AUTOOXIDATION OF CYSTEINE CATALYSED BY COBALT(II) TETRASULPHOPHTHALOCYANINE. MODELS OF OXIDASES V.

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The paper describes the kinetics of autooxidation of cysteine catalysed by cobalt(II) 4,4′,4″,4‴-tetrasulphophthalocyanine (CoTSP) in buffered solutions, pH 6·5 to 12·5. The results prove incompatible with the radical reaction mechanism previously propounded.

In preceding papers of this series[1,2] oxidation of hydrazine and hydroxylamine by molecular oxygen, catalysed by CoTSP, is interpreted by a mechanism based on the formation of a ternary complex substrate–catalyst–oxidant. The transfer of electrons is supposed to occur in the coordination sphere of the ternary complex, evidently without any liberation of free radicals from the complex. The catalyst thus works as a simple oxidase.

CoTSP also catalyses autooxidation of a number of thio compounds containing an S-H bond (cysteine, thioglycollic acid, butyl mercaptan). If the thiol hydrogen is substituted (methionine, dibutyl sulphide, thiodiglycollic acid) no autooxidation proceeds[3]. The mechanism (*I*) proposed[4–6] for the autooxidation of cysteine differs from that considered by us for the oxidation of hydrazine and hydroxylamine.

Mechanism *I*

(RSH = cysteine, RSSR = cystine)

$$RSH \rightleftharpoons RS^- + H^+ , \qquad (1)$$

$$Co^{(III)}TSP + RS^- \xrightarrow{slow} Co^{(II)}TSP + RS^\bullet , \qquad (2)$$

$$Co^{(II)}TSP + RS^- \xrightarrow{slow} Co^{(I)}TSP + RS^\bullet , \qquad (3)$$

$$RS^\bullet + RS^\bullet \xrightarrow{fast} RSSR , \qquad (4)$$

$$O_2 + 4\,Co^{(I)}TSP + 4\,H^+ \xrightarrow{fast} 4\,Co^{(II)}TSP + 2\,H_2O , \qquad (5)$$

$$O_2 + 4\,Co^{(II)}TSP + 4\,H^+ \xrightarrow{\text{fast}} 4\,Co^{(III)}TSP + 2\,H_2O\,. \qquad (6)$$

This mechanism is based largely on the interpretation of spectra of the systems CoTSP–cysteine–H_2O, CoTSP–O_2–H_2O, CoTSP–cysteine–cystine–O_2–H_2O, assuming a change in the oxidation state of cobalt. However, few kinetic measurements have been published. (The reaction rate at a constant concentration of oxygen at pH 7·8 is proportional to the concentrations of the catalyst and cysteine; the proportionality constant $k = 2{\cdot}1 \,.\, 10^3\ l\ mol^{-1}\ min^{-1}$, the activation energy $E_a = 7{\cdot}1$ kcal mol^{-1}. The rate passes throw a maximum at pH 9–10.)

As was shown previously[1,2], the catalytic activity of tetrasulphophthalocyanines is related to their capacity of a reversible binding of molecular oxygen[6–9]. The present paper advances a systematic study of the reaction kinetics in the pH region where the adduct CoTSP-O_2 is formed, and confronts the results with the radical mechanism *I*.

EXPERIMENTAL

Cobalt(II) 4,4′,4″,4‴-tetrasulphophthalocyanine was prepared by melting sodium 4-sulphophthalate, urea, ammonium chloride and cobalt(II) sulphate[10]. 0·1M solutions of cysteine were prepared by dissolution of L-cysteine (pure, Loba-Chemie, Vienna) in 0·1M-HCl (A.G.) that had been freed from oxygen by bubbling nitrogen through. The other chemicals were of A.G. purity.

