocytochrome *c* by dioxygen catalyzed by cytochrome *c* oxidase, is inhibited non-competitively towards cytochrome *c* by methanethiol, ethanethiol, 1-propanethiol and 1-butanethiol with K_i values of 4.5, 91, 200 and 330 μM, respectively.

2. The inhibition constant K_i of ethanethiol is found to be constant between pH 5 and 8, which suggests that only the neutral form of the thiol inhibits the enzyme.

3. The absorption spectrum of oxidized cytochrome *c* oxidase in the Soret region shows rapid absorbance changes upon addition of ethanethiol to the enzyme. This process is followed by a very slow reduction of the enzyme. The fast reaction, which represents a binding reaction of ethanethiol to cytochrome *c* oxidase, has a k_1 of 33 $M^{-1} \cdot s^{-1}$ and a dissociation constant K_d of 3.9 mM.

4. Ethanethiol induces fast spectral changes in the absorption spectrum of cytochrome *c*, which are followed by a very slow reduction of the heme. The rate constant for the fast ethanethiol reaction representing a bimolecular binding step is 50 $M^{-1} \cdot s^{-1}$ and the dissociation constant is about 2 mM. Addition of up to 25 mM ethanethiol to ferrocytochrome *c* does not cause spectral changes.

5. EPR (electron paramagnetic resonance) spectra of cytochrome *c* oxidase, incubated with methanethiol or ethanethiol in the presence of cytochrome *c* and ascorbate, show the formation of low-spin cytochrome a_3-mercaptide compounds with *g* values of 2.39, 2.23, 1.93 and of 2.43, 2.24, 1.91, respectively.

Introduction

Cytochrome *c* and cytochrome *c* oxidase catalyze the electron transfer in the mitochondrial respiratory chain from the bc_1 complex to molecular oxygen. Several ligands, such as cyanide, sulphide, carbon monoxide and azide are known to bind to cytochrome *c* oxidase and to inhibit its catalytic function during oxidation of ferrocytochrome *c* by dioxygen (for a review, see Ref. 1). Although these ligands may bind to cytochrome *c*, their inhibitory effect on the respiratory chain can be ascribed almost completely to ligand binding to cytochrome a_3. This conclusion is in agreement with the type of inhibition of the cytochrome *c* oxidase activity, which is non-competitive towards cytochrome *c*.

Recently, Vahlkamp et al. [2] reported that mercaptans affect the oxidative metabolism of rat liver and rat brain mitochondria. They suggested that inhibition of the terminal part of the mitochondrial electron transfer chain by mercaptans was related to the mechanism by which energy production in brain is depressed during hepatic failure. In this paper the effect of alkylthiols on the properties of cytochrome *c* and cytochrome *c* oxidase are studied.

Materials and Methods

Cytochrome *c* was prepared from horse heart according to the method of Margoliash and Walasek [3], cytochrome *c* oxidase was isolated from beef heart according to the methods of Fowler et al. [4] and MacLennan and Tzagoloff [5], as modified in our laboratory [6]. Absorption coefficients used were for cytochrome *c* (reduced minus oxidized) 21.1 $mM^{-1} \cdot cm^{-1}$ at 550 nm [7] and for cytochrome *c* oxidase (reduced minus oxidized) 24.0 $mM^{-1} \cdot cm^{-1}$ at 605 nm [8]. Stock solutions of methanethiol (Merck-Schuchardt), prepared by passing the gas for 15 min through a column (50 × 2 cm) of 0.1 M KOH at 0° C, were stored in liquid N_2.

Stock solutions of the other mercaptans (50—100 mM) were prepared freshly every day and kept at 0° C. Mercaptan concentrations were determined by the method of Ellman [9], using 5,5′-dithiobis(2-nitrobenzoate).

Ascorbic acid (BDH, biochemical grade) was neutralized with KOH. Stock solutions of 2.0 M were stored at −20° C.

Tween 80 was purchased from Sigma, ethanethiol (Analar grade) from BDH, other chemicals were mainly obtained from Merck ('zur Synthese' quality).

Steady-state activities of cytochrome *c* oxidase at 25° C were measured spectrophotometrically according to the method of Smith and Conrad [10], using a modified Durrum stopped-flow apparatus or a Zeiss PMQ II spectrophotometer, equipped with a log-converter and recorder. The reaction medium contained 100 mM potassium phosphate, 1% Tween 80. The pH was 7.0 with the exception of the experiments related to Fig. 5.

