BBA 46128

BIOCHEMICAL AND BIOPHYSICAL STUDIES ON CYTOCHROME aa_3

I. STEADY-STATE KINETICS OF CYTOCHROME aa_3

K. J. H. VAN BUUREN, B. F. VAN GELDER AND T. A. EGGELTE

Laboratory of Biochemistry, B.C.P. Jansen Institute, University of Amsterdam, Amsterdam (The Netherlands)*

(Received December 24th, 1970)

SUMMARY

1. The steady-state kinetics of the reaction in the system: ascorbate → cytochrome c → cytochrome aa_3 → O_2 were studied, measuring both the degree of reduction of cytochrome c and the rates of O_2 consumption.

2. In disagreement with some earlier reports it could be shown that the sole reaction of ascorbate is the reduction of ferricytochrome c. From the kinetics of the overall reaction, the rate constant for the reaction between ferricytochrome c and ascorbate could be calculated to be 23 $M^{-1} \cdot sec^{-1}$, in fair agreement with the value of 50 $M^{-1} \cdot sec^{-1}$ obtained from direct stopped-flow measurements.

3. Comparison of the rates of O_2 uptake at infinite cytochrome c and ascorbate concentrations for heart-muscle preparation and isolated cytochrome aa_3 revealed the presence of an inhibitor in the latter preparation. A mechanism of inhibition based on MINNAERT's Mechanism IV

$$(E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow[k_2]{(O_2)} EP \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} E + P),$$

in which the inhibitor reacts with E, ES and EP forming inactive complexes with about the same K_i, is in agreement with the experimental data.

4. The K_D for ES and EP, calculated from the kinetics of the overall reaction, was found to be 30 and 30–40 μM for the isolated and particulate cytochrome aa_3, respectively. By making use of the data of GIBSON *et al.* (*J. Biol. Chem.*, 240 (1965) 888) on the reaction between ferrocytochrome c and ferricytochrome a, values for k_{-1} and k_2 of 1200 and 300 sec^{-1}, respectively, were calculated.

5. MINNAERT's Mechanism IV (*Biochim. Biophys. Acta*, 50 (1961) 23) gives the simplest explanation of the observed steady-state kinetics.

INTRODUCTION

The steady-state kinetics in the system reducing agent → cytochrome c → cytochrome aa_3 → O_2 have been studied extensively[1–7]. It was clearly shown that the rate of O_2 uptake depends on the concentrations of cytochrome c and cytochrome aa_3,

* Postal address: Plantage Muidergracht 12, Amsterdam, The Netherlands.

and on the nature and concentration of the reducing agent. SLATER[3] emphasized the importance of the use of maximal rates, *i.e.* rates of O_2 uptake at infinite cytochrome *c* concentration and of the use of ascorbate as reducing agent.

The use of ascorbate as reducing agent is based on the premise that the ascorbate does not interfere in the mechanism of oxidation of cytochrome *c* by cytochrome aa_3. However, MINNAERT[4], who studied the kinetics of the reactions manometrically, found an effect of ascorbate on the maximal rate of O_2 consumption of a KEILIN–HARTREE heart-muscle preparation, and concluded that ascorbate could act as a second substrate for cytochrome aa_3. SMITH AND CAMERINO[6], on the other hand, did not find a significant effect.

In this paper it will be shown that the sole effect of ascorbate is the reduction of ferricytochrome *c*.

MATERIALS AND METHODS

Heart-muscle preparation

Heart-muscle preparations were isolated following the principles of the procedure of KEILIN AND HARTREE[8]. Fat-free beef-heart mince was washed 4 times with ice-cold water and ground with sand and 50 mM K_2HPO_4 in a mechanical mortar at 4° for 30 min. After removal of all heavy materials by centrifugation at 2000 × *g* for 15 min the pH of the turbid supernatant was brought from 7.2–7.4 to 5.6 with 10 % acetic acid. Crude heart-muscle preparation was collected after centrifuging at 1500 × *g* for 30 min. The final preparation was obtained after two washings with 0.66 M sucrose, 1 mM histidine and 50 mM Tris sulphate (pH 8.0). The material was collected by centrifugation at 78000 × *g* for 10 min. The Q_{O_2} (μl O_2 per h per mg protein) at infinite cytochrome *c* concentration was found to be about 2200 at 25°. The preparations can be kept in liquid N_2 for months without loss of activity.

