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THE REACTION OF FERROCYTOCHROME *c* WITH CYTOCHROME OXIDASE: A NEW LOOK

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1. Introduction

The reaction of purified cytochrome oxidase (ferrocytochrome *c*: O_2 oxidoreductase, E.C.1.9.3.1) with its natural substrate, cytochrome *c*, has for obvious reasons received a good deal of attention (see, for example, [1] for a review). Kinetic measurements in a stopped-flow apparatus of the direct reaction between the oxidized form of the oxidase and c^{2+} have, however, only been carried out to a limited extent, the most thorough study probably being that of Gibson et al. [2]. In "anaerobic" (about 0.1 μM O_2) experiments these authors found an initial rapid reaction, which they ascribed to the reduction of cytochrome *a* and one of the copper components, followed by slower changes. The second phase of the reaction was believed to be slow because of the formation of a complex between a^{2+} and c^{3+}, in which electrons are not readily transferred to a_3^{3+}.

For a number of reasons we have found it interesting to repeat and extend the type of stopped-flow measurements reported by Gibson et al. [2]. First, these authors did not include observations on the 830-nm band generally associated with one of the copper components of the enzyme (see [1]). Second, we have recently incorporated [3, 4] in our stopped-flow technique certain improvements which are deemed important for a complete description of the reaction. Thus, the auxiliary equipment of our apparatus allows accurate direct measurements of absolute, and not only difference, absorbance values. In addition, we can keep the concentration of O_2 in the system below 0.1 μM during the entire observation period, which is sometimes as long as 1800 sec.

Our measurements show that in the rapid phase of the anaerobic reaction only one mole of c^{3+} is formed per mole of oxidase (in terms of a unit containing two hemes, cytochrome *a* and a_3, and two copper atoms). The subsequent changes are so slow that we can get accurate estimations of the absorbance changes associated with the reduction of the initial electron acceptor in the oxidase, cytochrome *a*. This analysis shows, among other things, that a considerable part of the absorption at 830 nm is due to cytochrome *a* and not to copper, something which was pointed out by Gibson and co-workers [5, 6] but has often been neglected in recent studies (e.g. [7]). The reductive steps observed anaerobically subsequent to the rapid reduction of a^{3+} are too slow in relation to the turnover number of the enzyme to form part of the normal catalytic mechanism. If electrons should flow through any of these other reducible chromophores, our results in the presence of air can only be explained if O_2 interacts with the partially reduced enzyme in such a way as to change the rate constants for their reduction. The aerobic measurements also reveal spectral changes which cannot be described in terms of the absorption properties of fully oxidized and fully reduced enzyme.

A preliminary account of some of these results has been given at two recent conferences [8, 9]. At one of them, Antonini et al. reported some experiments of a similar nature [10].

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*Deceased 27th July 1972.

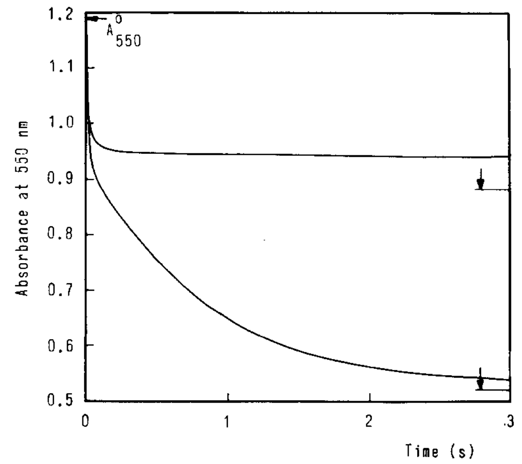

Fig. 1. Time course of the oxidation of cytochrome c^{2+} in the presence of cytochrome oxidase. The formation of $c^{3+}$was followed at 550 nm. The experiments were carried out in the stopped-flow apparatus (2-cm optical path) at 25° in 0.1 M phosphate, pH 7.4, containing 0.5% Tween 80. The concentrations of cytochrome oxidase and cytochrome *c* after mixing were 4.5 and 16 μM, respectively. The upper trace represents the anaerobic oxidation of cytochrome c^{2+}, while the lower trace was obtained in the presence of 125 μM O_2. Initial and final absorbance values for each curve are indicated (arrows).