Oxygen consumption was monitored with a Clark oxygen electrode via an appropriate circuit to a recorder. The polarographic assay vessel contained 100 mM potassium phosphate (pH 7.0), 1% Tween 80, 20 mM ascorbate, 0.75 mM *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine, 5—25 μM cytochrome *c* and 60 nM cytochrome *c* oxidase. For spectroscopic determinations a Cary

17R recording spectrophotometer was used. EPR spectra were obtained with a Varian E-9 spectrometer. Temperatures, magnetic field and microwave frequency were measured as described previously [11].

Results

Effect of ethanethiol binding to cytochrome c

Fig. 1A and B shows the difference spectra of ferricytochrome *c* upon addition of ethanethiol. In the Soret region a maximum at 422 nm and a minimum at 405 nm develop rapidly after addition of the mercaptan. At this rather high ethanethiol concentration the initial difference spectrum shifts slowly to the spectrum of ferro- minus ferricytochrome *c* (trace 6 in Fig. 1A and B). This secondary, reductive process interferes with the direct determination of the dissociation constant of the rapidly formed complex. It is possible to obtain a Hill plot by successive additions of ethanethiol to the same ferricytochrome *c* solution, though the maximal absorbance change, due to the rapid complex formation, can only be estimated.

The saturation degree α is calculated from $\Delta A_{422\ nm}$ minus $\Delta A_{405\ nm}$. The slope of the Hill plot ($n = 1.1$) suggests that a 1 : 1 complex is formed with a dissociation constant of about 2 mM. Some deviation from a straight line is observed at higher ethanethiol concentrations, indicating that the reductive reaction contributes to the absorbance change at this stage of the experiment.

Using the stopped-flow technique, the dissociation constant of the initially

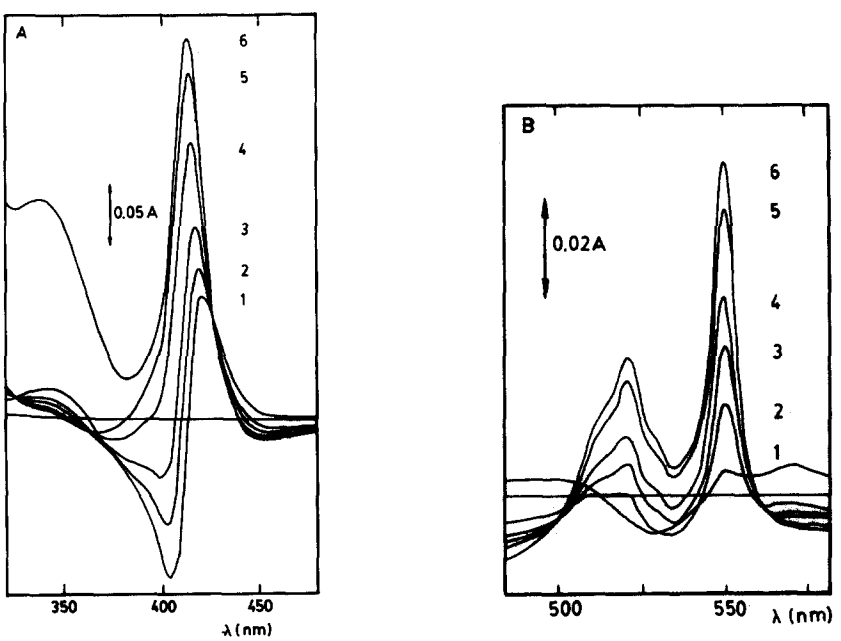


Fig. 1. Difference spectra of ferricytochrome c plus ethanethiol minus ferricytochrome c. (A) Soret region. (B) Visible region. Scan initiated: 1, immediately after mixing; 2, after 20 min; 3, after 60 min; 4, after 120 min; 5, after 200 min; 6, after addition of sodium dithionite. 4 μM ferricytochrome c and 20 mM ethanethiol, 25°C.

formed complex can be determined kinetically. When the observed reaction rate is plotted against the ethanethiol concentration a straight line is obtained (not shown). The rate of the binding reaction can be calculated from the slope of this line ($k_1 = 50\ M^{-1} \cdot s^{-1}$) and the dissociation constant from the intercept on the abscissa ($K_d = 2.0$ mM). This value is in good agreement with the K_d, obtained from the Hill plot.

