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THE EFFECT OF INHIBITORS ON THE OXYGEN KINETICS OF CYTOCHROME *c* OXIDASE

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SUMMARY

1. The oxygen kinetics of purified beef heart cytochrome *c* oxidase were investigated.

2. The effect of addition of various fixed concentrations of the inhibitors CO, HN_3, HCOOH, HCN and H_2S on the double reciprocal plot of respiration rate against oxygen concentration was studied.

3. CO is strictly competitive, azide and formate are uncompetitive, and cyanide and sulfide are non-competitive inhibitors towards oxygen.

4. Binding constants for the various inhibitors obtained from secondary plots of the oxygen kinetics at pH 7.4 are: CO: $K_i = 0.32$ μM, azide: $K_i = 33$ μM; formate: $K_i = 15$ mM; cyanide: $K_i = 0.2$ μM and sulfide: $K_i = 0.2$ μM.

5. The possible significance of these results in the elucidation of the reaction mechanism is discussed.

INTRODUCTION

In the discovery of the 'Atmungsferment' [1] and its identification with the cytochrome a_3 component of cytochrome *c* oxidase [2, 3], the use of inhibitors was of central importance. Since then a wealth of information about the properties of this enzyme has accumulated from ligand binding studies (see refs. 4 and 5 for review). To be able to gain information about the reaction mechanism of the cytochrome *c* oxidase from this knowledge, an obvious approach would be the conventional use of these inhibitors as tools in a study of the steady-state kinetics of the reaction. The effect of inhibitors on the cytochrome *c* kinetics has been studied quite extensively [6–9]. But as the ligand binding properties of the oxidase are closely associated with the component of the oxidase, cytochrome a_3, which in its reduced form reacts with O_2, a study of the effect of inhibitors on the oxygen kinetics might be expected to be especially informative. Only a few such studies have been reported. The investigations were concerned with CO [10] and HCN [11] inhibition of yeast cell respiration at low oxygen concentrations, and the observations may not be directly applicable to the isolated oxidase.

The effect of CO, azide, formate, cyanide and sulfide on the oxygen kinetics of the ascorbate → cytochrome *c* → cytochrome aa_3 → O_2 system is investigated in the present work. This represents an extension of our recent analysis of the mechanism of the cytochrome *c* oxidase reaction [12] based on measurements of the oxygen kinetics of the same ascorbate oxidase system at various fixed concentrations of cytochrome aa_3, cytochrome *c* and ascorbate. The proposed model is further developed to account for the results presented here in terms of the known ligand binding properties of the oxidase.

METHODS AND MATERIALS

The respirograph system [13] was extended as previously described [12]. A constant partial pressure of the gasous inhibitors CO, HCN and H_2S in the gas phase was obtained by means of an extra gas mixer [14]. This mixer adds a constant flow of inhibitor/nitrogen gas mixture of constant composition to the flow from the O_2/N_2 gas mixer, the composition of which is changing during the experiment. The O_2/N_2 composition in the gas phase is regulated by means of a computer system with feed-back control to give a linear increase with time in the oxygen concentration in the liquid. The respiration rate as a function at the oxygen concentration was calculated and plotted as Lineweaver-Burk plots by means of on-line computation during the experiments. Such plots were recorded at various fixed compositions of the inhibitor/nitrogen gas mixture.

Oxygen concentration was measured with Clark type electrodes (Radiometer, Copenhagen). Oxygen kinetics were measured in a stirred vessel open to diffusion from the gas phase, the composition of which was determined by the gasmixers. Cytochrome *c* kinetics were determined by measuring the oxygen consumption rate in a conventional 'closed' vessel. Both vessels were thermostated at 25 °C.

Purified cytochrome aa_3 was prepared as described by van Buuren [15]. Cytochrome *c* was Sigma type VI.