2. Materials and methods

Cytochrome oxidase was prepared from beef-heart particles of Keilin and Hartree essentially by the method of Yonetani [11]. The final fraction was dissolved in 0.1 M phosphate buffer, pH 7.4, containing 0.5% Tween 80 and stored frozen in liquid N_2. One set of experiments was also performed with an oxidase sample prepared according to van Buuren [12] (kindly provided by Drs. K. van Buuren and B. van Gelder, Amsterdam). The horse heart cytochrome *c* used was Sigma type VI, further purified by ion-exchange chromatography [13]. The reduced form was prepared by the addition of a small amount of sodium dithionite, the excess being removed by passage of the solution through a column of Sephadex G-25. The concentrations of oxidase and cytochrome *c* were determined spectrophotometrically on the basis of the molar absorption coefficients listed in [14]; the oxidase concentrations are expressed in terms of a unit containing 2 hemes and 2 copper atoms.

The anaerobic technique and the methods for recording and evaluating the stopped-flow traces have been described earlier [3, 4]. The slit widths of the monochromator were chosen to give the following spectral band widths: 60, 9, 7 and 7 nm at 830, 605, 550 and 445 nm, respectively. No significant stray light was found at any of the wavelengths.

The initial absorbance values at each wavelength were measured directly in the stopped-flow apparatus (see [3]). First, the potentiometer settings corresponding to 100% transmittance were determined with buffer alone. Then separate transmittance measurements on the oxidized oxidase and on the reduced cytochrome *c* were carried out. The stability of the instrument was such that a repeated measurement at any wavelength showed a deviation of at most a few tenths per cent transmittance.

3. Results

The reaction of cytochrome oxidase with 4 moles of cytochrome c^{2+} per mole of cytochrome *a* was followed in the stopped-flow apparatus both anaerobically and in the presence of 125 μM O_2. Cytochrome *c* oxidation was measured at 550 nm, as shown in fig. 1. In fig. 2 changes in the oxidase, observed at three wavelengths (830, 605 and 445 nm) are plotted. The relationship between the initial rapid changes at 550 and 830 nm is illustrated in fig. 3.

The rapid phase in the anaerobic reaction shown in fig. 1 corresponds to the formation of 1.1 – 1.2 mole of c^{3+} per mole of oxidase (this value should be decreased by at most 6% due to the change in oxidase absorption, see [11]). The second-order rate constant for this initial fast reaction between c^{2+} and the oxidase can be calculated to be $8 \times 10^6 M^{-1} sec^{-1}$. The same stoichiometry was found in an experiment with the Amsterdam preparation, but in this case the second-order rate constant was $> 10^7 M^{-1} sec^{-1}$.

The changes in the molar absorption coefficient of the oxidase, associated at the three wavelengths used with the rapid reaction, has been calculated from the data of figs. 2–3 (all values have been corrected for the contribution from cytochrome *c*): 0.98, 16 and 74 $mM^{-1} cm^{-1}$ at 830, 605 and 445 nm, respectively. These figures correpond to 39, 67 and 48%, respectively, of the total changes on full reduction of the oxidase.