Upon addition of dithionite to the ferricytochrome *c*-ethanethiol complex the spectrum of reduced cytochrome *c* is obtained. When ferrocytochrome *c* is incubated for several hours with 25 mM ethanethiol, no spectral change can be detected, indicating that no reaction occurs between ethanethiol and reduced cytochrome *c*.

Fig. 2A shows the pseudo first-order rate constant (k^*) of the reaction between ferricytochrome *c* and excess ascorbate at different ethanethiol concentrations. The apparent second-order rate constants (k_2^*), obtained from the slopes of the lines, are dependent on the concentration ethanethiol present, implying that the mercaptan inhibits the reduction of ferricytochrome *c* by ascorbate.

As the above-mentioned experiments suggest, ferricytochrome *c* and ethanethiol react readily to form a 1 : 1 complex,

$$\text{cyt. } c^{3+} + C_2H_5SH \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{cyt. } c^{3+}\text{—}C_2H_5SH \qquad (1)$$

Since both reactions are much faster than the reaction of ferricytochrome *c* with ascorbate (cf. Fig. 2A, upper line)

$$\text{cyt. } c^{3+} + \text{ascorbate} \overset{k_2}{\rightarrow} \text{cyt. } c^{2+} + \text{dehydroascorbate} \qquad (2)$$

the concentration of non-complexed ferricytochrome *c* can be represented by:

$$[\text{cyt. } c^{3+}_{\text{free}}] = \left(1 + \frac{[C_2H_5SH]}{K_d}\right) \cdot [\text{cyt. } c^{3+}_{\text{total}}] \qquad (3)$$

with

$K_d = k_{-1}/k_1$.

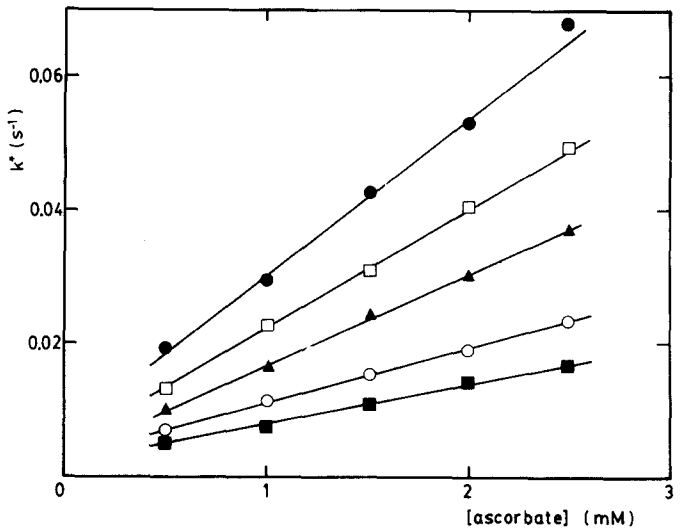
The assumption that the cytochrome *c*-ethanethiol complex is not reduced by ascorbate predicts a linear relationship between the reciprocal of the apparent second-order rate constant and the ethanethiol concentration:

$$\frac{1}{k_2^*} = \frac{1}{k_2} \cdot \left(1 + \frac{[C_2H_5SH]}{K_D}\right) \qquad (4)$$

k_2^* can be obtained from Fig. 2A, since $k^* = k_2^*[AH_2]$. When $1/k_2^*$ is plotted against the ethanethiol concentration (Fig. 2B) the intercept on the abscissa corresponds to a dissociation constant (K_d) of 1.8 mM. The straight line confirms the assumption that the cytochrome *c*-ethanethiol complex is not reducible by ascorbate.