Soluble cytochrome aa_3 preparations

Cytochrome aa_3 was isolated from heart-muscle preparations essentially according to the method of FOWLER *et al.*[9]. The soluble preparation was further purified by two cholate–$(NH_4)_2SO_4$ fractionations as described by MACLENNAN AND TZAGOLOFF[10].

The preparations have a $A^{red}_{444\ nm}/A^{ox}_{424\ nm}$ ratio of 1.30–1.37 and contain 9–11 μmoles heme per g protein.

The cytochrome aa_3 concentration was calculated[11] from $\Delta A_{605\ nm}$ (red—ox) using a ΔA of 24.0 $mM^{-1} \cdot cm^{-1}$. Small quantities of the enzyme were stored at liquid N_2 temperature and thawed just before use. The enzyme handled in this way does not show any decrease in activity after storing for 3 years.

Cytochrome c

Cytochrome *c* was isolated from the neutralized acid supernatant of the heart-muscle preparation by adsorbing it batchwise on Amberlite CG-50 (100–200 mesh), and further purified according to the method of MARGOLIASH AND WALASEK[12]. The preparations were free of polymeric contaminants as could be shown by the monophasic reduction by ascorbate[13].

The preparations had $A^{red}_{550\ nm}/A^{ox}_{280\ nm}$ ratios of 1.25–1.30. The concentration was calculated[14] from $\Delta A_{550\ nm}$ (red—ox) using a ΔA of 21.0 $mM^{-1} \cdot cm^{-1}$.

Assay system

O_2 uptake was measured with a Clark electrode mounted on a Gilson oxygraph. The reaction mixture contained: 65 mM phosphate, 1 mM EDTA, 67 μg/ml Asolectine, 0.5 % Tween 80, 8.3 mM sucrose, 2–30 mM ascorbate, and 10–100 μM cytochrome *c*. The pH of the mixture was 7.4 and the temperature 25°. The reaction was started by adding 60–350 nM cytochrome aa_3. The heart-muscle preparation or isolated cytochrome aa_3 was diluted in an ice-cold mixture containing 2 mg/ml Asolectine, 0.5 % Tween 80, 0.25 M sucrose and 10 mM phosphate (pH 7.4).

Rates are expressed as μM cytochrome *c* oxidized per sec, activities as mmoles cytochrome *c* per sec per g protein for heart-muscle preparations and for isolated cytochrome aa_3 as moles cytochrome *c* per sec per mole of cytochrome aa_3.

For the calculations the O_2 concentration at 25° was assumed to be 250 μM. Initial rates are taken and corrected for autooxidation.

Protein was determined according to the method of GORNALL *et al.*[15] as modified by YONETANI[16].

The degree of reduction of cytochrome *c* in the steady state was measured at 550 nm with a Zeiss PMQ spectrophotometer.

Materials

Asolectin (Associated Concentrates) sols were made according to WHARTON AND GRIFFITHS[7]. Stock sols of 50 mg/ml were stored at 0–5° and discarded after 2 days.

Ascorbate (British Drug Houses, biochemical grade) was dissolved in 30 mM EDTA and neutralized with KOH. Stock solutions of 0.9 M were stored at —20°.

Tween 80 was purchased from Sigma.

All other chemicals were of Analar Grade, mainly obtained from British Drug Houses.

RESULTS

Redox state of cytochrome c during the steady state

In the system ascorbate–cytochrome *c*–cytochrome aa_3–O_2 the O_2 consumption, as measured polarographically, is not proportional to the cytochrome aa_3 concentration. This is due to the fact that during the steady state cytochrome *c* is not completely reduced, as has been shown by MINNAERT[4] and YONETANI[17]. The degree of reduction of cytochrome *c* can be used to investigate the overall reaction mechanism.

In our opinion the mechanism proposed by MINNAERT[18] for the oxidation of ferrocytochrome *c* and designated as Mechanism IV is based on the simplest assumption, namely that the association and dissociation rate constants for the cytochrome *c*–cytochrome aa_3 complex do not change upon changing the valence state of the iron atom in cytochrome *c*. Therefore we use this mechanism to show the relation of the degree of reduction of cytochrome *c* in the steady state to the parameters of the system.