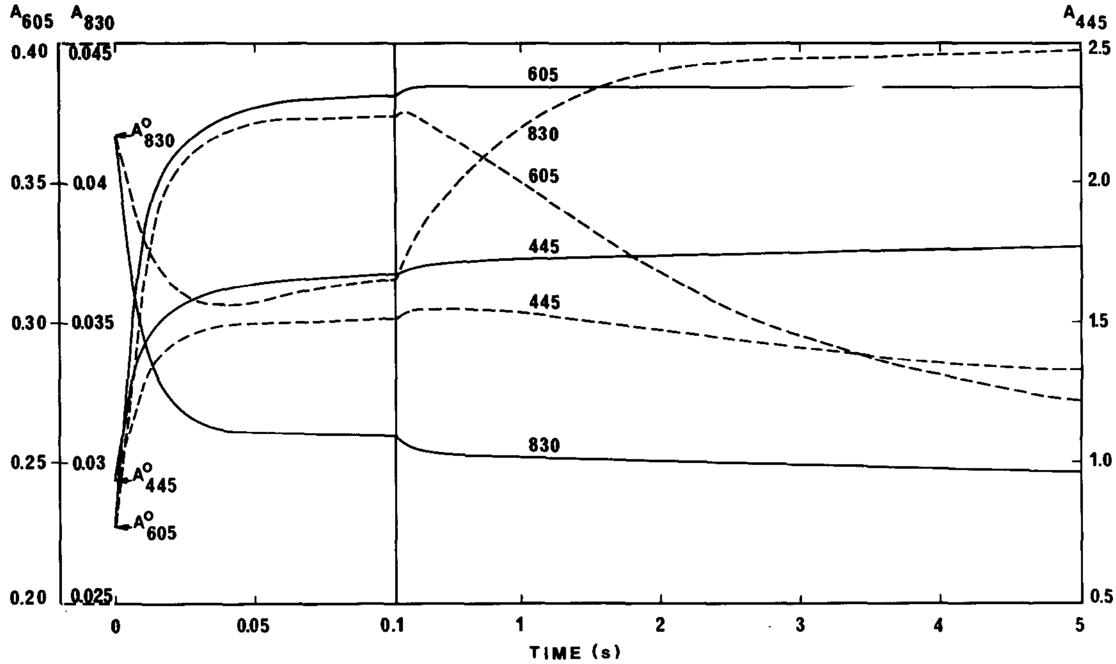

Fig. 2. Time course of the reduction of cytochrome oxidase by cytochrome c^{2+}. The reaction was followed at 445, 605 and 830 nm. Concentrations of reactants and other conditions were identical to those in fig. 1. Anaerobic and aerobic conditions are presented as full and dashed lines, respectively, on a continuous time scale which contracts at the vertical line. The absorbance values at 830 nm have been corrected for the contributions from cytochrome *c*, while the curves for the other wavelengths are uncorrected.

4. Discussion

In agreement with earlier results [2] we find that the anaerobic reaction between cytochrome oxidase and c^{2+} is biphasic. Gibson et al. [2] concluded that both a heme and a copper component accept electrons concomitantly in the rapid phase of the reaction. This conclusion was, however, based on an indirect reasoning which required an accurate knowledge of the absorption change associated with the reduction of one heme component, cytochrome *a*. In addition, the experiment was carried out in the presence of HCN, which is known to react with the oxidase in a particularly complex manner [15–17], even if it does not appear to affect the velocity of this initial step [2, 15]. In our experiments the number of electrons accepted by the oxidase is measured accurately by a direct determination in the stopped-flow apparatus of the absorbance change at 550 nm (fig. 1). The only necessary assumption in this estimation is that the absorbance change accompanying the oxidation of c^{2+} is not significantly affected by the presence of the oxidase. Thus, we can feel quite confident that the stoichiometry in the rapid phase is one. In fact, the absorbance change given by Gibson et al. for the burst in an aerobic experiment (fig. 8 in [2]) also corresponds to this stoichiometry.

Our results might appear to be in conflict with rapid-freeze EPR experiments of Beinert and Palmer [18], which show significant reduction of both heme and copper components in about 10 msec. The concentrations of both oxidase and cytochrome *c* were, however, 100-fold larger in these experiments, and the observations were made on frozen solutions.

The exact stoichiometry in our experiments would indicate that a single component in the oxidase, here called cytochrome *a*, is accepting electrons rapidly. This conclusion is supported by the linear relation between the absorbance changes at all four wavelengths, as illustrated for 830 and 550 nm in fig. 3. In our experiments cytochrome *a* must have an oxidation–reduction potential higher than that of cytochrome *c*, which presumably means that the effect of cytochrome *c* on the potentials of the oxidase components [19] is not manifested during our measurements.