Spectral effects of ethanethiol binding to cytochrome c oxidase

As can be seen in Fig. 3A, the spectrum of cytochrome *c* oxidase with



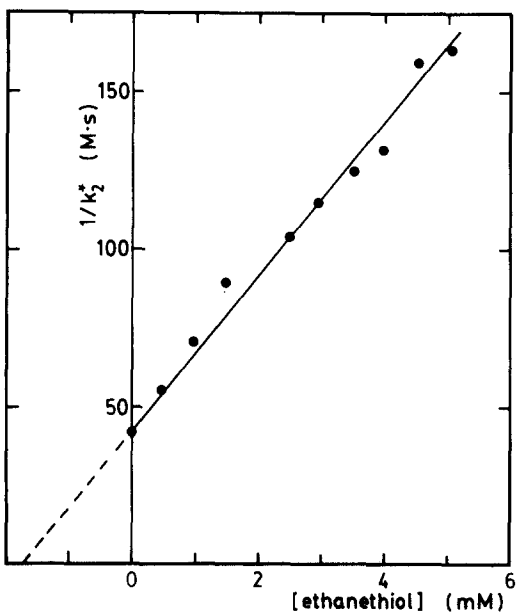


Fig. 2. (A) Effect of ethanethiol on the rate of reduction of ferricytochrome *c* by ascorbate. The apparent reaction rate (k^*) was determined from the exponential increase of the absorbance at 550 nm. The initial ferricytochrome *c* concentration was 20 μM. Ethanethiol was added before addition of ascorbate. ●——●, no ethanethiol; □——□, 0.5 mM ethanethiol; ▲——▲, 1.0 mM ethanethiol; ○——○, 2.5 mM ethanethiol; ■——■, 5 mM ethanethiol. (B) Effect of ethanethiol on the second-order rate constant of the reduction of ferricytochrome *c* by ascorbate. Apparent second-order rate constants were determined from the slope of the lines in Fig. 2A (not all experiments shown).

ethanethiol minus cytochrome *c* oxidase, recorded immediately after addition of ethanethiol, shows in the Soret region rapid formation of a minimum at 415 nm and a maximum at 439 nm, and in the α-band region a maximum at 595 nm and a minimum at 603 nm (Fig. 3B). This rapid reaction is followed by

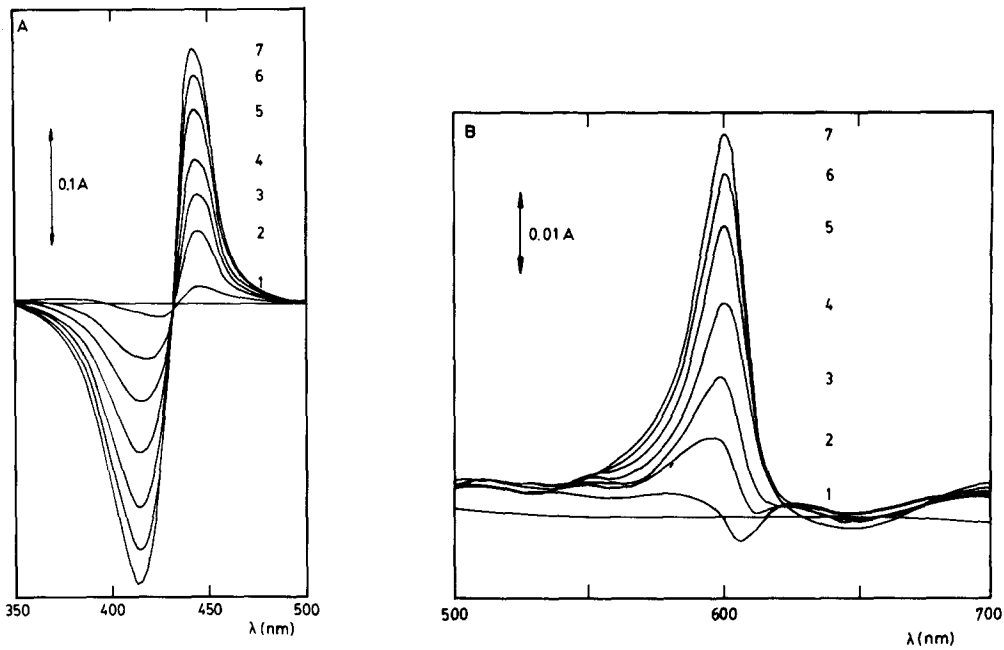


Fig. 3. Difference spectra of cytochrome *c* oxidase plus ethanethiol minus cytochrome *c* oxidase. (A) Soret region. (B) Visible region. Difference spectra were recorded: 1, immediately after mixing; 2, after 20 min; 3, after 40 min; 4, after 65 min; 5, after 95 min; 6, after 130 min; 7, after 170 min. 5 μM cytochrome *c* oxidase and 7 mM ethanethiol.

a much slower process, which causes further changes in the difference spectrum. The position of the maximum in the difference spectrum shifts from 439 to 444 nm and slowly to 442 nm; the minimum at 414 nm shifts to 418 nm and very slowly returns to its original position at 414 nm (Fig. 3A). The slow changes may be attributed to the reduction of cytochrome *c* oxidase by ethanethiol, although it is remarkable that the two extremes do not shift simultaneously. Due to these slow processes, a Hill plot of the first rapid complex formation shows a time-dependent curvature.