The general formulation of Mechanism IV is

$$S + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2(O_2)} EP \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} E + P \tag{1}$$

and the rate equation is

$$v = \frac{k_1 k_{-1} k_2 e[S]}{(k_{-1} + k_2)(k_1[S + P] + k_{-1})} \tag{2}$$

where e = [cytochrome aa_3], S = ferrocytochrome c, and P = ferricytochrome c.

Since the rate of reduction of ferricytochrome c by ascorbate is first order with respect to both cytochrome c and ascorbate[4], the rate equation may also be written

$$v = k_3[AH_2]\,[P] \tag{3}$$

where AH_2 = ascorbate.

By introducing the degree of reduction of cytochrome c

$$\rho = \frac{\text{ferrocytochrome } c}{\text{total cytochrome } c} = \frac{[S]}{[S + P]}$$

and equating the two expressions for v in the steady state, it can be derived that

$$\frac{1}{\rho} = 1 + \frac{k_2}{k_{-1} + k_2} \cdot \frac{k_1 k_{-1} e}{k_3[AH_2](k_1[S + P] + k_{-1})} \tag{4}$$

This equation predicts a straight-line relationship between $1/\rho$ and $1/[AH_2]$ for a given cytochrome c and enzyme concentration, with an intersection on the ordinate at $1/\rho = 1$. Fig. 1 shows that this is the case.

In accordance with Eqn. 4, the inverse of the slope of the lines for different cytochrome c concentration is linearly proportional to the cytochrome c concentration

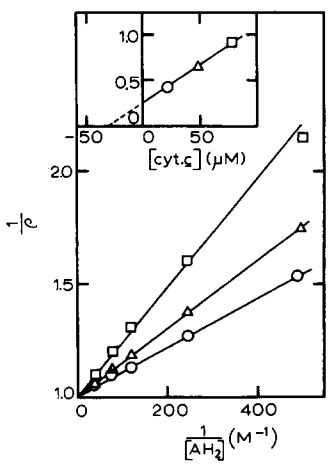

Fig. 1. Effect of ascorbate and cytochrome c concentrations on the degree of reduction of cytochrome c during the steady state. 61 nM isolated cytochrome aa_3; other conditions as described in MATERIALS AND METHODS. ○—○, 68 μM cytochrome c; △—△, 46 μM cytochrome c; □—□, 25 μM cytochrome c. The inset shows the effect of varying cytochrome c concentrations on the inverse slope of the lines.

(inset Fig. 1). The intercept on the abscissa of the inset represents k_{-1}/k_1, which is equal to the dissociation constant of the ES complex. According to the data of Fig. 1, this is 30 μM.

Effect of varying the ascorbate concentration

On eliminating $[S]$ and $[P]$ from Eqns. 2 and 3, the following expression for the molecular activity (MA $= v/e$) is obtained

$$\frac{1}{\mathrm{MA}} = \frac{e}{v} = \frac{e}{k_3[\mathrm{AH_2}]\,[S+P]} + \frac{k_{-1}+k_2}{k_2}\left(\frac{1}{k_1[S+P]} + \frac{1}{k_{-1}}\right) \qquad (5)$$

Eqn. 5 predicts straight lines in LINEWEAVER–BURK[19] type plots with $(S+P)$ and $\mathrm{AH_2}$ as parameters.