The concentration of oxygen in the course of the reaction was followed polarographically at a constant potential −0·72 V (saturated calomel electrode, Radelkis instrument OH 102). The reaction vessel (content 100 ml), was equipped with a thermostatic jacket ($t = 25{\cdot}0 \pm 0{\cdot}01$°C) and a silicone rubber stopper, through which were inserted the reference calomel electrode, the dropping mercury electrode, a gas inlet (O_2, air, N_2) and a closing valve. It was always fully filled in order to leave no space for a gas over the reaction mixture. The component starting the reaction was brought in from a syringe (in most cases a solution of cysteine).

The reaction was conducted in the Sörensen borate buffer, pH 6·5 to 12·5. The initial concentration of oxygen was $0{\cdot}5 \,.\, 10^{-4}$ to $6{\cdot}5 \,.\, 10^{-4}$ mol l^{-1}. Calibration was carried out with KCl solutions, for which the solubility of oxygen is tabulated. Concentration of CoTSP ranged from $5 \,.\, 10^{-7}$ to $1 \,.\, 10^{-5}$ mol l^{-1}, concentration of cysteine from $1 \,.\, 10^{-4}$ to $5 \,.\, 10^{-3}$ mol l^{-1}.

The absorption spectra were measured with a spectrophotometer Unicam SP 800 B in 1-cm cells equipped with silicone rubber stoppers. Through a hypodermic needle in the stopper a gas (oxygen, air, nitrogen) was introduced into the solution or another reagent was added. The nitrogen had been washed free of oxygen in chromous chloride and an alkaline solution of hydrazine and CoTSP.

The absorption spectra were recorded simultaneously with monitoring the oxygen concentration (the reaction vessel was connected with a through-flow cell). The solution was circulated with the aid of a peristaltic pump. The concentration of oxygen was monitored with an oxygen sensor SKT/03 (Chemoprojekt, Satalice), coupled to a recorder EZ 11.

In measuring the dependence of the initial reaction rate on the relative amount of the CoTSP-O_2 adduct in the catalyst the buffered solutions of CoTSP (in the concentration range $5 \,.\, 10^{-6}$ to $5 \,.\, 10^{-5}$ mol l^{-1}), pH 12·3, had always been saturated with oxygen for 20 min. Changes in absorption spectra were investigated in the region 400 to 700 nm. The reaction was started by the

addition of cysteine. In reference experiments, in which only the buffer was saturated with oxygen to the same concentration, the reaction was started by the simultaneous addition of CoTSP and cysteine.

In a polarographic monitoring of the CoTSP–cysteine interaction in oxygen-free media the buffered solutions of CoTSP ($6 . 10^{-5}$ to $1 . 10^{-3}$ mol l^{-1}), pH 12·5, were always presaturated with nitrogen till maximum suppression of the oxygen polarographic wave was reached. Then, under a constant introduction of nitrogen, a solution of cysteine of the desired concentration was added from the syringe. Calibration was carried out with solutions of cystine (molarity range $5·5 . 10^{-4}$ to $2·1 . 10^{-3}$) $E_{1/2} = 1·3$ V/S.C.E.

RESULTS AND DISCUSSION

In Fig. 1 are plotted the initial reaction rates *vs* pH for a catalyst concentration $2·06 . 10^{-6}$ mol l^{-1} and for zero concentration of the catalyst. As it is seen, the non-catalysed reaction had a maximum rate at pH ~ 7; at pH > 9 the rate was nearly two orders of magnitude lower than that of the reaction catalysed by 10^{-5}M-CoTSP. However, in the whole range of the catalyst concentrations the non-catalysed reaction was kinetically significant. The initial rate of the CoTSP-catalysed reaction passes throw a maximum at pH 9·7.