To determine the dissociation constant kinetically the rate of formation of the initial cytochrome *c* oxidase-ethanethiol complex was monitored at 439 nm with a stopped-flow apparatus. When the observed rate constant was plotted against the ethanethiol concentration a straight line was obtained which suggests a simple bimolecular reaction. From the slope of the line the value of the rate of complex formation is calculated ($33\ M^{-1} \cdot s^{-1}$), and from the intercept on the ordinate a dissociation rate constant of $0.13\ s^{-1}$ is obtained; from these values a dissociation constant of 3.9 mM can be calculated.

The effect of mercaptans on the steady-state activity of cytochrome c oxidase

The effect of increasing concentrations of several mercaptans on the enzymic activity of cytochrome *c* oxidase is measured spectrophotometrically at various ferrocytochrome *c* concentrations. In the absence of the mercaptans an exponential decrease of the ferrocytochrome *c* concentration is found after addition of cytochrome *c* oxidase, as has been reported frequently. When

mercaptans are present, this decrease is still exponential except for the first 5—20 s. From the second part of the curve, which represents over 90% of the total reaction, the molecular activity of cytochrome *c* oxidase is calculated in the usual manner. The Lineweaver-Burk plot of Fig. 4A shows that for all 1-propanethiol concentrations used, a linear relationship is obtained between

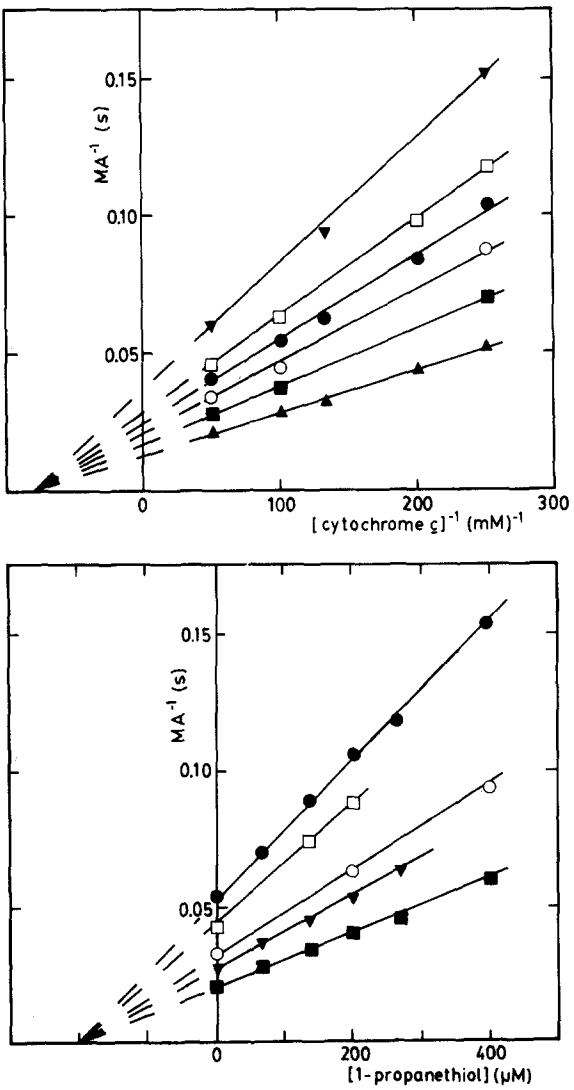


Fig. 4. (A) Lineweaver-Burk plot of the enzymic oxidation of ferrocytochrome *c* at various 1-propanethiol concentrations. Molecular activity (MA) was calculated as the number of ferrocytochrome *c* molecules oxidized/s per molecule cytochrome *c* oxidase and was determined from the exponential decrease of the absorbance at 550 nm. The enzymic reaction was started by the addition of cytochrome *c* oxidase to a final concentration of 16 nM. ▲———▲, no 1-propanethiol; ■———■, 67 μM 1-propanethiol; ○———○, 133 μM; ●———●, 200 μM; □———□, 267 μM; ▼———▼, 400 μM. (B) Dixon plot of the inhibition of cytochrome *c* oxidase activity by 1-propanethiol. Molecular activities were determined at the following ferrocytochrome *c* concentrations. ●———●, 4 μM; □———□, 5 μM; ○———○, 7.5 μM; ▼———▼, 10 μM; ■———■, 20 μM.