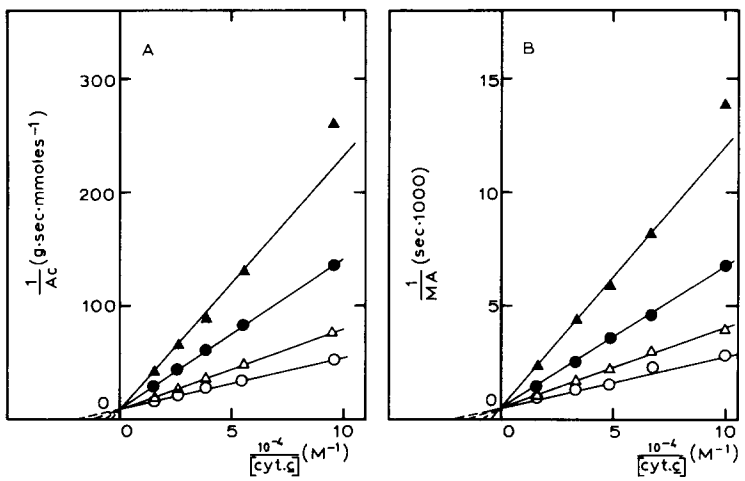

Fig. 2. Effect of ascorbate and cytochrome *c* concentration on the enzyme activity. A. 160 μg heart-muscle preparation per ml. ○—○, 30 mM ascorbate; △—△, 10 mM ascorbate; ●---●, 4 mM ascorbate; ▲—▲, 2 mM ascorbate. B. 63 nM cytochrome aa_3. ○—○, 30 mM ascorbate; △—△, 12 mM ascorbate; ●---●, 5 mM ascorbate; ▲—▲, 2 mM ascorbate. Experimental conditions as described in MATERIALS AND METHODS.

Fig. 2A shows the effect of varying amounts of ascorbate on the activity measured at different cytochrome *c* concentrations when a KEILIN–HARTREE heart-muscle preparation was used. In this plot the lines intersect at the ordinate showing no effect of ascorbate on the specific activity at infinite cytochrome *c* concentration. Similar results are obtained using isolated cytochrome aa_3 (Fig. 2B). From the fact that no effect of ascorbate on the molecular activity at infinite cytochrome *c* concentration is found, it is concluded that ascorbate does not act as a second substrate for cytochrome aa_3 and that the only role of ascorbate in this system is the reduction of ferricytochrome *c*.

Effect of varying the cytochrome aa_3 concentration

In a LINEWEAVER–BURK type plot (Fig. 3A) the straight lines for different concentrations of heart-muscle preparation intersect on the ordinate. Thus the activity

at infinite cytochrome *c* concentration is proportional to the cytochrome aa_3 concentration.

However, when isolated cytochrome aa_3 is used the LINEWEAVER–BURK type plot (Fig. 3B) shows lines which intersect in the second quadrant. Thus MA_{max} depends on the enzyme concentration used, being smaller at higher enzyme concentrations.

As can be seen in Fig. 4 which is in fact a DIXON type plot (ref. 20), MA_{max} is inversely proportional to the enzyme concentration. This phenomenon can only be explained by assuming the presence of an inhibitor in purified cytochrome aa_3

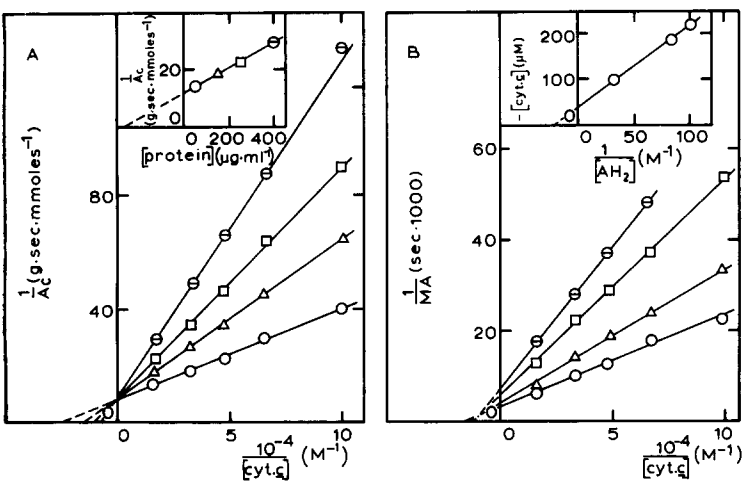

Fig. 3. Effect of enzyme and cytochrome *c* concentration on the enzyme activity. A. Heart-muscl preparation. ○—○, 50 μg protein per ml; △—△, 150 μg protein per ml; □—□, 250 μg protein per ml; ⊖—⊖, 400 μg protein per ml. B. Isolated enzyme. ○—○, 63 nM cytochrome aa_3; △—△, 123 nM cytochrome aa_3; □—□, 192 nM cytochrome aa_3; ⊖—⊖, 302 nM cytochrome aa_3; 30 mM ascorbate; other conditions as in MATERIALS AND METHODS. Inset of A. The effect of concentration of heart-muscle preparation on the inverse of the activity. The points are calculated from A. 60 μM cytochrome *c*, 30 mM ascorbate. Inset of B. Dependence of the distance to the ordinate of the point of intersection in B on the ascorbate concentration. The points are obtained from two additional plots similar to the one shown in B but with different ascorbate concentrations (see text).