The kinetical data reveal that the initial reaction rate is directly proportional to the cysteine concentration throughout the investigated ranges of cysteine concentration and pH. Measurements at different concentrations of oxygen and CoTSP

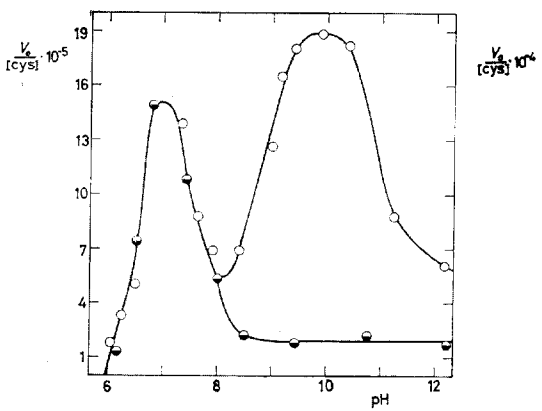

FIG. 1

Initial Reaction Rate Divided by the Starting Concentration of Cysteine in Relation to pH

◒ Absence of the catalyst, ○ catalyst concentration CoTSP = $2·06 . 10^{-6}$ mol l^{-1}.

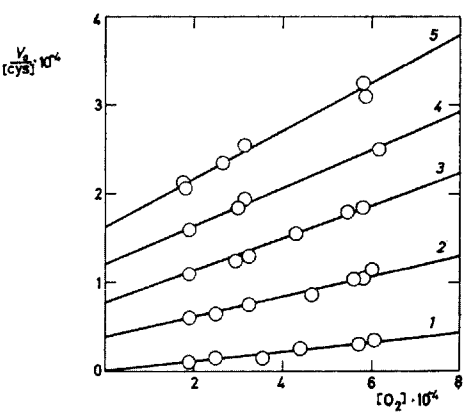

FIG. 2

Initial Reaction Rate at pH 10·7 Divided by the Starting Concentration of Cysteine in Relation to Concentration of Oxygen

Molar concentrations of CoTSP: *1* zero, *2* $1·03 . 10^{-6}$, *3* $2·06 . 10^{-6}$, *4* $3·09 . 10^{-6}$, *5* $4·12 . 10^{-6}$.

have shown that the initial reaction rate can formally be related to the reactant concentrations by the equation

$$v_0 = k_1[\text{cyst.}]\,[O_2] + k_2[\text{cyst.}]\,[\text{CoTSP}] + k_3[\text{cyst.}]\,[\text{CoTSP}]\,[O_2]\,. \qquad (7)$$

The values of k_1, k_2 and k_3 for four pH's are listed in Table I (The value of k_1 for pH 7·0 was determined directly by measuring the rate of the non-catalysed reaction.) The initial reaction rate as a function of oxygen concentration for different concentrations of CoTSP is shown in Fig. 2.

An essential feature of mechanism *I* is the fact that the function of the catalyst is conditioned by a change in the oxidation state of cobalt. The rate determining step is here a one-electron oxidation of cysteine by the catalyst. The reaction intermediates would then be the cobalt(I) tetrasulphophthalocyanine and RS$^\bullet$ radicals. The reoxidation of the catalyst by oxygen is regarded as fast enough not to affect the overall reaction rate.

The authors[4–7] of mechanism *I* assign the individual spectral bands, in the region 400–700 nm, of tetrasulphophthalocyanines of Co(II), Co(I), and Co(III) as follows:

Co(II) (λ_1 634 nm, ε_1 38000)

Co(I) (λ_2 454 nm, ε_2 26500, λ_3 692 nm, ε_3 18000)

Co(III) (λ_4 680 nm, ε_4 123000, λ_5 612 nm, ε_5 32000)

From our measurements[7,8], corroborated in ref.[9], it appears, however, that the complex characterized by absorptions at 670 nm and 600 nm is a reversible adduct CoTSP-O_2, and not Co(III) tetrasulphophthalocyanine. The assignment of the absorption band at ~450 nm to Co(I)TSP is not quite safe. This band is also exhibited,

