Biochimica et Biophysica Acta, 502 (1978) 248—254

BBA 47489

ANOMALIES IN THE POLAROGRAPHIC MEASUREMENT OF CYTOCHROME OXIDASE ACTIVITY *

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(Received October 14th, 1977)

Summary

Reaction kinetics of the reduction of O_2 by cytochrome oxidase follow essentially the same rate equation as that proposed for the oxidation of cytochrome *c*. However, the apparent second order rate constant varies with the oxidase concentration. The redox level of cytochrome *c* at the steady state was found to be essentially temperature-independent. Currently recognized pathways (or mechanisms) of electron transport from cytochrome *c* to O_2 do not predict, and cannot account for the occurrence of these phenomena.

Introduction

The kinetics of beef heart cytochrome *c* oxidase (EC 1.9.3.1) have been investigated for an extensive period of time, but the correct reaction mechanism remains rather controversial [1,2]. Minnaert [3] empirically formulated a rate equation for the spectrophotometrically-followed oxidation of cytochrome *c*. Ascorbate has often been used to obtain and maintain a steady state level of reduced cytochrome *c* when polarographic determinations of cytochrome oxidase activity are made [4—8]. It has been proposed that the introduction of ascorbate into the reaction medium will introduce further complications if the reduction of cytochrome *c* by ascorbate is relatively slow, and the rate equation should consequently be altered [7,8]. The intention of this communication is to report several apparent anomalies detected in polarographic measurements of cytochrome oxidase activity, which the currently proposed rate equations [7,8] cannot account for.

* Department of Agronomy Series Paper 1223.

Materials and Methods

Cytochrome oxidase was obtained from mitochondrial preparations of bovine heart muscle [9] according to the procedure of Yonetani [10]. Cytochrome oxidase preparation was initiated the same day fresh beef hearts arrived at a local supermarket. The spectral characteristics met the criterion for purity established by Yonetani [10]. The concentration was calculated from the extinction coefficient of 79 mM^{-1} at 422 nm [10]. This preparation was stored at −40° C until use.

Cytochrome oxidase activity was determined polarographically with a Clark-type oxygen electrode in a reaction medium of 50 mM potassium phosphate, pH 6.0, 1 mM EDTA, 25 mM sodium ascorbate, and varying amounts of cytochrome oxidase and Sigma type VI cytochrome *c* in final volumes of 3.011—3.055 ml. Temperature was controlled with a circulating, refrigerated water bath.

Steady state levels of cytochrome *c* were determined using the reaction medium described above. The reaction was followed with a single beam spectrophotometer at 550 nm. The contribution of oxidase to the absorption at this wavelength is negligible. The relatively significant contribution of oxidized cytochrome *c* was negated by considering both extinction coefficient formulas for cytochrome *c*, $\epsilon^{550}_{red} = 0.0277\ \mu M^{-1}$ and $\epsilon^{550}_{oxid} = 0.0092\ \mu M^{-1}$ [11].

Results and Discussion

The rate of oxidation of cytochrome *c* by cytochrome oxidase can be described by the relation [3]:

$$V = \frac{k_1 k_2 [c^{2+}] [e]}{k_1 [\text{total } c] + k_2} \tag{1}$$

where V is the rate of cytochrome *c* oxidation, $[c^{2+}]$ is the concentration of reduced cytochrome *c*, [total *c*] is the total concentration of cytochrome *c* and $[e]$ is the oxidase concentration. The apparent second order rate constant for the reaction between cytochrome *c* and the oxidase, k_1, is analogous to V/K_m in Michaelis-Menten terminology. The apparent first order rate constant for the reduction of oxygen, k_2, is analogous to V in Michaelis-Menten terminology.

Introduction of ascorbate into the reaction medium will introduce further complications if the reduction of cytochrome *c* by ascorbate is relatively slow [7,8]. Introducing the ascorbate reaction into the rate equation yields a modified equation [7] which can be transformed into a form suitable for double-reciprocal plots. Points on these plots, according to Yonetani [5], "fall on an almost straight line". The modified equation also predicts a straight-line relationship between (% reduced cytochrome *c*)$^{-1}$ and enzyme concentration for any constant ascorbate concentration [8], although no such plot was depicted in their paper. Fig. 1 demonstrates such plots, which are obviously non-linear. Replotting data of Yonetani [6] in this manner similarly yields hyperbolic curves rather than straight lines. It is therefore apparent that the modified rate equation currently proposed [7,8] is incorrect.