the reciprocal molecular activity and the reciprocal ferrocytochrome *c* concentration and furthermore that an increase of 1-propanethiol concentration results in an increase of the slope of the line. At these 1-propanethiol concentrations the lines coincide at the abscissa, corresponding to an apparent K_m value of 12 μM. It is clear from these results that 1-propranethiol inhibits the enzymic activity of cytochrome *c* oxidase non-competitively towards cytochrome *c*. The data from Fig. 4A are replotted in a Dixon plot (Fig. 4B). From the common intercept on the abscissa an inhibition constant K_i of 200 μM for 1-propanethiol is obtained.

Measurements of the enzymic activity of cytochrome *c* oxidase in the presence of other mercaptans show the same type of non-competitive inhibition towards cytochrome *c*. The inhibition constants of these mercaptans are presented in Table I. It is obvious that the inhibiting capability of the mercaptans decreases when the alkyl chain gets more bulky. Also shown in Table I are the K_i values of secondary thiols (2-propanethiol and 2-butanethiol) which are much higher than those of their primary isomers.

The effect of ethanethiol on the enzymic activity of cytochrome *c* oxidase was also determined polarographically. In this assay almost all cytochrome *c* is reduced [13] and not able to form a complex with ethanethiol. Again a non-competitive inhibition with K_i = 90 μM is found, so that the inhibitory action of the mercaptan may be completely attributed to its effect on cytochrome *c* oxidase.

In order to determine whether the protonated, uncharged mercaptan (RSH) or the unprotonated mercaptide (RS^-) is the inhibitory form, the inhibition constant K_i of ethanethiol (pK_a = 10.6 [14]) was determined in the pH range 5—8. If the mercaptide species would be responsible for the inhibition of cytochrome *c* oxidase activity, a decrease in K_i by a factor 10^3 may be expected over the pH range investigated. However, neither the non-competitive character of the inhibition is altered, nor a significant change in the value of the inhibition constant is observed in this pH range (Fig. 5). Assuming that the

TABLE I

INHIBITION OF CYTOCHROME *c* OXIDASE ACTIVITY BY VARIOUS THIO-COMPOUNDS (RSH)

Compound	R-SH	K_i
Hydrogen sulphide	H-	<0.1 μM *
Methanethiol	CH_3-	4.5 μM **
Ethanethiol	CH_3-CH_2-	91 μM
1-Propanethiol	CH_3-CH_2-CH_2-	200 μM
2-Propanethiol	CH_3 >CH- CH_3	1500 μM
1-Butanethiol	CH_3-CH_2-CH_2-CH_2-	330 μM
2-Butanethiol	CH_3-CH_2 >CH- CH_3	6.0 μM
Cysteamine	NH_2-CH_2-CH_2-	>50 mM
Thioglycolic acid	COOH-CH_2-	>50 mM

* Ref. 12.

** Determined at 10°C.

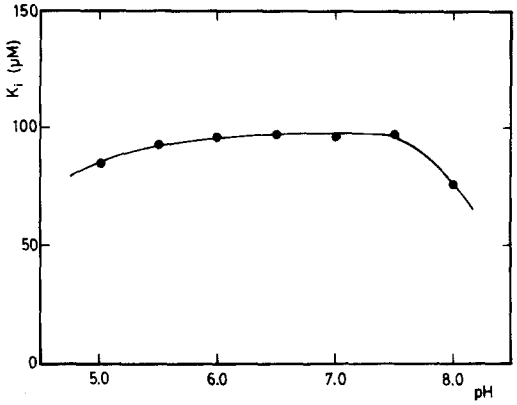


Fig. 5. Effect of pH on the inhibition constant of ethanethiol. The inhibition constant (K_i) was determined from at least three experiments, using ethanethiol concentrations ranging from 33 to 267 μM. The non-competitive character of the inhibition was confirmed at pH 5.5 and 7.0. Experimental conditions: 20 nM cytochrome *c* oxidase; 25 μM cytochrome *c*; 100 mM potassium phosphate, 1% Tween 80, 25°C.