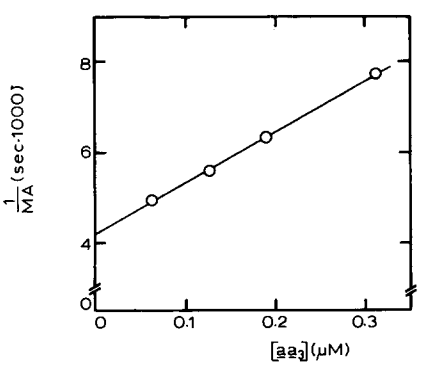

Fig. 4. Effect of cytochrome aa_3 concentration on the molecular activity at infinite cytochrome *c* concentration. The points are obtained from Fig. 3B.

preparations. Since the enzyme is isolated in the presence of bile salts, which are known inhibitors of cytochrome aa_3, it is possible that the inhibition is caused by cholate and/or deoxycholate[21–25]. The nature of the inhibitor will be further investigated.

If the inhibitor forms inactive complexes with E, EP and ES with the same K_i, the rate equation becomes

$$v = \frac{k_1k_{-1}k_2k_3e[AH_2][S + P]}{k_1k_{-1}k_2e + \left(1 + \frac{ae}{K_i}\right)(k_{-1} + k_2)(k_1[S + P] + k_{-1})k_3[AH_2]} \quad (6)$$

where the concentration of the inhibitor is ae (a is a constant for the preparation used),

$$\frac{1}{MA} = \frac{e}{v} = \frac{e}{k_3[AH_2][S + P]} + \frac{k_{-1} + k_2}{k_2}\left(1 + \frac{ae}{K_i}\right)\left[\frac{1}{k_1[S + P]} + \frac{1}{k_{-1}}\right] \quad (7)$$

At infinite cytochrome c concentration:

$$\frac{1}{MA_{max\ (cyt.\ c)}} = \frac{k_{-1} + k_2}{k_{-1}k_2}\left(1 + \frac{ae}{K_i}\right) \quad (8)$$

Eqn. 8 is consistent with the findings that $1/MA_{max}$ is independent of the ascorbate concentration (Fig. 2B), and is linearly dependent on the enzyme concentration (Fig. 4). From the point of intersection of the straight line on the ordinate in Fig. 4 $k_{-1}k_2/(k_{-1} + k_2)$ can be calculated to be 240 sec^{-1}.

At infinite ascorbate concentration the rate equation is:

$$\frac{1}{MA_{max\ (ascorbate)}} = \frac{k_{-1} + k_2}{k_2}\left(1 + \frac{ae}{K_i}\right)\left[\frac{1}{k_1[S + P]} + \frac{1}{k_{-1}}\right] \quad (9)$$