TABLE I

Formal Rate Constants from Equation (7) $t = 25°C$

pH	k_1 1 mol^{-1} s^{-1}	k_2 1 mol^{-1} s^{-1}	k_3 1 mol^{-2} s^{-1}
12·5	$3{\cdot}5 \cdot 10^{-2}$	$2{\cdot}20 \cdot 10^{1}$	$1{\cdot}00 \cdot 10^{4}$
10·7	$5{\cdot}5 \cdot 10^{-2}$	$3{\cdot}87 \cdot 10^{1}$	$6{\cdot}15 \cdot 17^{4}$
9·2	$1{\cdot}4 \cdot 10^{-1}$	$3{\cdot}40 \cdot 10^{1}$	$4{\cdot}53 \cdot 10^{4}$
7·0	$3{\cdot}1 \cdot 10^{-1}$[a]		

[a] The value was determined from the rate of the reaction in the absence of CoTSP.

in an inert atmosphere, by mixtures of CoTSP with hydrazine, hydroxylamine, ascorbic acid, thiols, thioglycollic acid, cysteine, Na_2S (ref.[5]), $Na_2S_2O_4$ (ref.[5]) and with Na_2SO_3 (ref.[11]). The absorption band appears after the reduction of CoTSP by titanium trichloride[5] or sodium borohydride[11], or electrolytic reduction of CsTSP in dimethyl sulphoxide[12]. If the interaction of CoTSP and cysteine in an inert atmosphere effected a complete one-electron transfer (equation (*3*)), the fast recombination of radicals would necessarily produce cystine, which would be easily detected at pH 12·3. However, our polarographic analysis revealed no wave of cystine under conditions where a detectable quantity of cystine would have to be formed by the reaction considered. The cystine wave was not present until oxygen had been bubbled through the solution. Hence it seems unlikely that the interaction of cysteine with CoTSP should entail reduction to Co(I)TSP; in this case the band at 450 nm probably belongs to an adduct (complex) CoTSP–cysteine.

According to the hypothesis of the oxidation change in cobalt the reaction rate should be decreased by an increase in the relative concentration of Co(III) (absorbing at 670 and 600 nm)[6]. The assumed, relatively slow reaction 2 should play a role in the kinetics. In view of these ideas we carried out several experiments in which the catalyst solution was saturated with oxygen for several minutes at pH 12·3 before the reaction; the increase in absorption at 670 nm was measured. However, the reaction rate was the same in these experiments (within the range of experimental error) as in the reaction catalysed by CoTSP, in the spectrum of which the band at 670 nm is absent. Consequently, the relative amount of the CoTSP-O_2 adduct had no influence on the reaction rate.

In several experiments we also followed simultaneously the decrease in the content of oxygen and the changes in absorption spectra in the course of the reaction. The decrease in oxygen did not affect the spectra much. If the spectrum of the starting catalyst has a marked peak at 670 nm it steadily decreased during the reaction, but no quantitative conclusion can be drawn because several bands overlap in this region. When the reaction had ended an absorption peak at 450 nm soon appeared if the solution contained an excess of cysteine.

The experimental data allow us to draw several conclusions on the mechanism of the reaction. The overall process in the region pH 6·5 – 12·5 consists of two simultaneous reactions. One is the so-called non-catalysed reaction, which is first order with respect to both cysteine and oxygen; it is probably catalysed by traces of metallic ions (especially Cu and Fe)[13,14]. The other reaction, catalysed by CoTSP, has a rate defined by the equation

$$v_0 = k_2[\mathrm{CoTSP}]\,[\mathrm{Cys}] + k_3[\mathrm{CoTSP}][\mathrm{Cys}][\mathrm{O_2}]\,. \qquad (8)$$

The dependence on oxygen concentration shows the impossibility of mechanism *I* in its original form, *i.e.* with the rate-determining step (*2*) or (*3*) and a sufficiently

fast reoxidation of the catalyst, so that the reaction rate would be independent of the oxygen concentration.