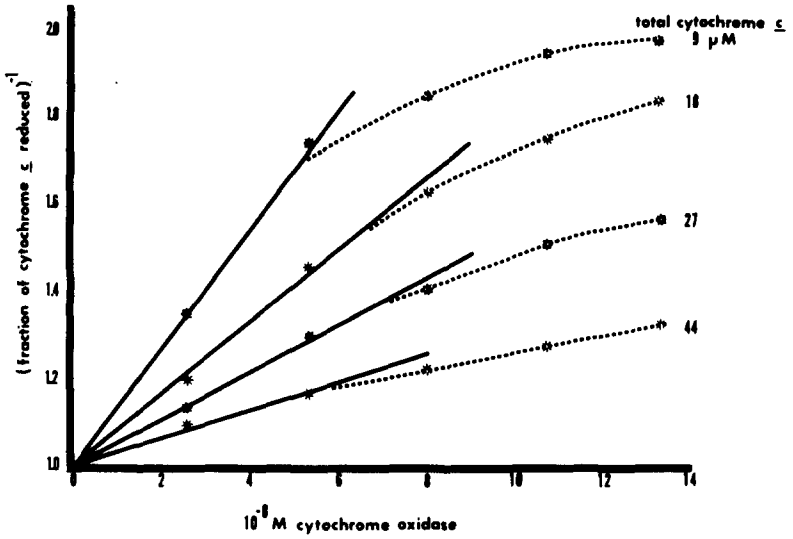

Fig. 1. The relationship between oxidase concentration and the degree of substrate reduction at the steady state. The assay was performed at 20° C under conditions described in Materials and Methods.

Since ascorbate does not play a direct role in the catalytic reduction of O_2 [5,8], the steady state rate equation (Eqn. 1) for the reaction of cytochrome *c* with the oxidase should hold for polarographic measurements as well. The rate of O_2 uptake should be solely a function of the rate of cytochrome *c* oxidation. Plots of Eqn. 1, transformed to a linear form:

$$\frac{[c^{2+}][e]}{v} = \frac{1}{k_2}\,[\text{total } c] + \frac{1}{k_1} \qquad (2)$$

are given in Fig. 2 for varying enzyme concentrations. It is apparent that the data fit this equation quite well with the exception that values of k_1 vary with the enzyme concentration. This is in agreement with the observations of Slater [4] and Yonetani [6], who found that the "k_m" for cytochrome *c* varies with the enzyme concentration. This deviation may be due to the effects of cholate on the oxidase activity [12]. However, we have not observed a significant variation of k_2 with oxidase concentration, as is predicted by cholate inhibition [12].

The temperature-dependence of the steady state redox level of cytochrome *c* is presented in Fig. 3. The redox level of cytochrome *c* at any given enzyme concentration is essentially temperature-independent. It is interesting to note that Chance [13] similarly recorded in vivo steady state levels of cytochromes that were essentially temperature-independent. The steady state level of the cytochrome will be a function of the overall reaction, from the reduction of O_2 to the oxidation of ascorbate. Therefore, the rate constants for each elementary reaction would appear to have about the same temperature coefficient.

At the steady state the reduction of cytochrome *c* by ascorbate, $V = k_0[\text{ascorbate}]\,[c^{+3}]$ should be equal to the velocity of oxygen uptake [8] and

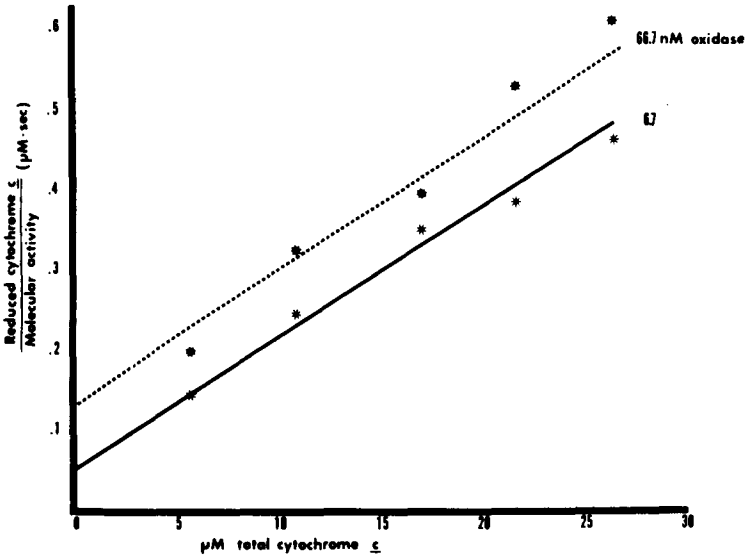

Fig. 2. The relationship between cytochrome *c* concentration and molecular activity (turnover number) of cytochrome oxidase. The assay was performed at 25° C under conditions described in Materials and Methods. The slope of the line represents $1/k_2$. *The* Y-intercept represents $1/k_1$.