heme-thiolate binding site is in pH equilibrium with the solvent, it may be concluded that the uncharged mercaptan inhibits cytochrome *c* oxidase. It is interesting to note that charged mercaptans like thioglycolic acid or cysteamine are unable to inhibit cytochrome *c* oxidase (Table I), although the size of these ligands is comparable to the size of propane- or butanethiol.

EPR spectra of cytochrome *c* oxidase also demonstrate that thiols form complexes with cytochrome *c* oxidase. When the enzyme is incubated (1 min) with ethanethiol (50 mM) the low-spin heme signal at $g = 3$ and the signal at $g = 2$ originating from low-spin cytochrome *a* and copper, respectively, decrease in intensity. This shows that ethanethiol reduces the enzyme in line with the optical experiments. At the same time two minor low-spin heme signals appear at $g = 2.43$, 2.24 and 1.91 and at 2.36, 2.24 and 1.94, respectively. Prolonged incubation of the enzyme with ethanethiol had no further effect on the spectrum. Since most cytochrome a_3-ligand complexes become clearly visible only in the partially reduced enzyme, ascorbate and a catalytic amount of cytochrome *c* were added. Fig. 6 (A and B) shows that after 1 min incubation the new low-spin resonances intensify while both the copper signal and the low-spin cytochrome *a* signal decrease in intensity. Upon prolonged incubation (Fig. 6, trace C) the latter signals have disappeared nearly completely and the low-spin heme signal at $g = 2.43$, 2.24 and 1.91 shows a further intensification. The *g* values of these ethanethiol-induced species correspond to those reported for other mercaptide-hemoprotein complexes [15]. Experiments similar to those shown in Fig. 6 were carried out with methanethiol instead of ethanethiol. The EPR spectrum of cytochrome *c* oxidase incubated with this ligand and subsequently reduced with ascorbate and cytochrome *c* shows again formation of a typical low-spin heme-mercaptide compound with $g_{z,y,x} = 2.39$, 2.23 and 1.93.

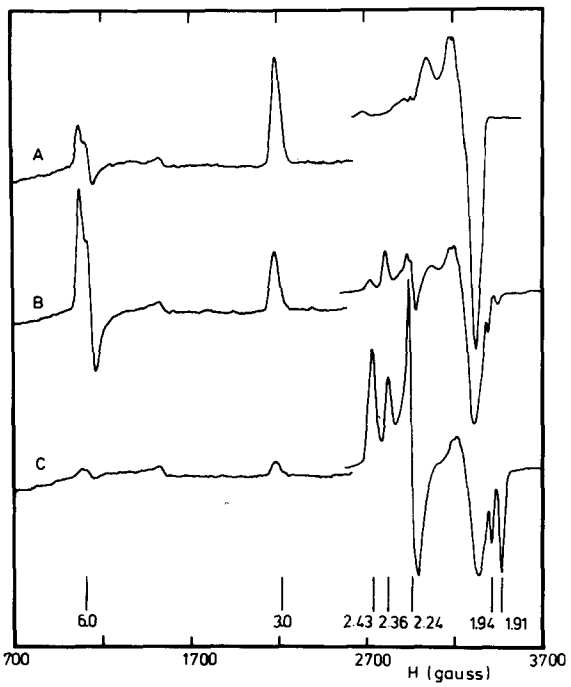


Fig. 6. The effect of ethanethiol on the EPR spectrum of cytochrome *c* oxidase. (A) 0.27 mM cytochrome *c* in 50 mM potassium phosphate (pH 7.0), 0.5% Tween 80. (B) After addition of 50 mM ethanethiol and incubation (1 min) at room temperature, 20 μM cytochrome *c* and 40 mM ascorbate were added and incubated for 1 min at room temperature. (C) After incubation of (B) at room temperature for 4 min. Conditions of EPR spectroscopy were: frequency: 9.312 GHz; microwave power, 2 mW; modulation amplitude, 10 G; scanning rate, 500 G · min^{-1}; time constant 1 s; temperature 14 K. The copper signal in (A) and (B) was recorded at a 5-fold lower receiver gain, whereas that in (C) was recorded at a 2-fold lower gain.