RESULTS

Qualitatively the steady-state kinetics of cytochrome *c* oxidase, measuring the oxygen consumption rate, v, of the ascorbate → cytochrome *c* → cytochrome aa_3 → O_2 system as a function of $[O_2]$, does not change its character during the purification procedure. The Lineweaver-Burk plots of $1/v$ against $1/[O_2]$ with the present purified cytochrome *c* oxidase preparation at various concentrations of the enzyme (shown in Fig. 1) are very similar to those obtained [12] with a fraction taken at an early stage of the purification. Also in accordance with the measurements on the partly purified preparation, plots of $1/v$ against $1/[O_2]$ at various fixed concentrations of cytochrome *c* and ascorbate are linear and parallel with the purified enzyme (experiments not shown). Some quantitative differences are however observed.

The most pronounced effect is the decline in molecular activity during purification [16, 17]. This enzyme preparation has a maximal turnover (TN_{max}) of 210 electrons/s per cytochrome aa_3 compared to $TN_{max} = 400$ electrons per cytochrome aa_3 for the partly purified oxidase preparation [12]. The apparent K_m for oxygen of

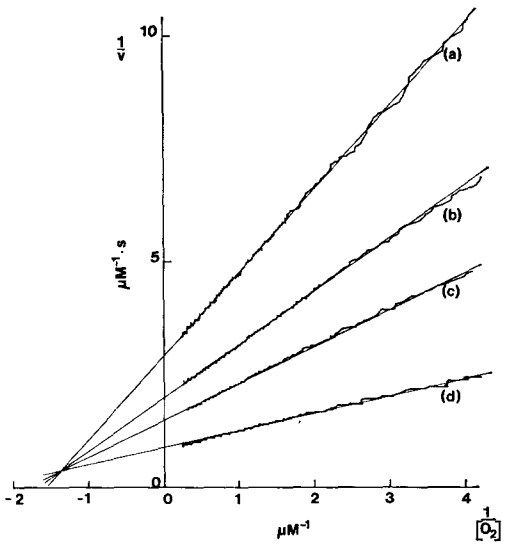


Fig. 1. Reciprocal plots of oxygen consumption rate against $[O_2]$ concentration. The effect of varying enzyme concentration. (a) 8.5 nM, (b) 12.8 nM, (c) 17 nM, (d) 34 nM cytochrome aa_3. The medium contained 23 mM ascorbate, 68 μM cytochrome c, 67 mM potassium phosphate, 1 mM EDTA, 0.5 % Tween-80 pH 7.4, 25 °C.

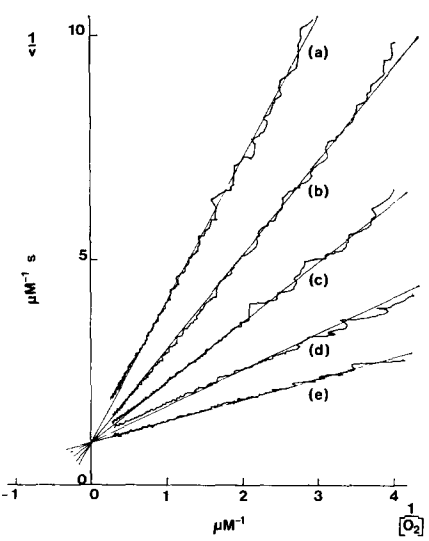


Fig. 2. The effect on the oxygen kinetics of various concentrations of carbon monoxide. (a) 1.73 μM, (b) 1.15 μM, (c) 0.58 μM, (d) 0.23 μM, (e) 0 μM of CO. The medium contained 23 mM ascorbate, 68 μM cytochrome c, 34 nM cytochrome aa_3, 67 mM potassium phosphate, 1 mM EDTA, 0.5 % Tween-80, pH 7.4, 25 °C.

the present preparation (0.75 μM) is a little lower than that of the partly purified enzyme (0.95 μM) [12].

Preferably the effect of a terminal inhibitor on the oxygen kinetics should be studied at a constant saturating concentration of ferrocytochrome *c*. This requirement is met in the present study by addition of cytochrome *c* at a concentration which is high, 68 μM, relative to the apparent K_m of 20 μM. A rapid regeneration of reduced cytochrome *c* is secured by a high concentration, 23 mM, of ascorbate. At the highest oxidation rates reported, maximally 8 % of ferricytochrome *c* will accumulate.