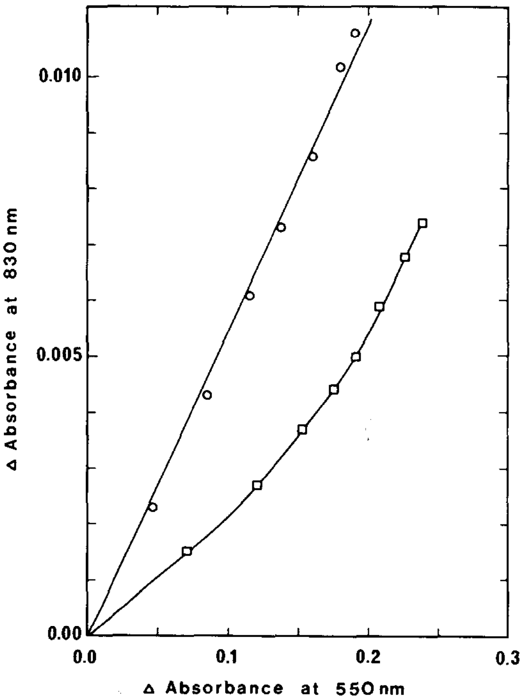

Fig. 3. The relationship between the changes in absorbance at 830 and 550 nm under anaerobic (○—○—○) and aerobic (□—□—□) conditions. Concentrations and other conditions were identical to those presented in fig. 1. The points were obtained by correlating the observed change in absorbance at one wavelength with the corresponding change at the other wavelength during the first 25 msec of the reaction. The absorbance changes at 830 nm are not corrected for the contributions from cytochrome *c*.

The clear separation in time between the reduction of cytochrome *a* and the other components allows us to make accurate determinations of the absorbance changes associated with the reduction of a^{3+} alone (see Results). It is noteworthy that about 40% of the change at 830 nm can be ascribed to cytochrome *a*, something which must be remembered when the near-infrared band is used to monitor changes in the redox state of a copper component. The change at 605 nm is considerably lower than that which has earlier been attributed to cytochrome *a* (see [1]). The most common way to determine this contribution is to compare the spectral changes on reduction in the presence and absence of HCN. This indirect method presupposes that the absorption properties of *a* are unaffected by the redox state of other components and by the presence of HCN. In fact, recent redox studies [19, 20] have also indicated a smaller contribution from cytochrome *a* compared to earlier values.

All changes after the initial burst are extremely slow in our experiments. This cannot be due simply to an unfavourable oxidation–reduction potential of the other reducible centres. This is seen from the fact that about 2 electrons are accepted by the oxidase during the observation time of our experiments, as judged from the absorbance change at 550 nm (fig. 1), and that the absorbance changes at the other wavelengths all correspond to reduction of the oxidase. The slowness does not appear to be a property of our particular oxidase preparation since it is found also with the Amsterdam enzyme.

Despite the slowness of the reactions subsequent to the burst in the anaerobic experiments, all c^{2+} is rapidly oxidized in the presence of O_2 (fig. 1). This means that if electrons pass through any of the observed slow-reacting centres as part of the catalytic mechanism, O_2 must interact with partially reduced enzyme in such a way as to change the rate constants for these reductive steps.

Gibson et al. [2] ascribed the slow changes to the reduction of a_3^{3+}, which they considered rate-determining in the experiments in the presence of O_2. However, their half-time for the appearance of $a_3{}^{2+}$ (about 1 sec), determined in the presence of CO, is short compared to our slow phases. They pointed to the similar half-time for the aerobic oxidation of c^{2+}. This must, however, be a coincidence, as the aerobic rate with our slowly reducible enzyme is almost identical (cf. fig. 1 with fig. 8 in [2]). Thus, no matter what the cause of the slowness is (Gibson et al. [2] postulated an effect of an a^{2+}–c^{3+} complex), it appears necessary to consider an effect of O_2 on the rate constants. This possibility was indeed mentioned by Keilin already in 1939 (see [21]).

Finally we would like to point out that the spectral changes at 830 nm in the presence of O_2 cannot be accounted for in terms of the absorbance differences seen anaerobically. Thus, a^{3+} is almost completely reduced in the burst even in the aerobic experiment, but the decrease at 830 nm associated with this reduction does not appear complete (fig. 2). In fig. 3 it is also seen that a linear relation between the absorbance changes at the two wavelengths is no longer obtained in the presence of air. The chemical nature of the species responsible for these new spectral contributions must be a matter for future investigations.

Acknowledgements

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