Apart from the dependence on oxygen concentration, there have already been mentioned some experimental facts incompatible with mechanism *I*: *a*) Co(III)TSP does not exist under the experimental conditions used; the compound in question is a Co(II)TSP-O_2 adduct. *b*) The reaction rate depends on the total concentration of CoTSP and is independent of the relative content of the CoTSP-O_2 adduct. *c*) Co(I)TSP is hardly a possible reaction intermediate. From all these data it can be judged that at least in the first step the reaction proceeds by the mechanism involving the ternary complex, like in the oxidation of hydrazine and hydroxylamine[1,2].

The dependence of the rate of the catalysed reaction upon pH (Fig. 1) can be interpreted like that of an enzyme-catalysed reaction, where the effect of deprotonization of functional groups of the enzyme is considered[15]. The dissociation constants of cysteine are: $pK_{a1} = 2{\cdot}3$(—COOH), $pK_{a2} = 8{\cdot}3$(—SH) and $pK_{a3} = 10{\cdot}4$ ($—NH_3^+$) (ref.[16]). In the alkaline region the groups —SH and NH_3^+ dissociate, so that three differently protonized forms of cysteine can be operative. If we assume that the binary complex with CoTSP is formed mainly by cysteine with a dissociated mercapto group and an undissociated ammonium group, the catalysed autooxidation of cysteine can be described by the scheme

$$\begin{array}{ccccccc} \mathrm{HRSH} & & & & & & \\ \Big\downarrow\uparrow K_{a2} & & & & & & \\ \mathrm{HRS^-} & \overset{K_1}{\rightleftharpoons} & \mathrm{HRS^-\,.\,CoTSP} & \overset{K_2}{\rightleftharpoons} & \mathrm{HRS^-\,.\,CoTSP\,.\,O_2} & \xrightarrow{k} & \\ \Big\downarrow\uparrow K_{a3} & & & & & & \\ \mathrm{RS^{2-}} & & & & & & \end{array}$$

$$\longrightarrow \text{ products} \qquad (II)$$

where K_1 and K_2 are equilibrium constants of the binary and the ternary complexes, respectively. If the concentrations of the single forms of cysteine are expressed by equilibrium and dissociation constants in the mass balance for cysteine, the concentration of the reactive form, HRS^-, is

$$[\mathrm{HRS^-}] = \frac{[\mathrm{Cys}]_0}{1 + \dfrac{[\mathrm{H^+}]}{K_{a2}} + \dfrac{K_{a3}}{[\mathrm{H^+}]} + K_1[\mathrm{CoTSP}] + K_1K_2[\mathrm{CoTSP}][\mathrm{O_2}]}, \qquad (9)$$

where $[Cys]_0$ is the analytical concentration of cysteine. It is evident that under the given conditions the reaction rate will pass through a maximum at a pH at which

$[HRS^-]$ reaches its maximum as well. Analysis for the extreme of function (9) reveals that $[HRS^-]$ will exhibit a peak at $pH_{max} = 0{\cdot}5(pK_{a2} + pK_{a3})$. In our case, consequently, the reaction rate should be maximum at pH_{max} 9·35. The experimental value was pH_{max} 9·7. As is seen, the dependence of v_0 on pH accords with the assumption that the catalysed reaction prceeds by the mechanism involving the ternary complex.

Provided the rate-determining step is decomposition of the ternary complex, the relation of the initial rate, v_0, to concentration of cysteine should obey the Michaelis–Menten equation. The experimental course, in the measured concentration range, was linear. This may be consistent with the Michaelis–Menten equation, but is by no means a proof of its validity. The typical curvature may occur in a region of substrate concentrations difficult to realize experimentally.

Cysteine may be linked to CoTSP through the sulphur atom or through the nitrogen of the amino group. The former possibility seems much more likely, since CoTSP also catalyses autooxidation of other thio compounds, with sulphur as the only donor atom. Besides, the linkage *via* sulphur has been demonstrated in the reactions of cysteine and some mercaptans with ions of transition metals[17]. Considering that the oxidation product is cystine, so that the reaction must produce the S—S bond, it seems probable that the transfer of the first electron in the ternary complex is followed by liberation of an $RS^{\bullet}$ radical and dimerization.

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