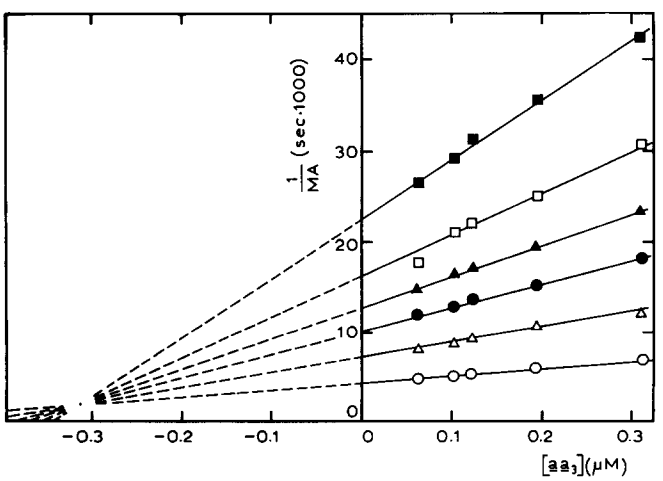

Fig. 5. Effect of cytochrome aa_3 concentration on the molecular activity at infinite ascorbate concentration. The points are obtained by extrapolation in 1/MA *vs.* 1/[ascorbate] plots (not shown). ○—○, infinite cytochrome *c*; △—△, 60 μM cytochrome *c*; ●—●, 30 μM cytochrome *c*; ▲—▲, 21 μM cytochrome *c*; □—□, 15 μM cytochrome *c*; ■—■, 10 μM cytochrome *c*. Experimental conditions as described in MATERIALS AND METHODS.

This equation predicts straight lines when the inverse of $MA_{max\ (ascorbate)}$ is plotted against enzyme concentration, and that the lines for different cytochrome *c* concentrations should intersect on the ordinate. However, in Fig. 5 the point of intersection lies somewhat above the enzyme axis, indicating a slight difference in the inhibition constants.

According to Eqn. 7, the straight lines in the plot of 1/MA *vs.* $1/[S+P]$ intersect at

$$-\frac{1}{[S+P]} = \frac{(k_{-1}+k_2)}{k_{-1}} \cdot \frac{k_1 k_3 a[AH_2]}{(k_{-1}+k_2)k_3 a[AH_2] + k_1 k_2 K_i} \qquad (10)$$

Thus, the value of $[S+P]$ calculated from the point of intersection should be a linear function of the inverse ascorbate concentration, the line intercepting the ordinate of a plot of $[S+P]_{intersect}$ *vs.* $1/[AH_2]$ at k_{-1}/k_1, the dissociation constant of the cytochrome *c*–cytochrome aa_3 complex. The inset of Fig. 3B shows that this is the case, and the value of k_{-1}/k_1 calculated from the straight line is 40 μM, in good agreement with the value calculated from Fig. 1.

The reduction of ferricytochrome c by ascorbate

As already mentioned ascorbate reduces only ferricytochrome *c*. The kinetics of this reaction can be studied at infinite cytochrome aa_3 concentration, when the rate-limiting step of the overall reaction is the reaction between ascorbate and ferricytochrome *c*.

Fig. 6A shows a plot of the inverse velocity against the inverse enzyme concentration using KEILIN–HARTREE heart-muscle preparation. Straight lines appear for each cytochrome *c* concentration used, intersecting at the third quadrant. A similar plot ($1/v$ *vs.* $1/e$) (Fig. 6B) with different ascorbate concentrations shows parallel lines.

The data of Fig. 6 are used for plotting the rate at infinite cytochrome aa_3

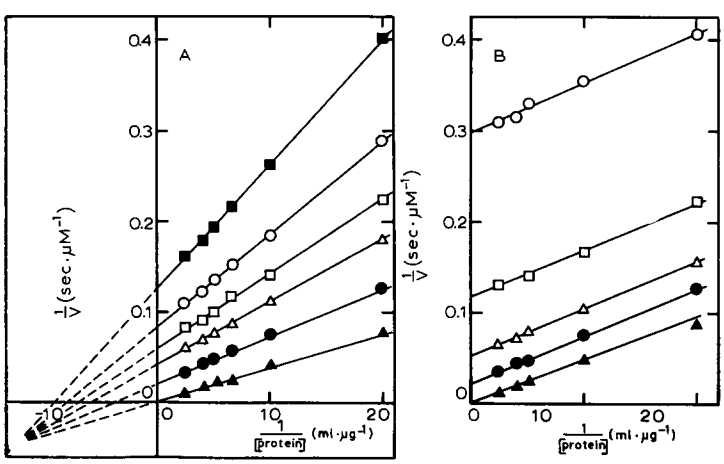

Fig. 6. Effect of concentration of heart-muscle preparation on the reaction rate. A. 30 mM ascorbate. ▲—▲, infinite cytochrome *c*; ●—●, 60 μM cytochrome *c*; △—△, 30 μM cytochrome *c*; □—□, 21 μM cytochrome *c*; ○—○, 15 μM cytochrome *c*; ■—■, 10 μM cytochrome *c*. B. 60 μM cytochrome *c*. ▲—▲, infinite ascorbate; ●—●, 30 mM ascorbate; △—△, 12 mM ascorbate; □—□, 5 mM ascorbate; ○—○, 2 mM ascorbate. Experimental conditions as in MATERIALS AND METHODS.