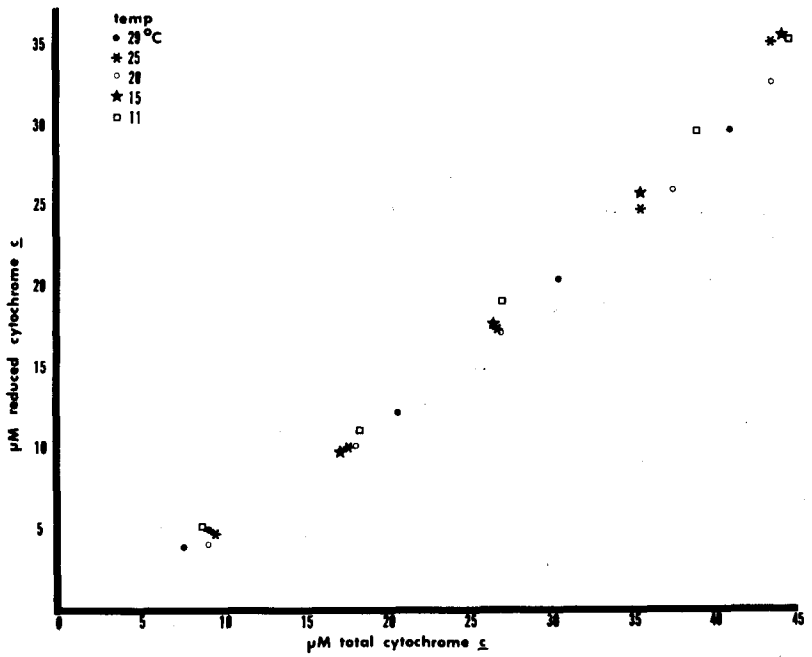

Fig. 3. The temperature-dependence of the steady state level of reduced cytochrome *c*. The spectrophotometric assays were performed with 107 nM oxidase under conditions described in Materials and Methods. Redox levels of cytochrome *c* were determined at various concentrations of total cytochrome *c* and at various temperatures.

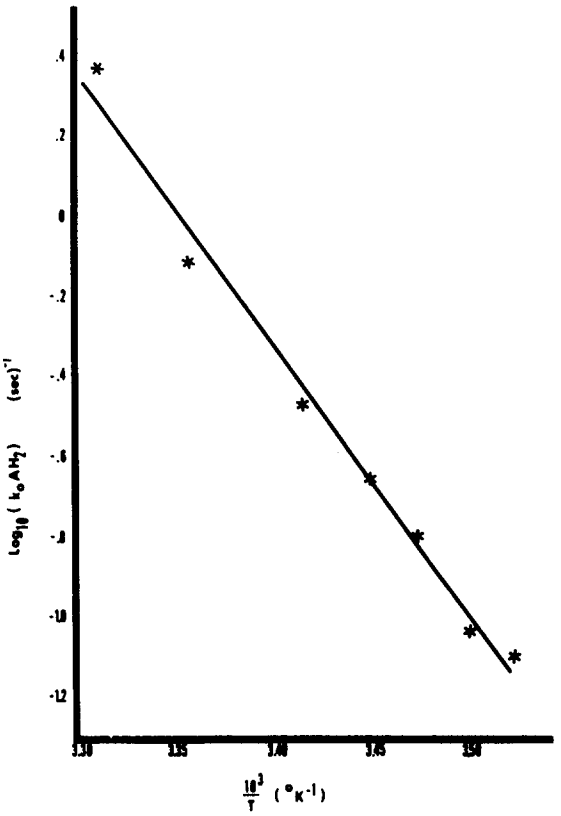

Fig. 4. Arrhenius plot of the apparent rate constant for the reduction of cytochrome *c* by ascorbate at pH 6.0. The rate constants were determined by the method of Minnaert [7].

independent o the mechanism of cytochrome oxidase activity. The values of k_0[ascorbate] can be calculated from the rate of reduction of oxidized cytochrome *c* [7] at various temperatures (Fig. 4). From these values and the polarographically measured enzyme rates it is possible to calculate what the steady state level of oxidized cytochrome *c* should be (Fig. 5). At relatively low temperatures the values agree fairly well with the observed redox level while at higher temperatures there is pronounced disagreement. At higher temperatures the observed redox levels of oxidized cytochrome *c* are much higher than would be predicted. This indicates that at the steady state the rate of reduction of cytochrome *c* by ascorbate, measured in the absence of cytochrome oxidase, is not equal to the rate of oxidation of cytochrome *c* by cytochrome oxidase, but the steady state is maintained. The possibility exists that there is a temperature-dependent enhancement of the ascorbate reaction by cytochrome oxidase. If this were the case, however, the temperature-dependence of this process must be such that the steady state level of oxidized cytochrome *c* is, as observed, temperature independent (Fig. 3).