In Fig. 2 the effect of CO on the oxygen kinetics is investigated. The double reciprocal plot of respiration rate against oxygen concentration is shown for the uninhibited enzyme (curve e) and in the presence of various fixed partial pressures of CO in the gas phase (curves a–d). CO is a strictly competitive inhibitor towards oxygen. The secondary plot of slope against inhibitor concentration yields a straight line, from which an apparent K_i equivalent to 0.32 μM of CO is found.

In Figs. 3 and 4 the effect of azide and formate at various fixed concentrations is shown. Both inhibitors are of the uncompetitive type. Secondary plots of intercept against inhibitor concentration yield straight lines. K_i = 33 μM for azide and K_i = 15 mM for formate, at pH 7.4.

The effect of fluoride on the oxygen kinetics could not be determined, since ascorbate oxidation was only slightly inhibited at high concentrations of this substance. When the same concentration of chloride was added to the control, to compensate for the effect imposed by the change in ionic strength of the medium, the inhibitory effect of fluoride was extremely small (K_i > 0.2 M at pH 7.4).

The effect of cyanide and sulfide is shown in Figs. 5 and 6. The effect of these inhibitors on the steady-state kinetics seems to be complicated by the slow rate of binding to the oxidase of these inhibitors [18, 19]. The following procedure was

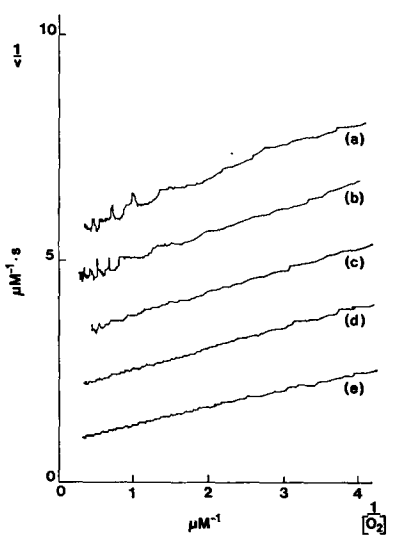


Fig. 3. The effect on the oxygen kinetics of various concentrations of azide. (a) 176 μM, (b) 132 μM, (c) 88 μM, (d) 44 μM, (e) 0 μM of NaN_3. Other conditions as described in Fig. 2.

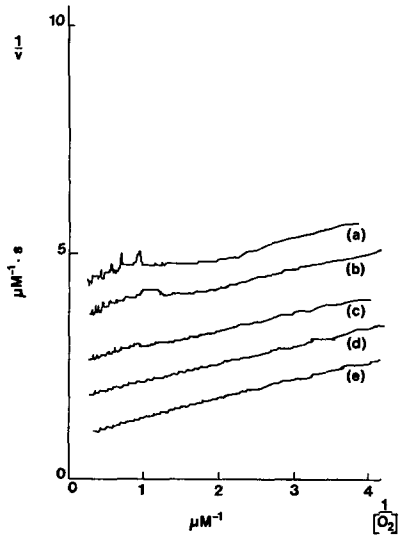


Fig. 4. The effect on the oxygen kinetics of various concentrations of formate. (a) 44.5 mM, (b) 31.0 mM, (c) 27.3 mM, (d) 13.6 mM, (e) 0 mM of HCOONa. Other conditions as described in Fig. 2.

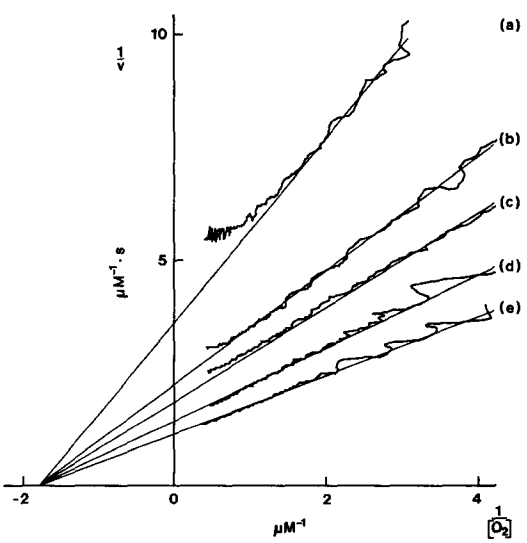


Fig. 5. The effect on the oxygen kinetics of various concentrations of cyanide. (a) 0.30 μM, (b) 0.23 μM, (c) 0.15 μM, (d) 0.075 μM, (e) 0 μM of HCN. Other conditions as described in Fig. 2. To allow cyanide binding under turnover conditions, the procedure described in the text was followed.