Discussion

The results obtained in this study show that ethanethiol reacts with oxidized cytochrome *c* to form a spectroscopically distinct compound which is not reducible by ascorbate, whereas no compound is formed with reduced cytochrome *c*. The dissociation constant of the ferricytochrome *c*-ethanethiol complex (K_d = 2 mM) is much larger than the inhibition constant of the cytochrome *c* oxidase activity by ethanethiol (K_i = 91 μM). This demonstrates that inhibition of the respiratory chain by this mercaptan is primarily due to reaction with cytochrome *c* oxidase. Similarly, azide and cyanide have been reported [16] to react with oxidized cytochrome *c* with a dissociation constant which is large compared to the respective inhibition constants of the cytochrome *c* oxidase activity.

The affinity of ethanethiol towards oxidized cytochrome *c* oxidase is even lower than towards cytochrome *c*. However, as is observed with cyanide [17], the affinity for cytochrome *c* oxidase increases when the enzyme is under turnover conditions. Preliminary experiments show that in the presence of cytochrome *c*, ascorbate and oxygen the inhibited cytochrome *c* oxidase-

thiol complex is formed during turnover with a second-order rate constant of $50\ M^{-1} \cdot s^{-1}$ and a dissociation rate constant of $4.5 \cdot 10^{-3}\ s^{-1}$. From these rate constants an inhibition constant ($K_i = 90\ \mu M$) can be calculated which is the same as that obtained directly from inhibition studies analogous to those shown in Fig. 4. These results suggest that in partially reduced enzyme the ligand-binding site becomes more exposed, in line with a proposal by Van Buuren et al. [18].

Our inhibition studies of cytochrome *c* oxidase activity by various mercaptans (Table I) demonstrate that the inhibitory action of the SH group is strongly affected by size and shape of the alkyl chain. Thus, steric hindrance by this chain might preclude the mercaptans from approaching the vicinity of the inhibitory site on cytochrome *c* oxidase. Furthermore, only neutral mercaptans are able to inhibit cytochrome *c* oxidase. This finding is in agreement with previous observations [16,19] on the inhibition of cytochrome *c* oxidase by cyanide and azide, which also demonstrates that the uncharged ligand inhibits cytochrome *c* oxidase. These observations may suggest that the site of inhibition, presumably the heme iron of cytochrome a_3, is situated in a hydrophobic environment or is only accessible via a hydrophobic region. Charged molecules are therefore unable to penetrate to the ligand-binding site. This hypothesis is supported by the infrared studies on carboxycytochrome *c* oxidase, which demonstrate that the CO molecule bound to cytochrome a_3 resides in a hydrophobic environment [20].

The EPR spectra of cytochrome *c* oxidase, partially reduced in the presence of ethane- or methanethiol, demonstrate that these thiols form low-spin complexes with cytochrome a_3. This was also concluded by Nicholls and Hildebrandt [21] from their study of the effect of alkyl thiols on the optical spectrum of partially reduced enzyme. The *g* values of these compounds are very close to those reported for other heme-thiol complexes [15] and this suggests that the sulphur atom is ligated to the heme iron of cytochrome a_3. Chevion et al. [15] have made a crystal-field analysis of several thiol compounds and were able to assign a domain in the 'truth' diagram [22], which corresponds to a ligand field of the heme iron in which the counter ligand to sulphur was imidazole. A similar analysis carried out for the thiol complexes of cytochrome *c* oxidase shows that the crystal-field parameters, calculated according to Taylor [23], of the signals observed in the ethanethiol-treated enzyme and that of the methanethiol-treated enzyme fall in this region. This suggests that in cytochrome *c* oxidase one of the axial ligands in cytochrome a_3 is a nitrogen from imidazole. This suggestion is in line with the observations of Blokzijl-Homan and Van Gelder [24] on NO-treated cytochrome *c* oxidase. This NO-bound form shows a nitrogen hyperfine pattern, which is consistent with imidazole as the axial ligand *trans* to the NO. In addition the low-spin azide, cyanide and sulphide forms of cytochrome a_3 have also EPR properties [25] which are very similar to those of the corresponding derivatives of myoglobin in which the axial ligand is imidazole.

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