The above analysis assumes that the reduction of cytochrome *c* by cytochrome *a* necessitates the reduction of O_2 by cytochrome a_3. A fairly large amount of evidence has accumulated suggesting that this is not the case. Yone-

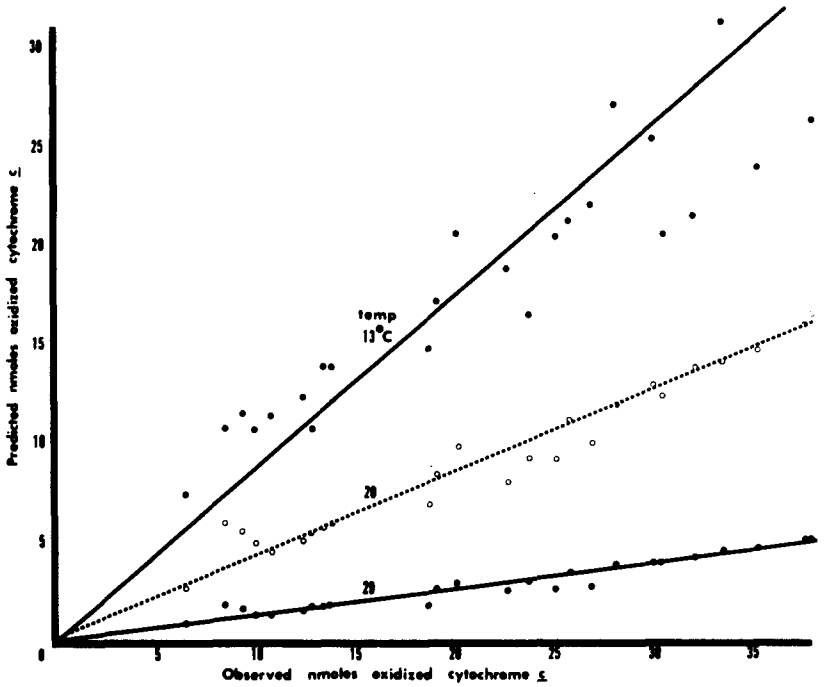

Fig. 5. Prediction of the steady state level of oxidized cytochrome *c*. Predicted values were calculated from the apparent rate constant for the reduction of cytochrome *c* by ascorbate (k_0[ascorbate]) and the velocity of the cytochrome oxidase reaction (v) by the relationship:
[oxidized cytochrome *c*] = $v./k_0$[ascorbate].

tani [5] has stated that reduced cytochrome a_3 can react with O_2 in the presence or absence of cytochrome *c*, and that the flow of electrons may be represented as:

Ascorbate → cytochrome *c* → cytochrome a_3 → O_2
↑↓
cytochrome *a*

In support of this pathway it was found that the steady state level of reduced cytochrome *a* does not correspond to the rate of reduction of O_2 [5]. This mechanism, however, cannot explain the observed (Fig. 5) lack of correlation between the rate of O_2 reduction and the steady state level of reduced cytochrome *c*.

Vanneste et al. [14] have suggested that the existance of inactive or latent cytochrome oxidase molecules in solubilized preparations could account for the type of behavior which led to the pathway shown above. If this were the case, then the fraction of oxidase that is inactive would necessarily be temperature-dependent in a manner such that the steady-state level of reduced cytochrome *c* is temperature-independent.

The above-mentioned results suggest that the recent statement concerning the cytochrome oxidase reaction, that "both the cytochrome *c* kinetics and the O_2 kinetics seem to indicate a rather simple reaction mechanism for the enzyme ..." [15] is an oversimplification. The temperature-independence of

the steady state level of reduced cytochrome *c* cannot be accounted for by any of the currently accepted mechanisms or pathways of electron transport. Resolution of this phenomenon must precede any new proposals of the mechanism of electron transport in site III of oxidative phosphorylation.

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