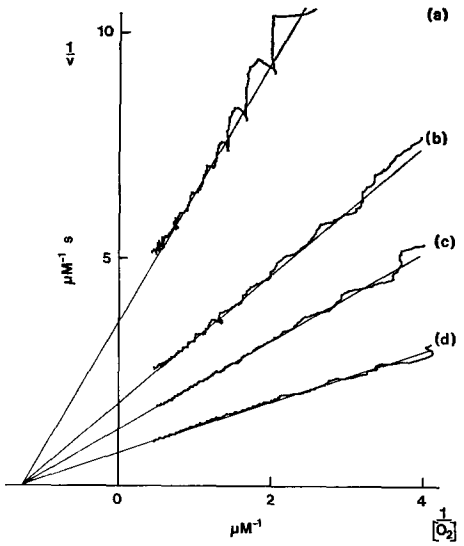


Fig. 6. The effect on the oxygen kinetics of various concentrations of sulfide. (a) 0.32 μM, (b) 0.22 μM, (c) 0.12 μM, (d) 0 μM of H_2S. Other conditions as described in Fig. 2. To allow sulfide binding under turnover conditions, the procedure described in the text was followed.

followed in those experiments to allow binding to take place under turnover conditions. At a fixed partial pressure of the inhibitor an ordinary gradient from anaerobiosis to about 3 μM of oxygen in the liquid was produced. At this value the gas composition was kept constant for 10 min. The oxygen in the gas phase was then replaced by nitrogen, and immediately after the return of the system to anaerobic conditions a linear gradient in the liquid oxygen concentration was again produced.

Double reciprocal plots of v_R against $[O_2]$ monitored during the second gradient of such experiments are shown in Figs. 5 and 6. These plots did not change significantly as a third gradient was produced, except for a slight inactivation of the enzyme, which was also observed in the uninhibited ascorbate oxidase system. Cyanide and sulfide inhibition is of the purely non-competitive type. The apparent K_i found from secondary plots, intercept as well as slope, is 0.2 μM for both cyanide and sulfide.

The effect of the inhibitors HN_3, HCOOH, HCN and H_2S on the cytochrome *c* kinetics of the oxidase was also tested. The oxygen uptake of the system: ascorbate → cytochrome *c* → cytochrome aa_3 → O_2 was measured as a function of added cytochrome *c* at various fixed concentrations of inhibitor at high oxygen concentrations. HCN an H_2S were allowed to bind to the oxidase under turnover conditions for 10 min at a low cytochrome *c* concentration, before further cytochrome *c* was added. Azide, formate, cyanide and sulfide are all purely non-competitive inhibitors towards cytochrome *c*. Within experimental error the values of K_i found from secondary plots of the cytochrome *c* kinetics are equal to the corresponding K_i values found from the oxygen kinetics. Azide: $K_i = 55$ μM; formate: $K_i = 13$ mM; cyanide: $K_i = 0.15$ μM; sulfide: $K_i = 0.20$ μM.

DISCUSSION

That CO is a competitive inhibitor towards oxygen was postulated in 1927 by Warburg [20] to be a characteristic feature of the 'Atmungsferment'. The present investigation is however the first to demonstrate the effect of CO on the oxygen steady-state kinetics of the purified cytochrome *c* oxidase in a direct way. The apparent K_i at maximal turnover of the enzyme is found to be in reasonable agreement with the dissociation constant given by k_{off} and k_{on}, measured from CO binding studies on the reduced oxidase [21].

The observation that CO is a competitve and that azide and formate are uncompetitive inhibitors towards oxygen can easily be accommodated by the model proposed to account for the oxygen kinetics of the solubilized oxidase [12]. The assumptions to be made are in accordance with observations on the ligand induced changes in the absorption spectrum of cytochrome aa_3, namely that CO binds to the reduced cytochrome a_3 and prevents the oxidation by oxygen [3]; and that azide [3] and formate [9, 22] bind to the oxidized form and prevent the reduction of this group.

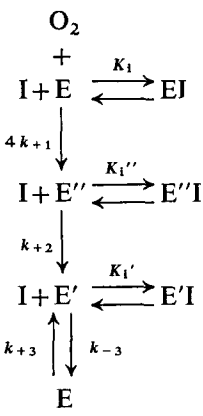
Azide [18] and sulfide [8] can be replaced by cyanide, and formate can be replaced by azide [22] and cyanide [9]. These observations indicate that the four inhibitors are all competing for the same oxidized form of the enzyme or various oxidized forms, which are reversibly connected.

The dissociation constant for the cyanide complex of the fully oxidized oxidase is 1 μM, and it has been proposed that the binding of cyanide to the half reduced oxidase ($a^{2+}\, a_3{}^{3+}$) is responsible for cyanide inhibition of the catalytic reaction [19]. Heme-heme interaction is proposed as the reason why ferricytochrome a_3 has a higher affinity for cyanide in this state of the oxidase as compared to the fully oxidized state [19]. Measurements of the cyanide binding to the oxygenated cytochrome aa_3 [23] indicate that the affinity of this form for cyanide might also be sufficiently high to account for the inhibition of the catalytic reaction.

The non-competitive inhibition towards oxygen of cyanide and sulfide can be accommodated by the model too, if it is assumed that they bind to oxidized and reduced cytochrome a_3 with equal affinity. However, this assumption is apparently in conflict with the observation, based on ligand induced spectral changes, that cyanide binds to ferrocytochrome a_3 with a much lower affinity ($K_D = 100$ μM) than to ferricytochrome a_3 ($K_D = 1$ μM) [18], and also fails to account for the observation that the cyanide complex of the fully reduced oxidase can react with oxygen [19].

In order to account for a slope effect of an inhibitor on a Lineweaver-Burk plot, binding of the inhibitor to (1) the enzyme form, which reacts with the variable substrate, or (2) an enzyme form, which is reversibly connected to this form should be considered [24]. Ligand binding studies strongly argue against the first possibility as discussed above. We conclude that in order to account for the non-competitive inhibition by cyanide and sulfide it is necessary to postulate an intermediate, E′, in the reduction of the oxidized enzyme, E″, to the form, E, which reacts with oxygen. The intermediate has a high affinity for cyanide and sulfide, but does not bind azide and formate. It is reversibly connected to E and CO is expected to be able to replace these inhibitors from E′ by its binding to E.

The simplest model capable of accounting for the three different ways, that terminal inhibitors can affect the oxygen kinetics of the cytochrome *c* oxidase reaction, seems thus to be the one described in Eq. 1a. The rate equation for this model is given in Eqn. 1b:



(1a)

$$\frac{e}{v} = \frac{1}{k_{+2}}\left(1+\frac{[I]}{K_i''}\right) + \frac{1}{k_{+3}}\left(1+\frac{[I]}{K_i'}\right) + \frac{k_{-3}}{k_{+3}}\left(1+\frac{[I]}{K_i'}\right)\frac{1}{4k_{+1}}\frac{1}{[O_2]} + \left(1+\frac{[I]}{K_i}\right)\frac{1}{4k_{+1}}\frac{1}{[O_2]} \qquad (1b)$$

To account for the various types of inhibition it is assumed that CO binds to E, ($K_i' = \infty$; $K_i'' = \infty$), that azide and formate bind to E'' ($K_i = \infty$; $K_i' = \infty$) and that cyanide and sulfide bind to both E' and E'' ($K_i' = K_i''$; $K_i = \infty$). Pure non-competitive inhibition with respect to oxygen is obtained in this case if in addition $k_{-3} \gg k_{+3}$.

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