concentration ($v_{e\to x}$) against the cytochrome *c* concentration (circles in Fig. 7A) or ascorbate concentration (circles in Fig. 7B). In agreement with the observations of MINNAERT[18] straight lines are found, indicating first-order kinetics with respect to the cytochrome *c* and ascorbate concentration. From the slope of the lines k_3 can be calculated to be 23 $M^{-1}\cdot sec^{-1}$. This value of k_3 can be compared with the value of 50 $M^{-1}\cdot sec^{-1}$ obtained from direct stopped-flow measurements under the conditions of the polarographic assay.

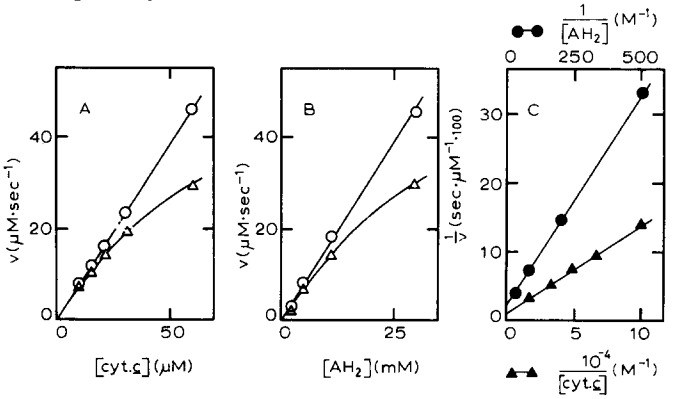

Fig. 7. Rates of cytochrome *c* reduction at infinite enzyme concentration. For heart muscle preparation (○---○) the points were obtained from Fig. 6. For isolated cytochrome aa_3 (△—△) the points were obtained from similar plots (not shown). A. Effect of cytochrome *c* concentration at 30 mM ascorbate. B. Effect of ascorbate concentration at 60 μM cytochrome *c*. C. The data of A and B for the isolated cytochrome aa_3 replotted as the inverse of the rate against the inverse of either the cytochrome *c* (▲—▲) or the ascorbate (●—●) concentration.

Using isolated cytochrome aa_3, $1/v$ *vs.* $1/e$ plots have the same appearance as with the heart-muscle preparation (not shown). However, the plots of $v_{e\to x}$ *vs.* cytochrome *c* (Fig. 7A) or ascorbate (Fig. 7B) concentrations show a deviation of the straight-line relationship. This is due to the presence of the inhibitor in the cytochrome aa_3 preparations as can be seen in the rate equation

$$v^*_{e\to x} = \frac{k_1k_{-1}k_2k_3K_i[AH_2^-][P]}{(k_{-1}+k_2)k_3a[AH_2^-]\{k_1[P]+k_{-1}\}+k_1k_{-1}k_2K_i}$$

for isolated cytochrome aa_3.

However, when $1/v^*_{e\to x}$ is plotted against the inverse either of ascorbate or cytochrome *c* concentration straight lines appear (Fig. 7C), as is to be expected from Eqn. 12.

$$\frac{1}{v^*_{e\to\infty}} = \frac{1}{k_3[AH_2^-][P]} + \frac{(k_{-1}+k_2)a}{k_2K_i}\left[\frac{1}{k_1[P]}+\frac{1}{k_{-1}}\right] \qquad (12)$$

From the slope of the line with different ascorbate concentrations a value for k_3 can be calculated equal to 24 $M^{-1}\cdot sec^{-1}$.

K_m values

From the LINEWEAVER–BURK type plots (Fig. 3) the apparent K_m values for cytochrome *c* can be calculated. The relationship of the K_m derived from the rate

* At infinite enzyme concentration, $[S] = 0$.

equation for the particulate and isolated preparations, respectively, are as follows:

$$K_{m(\text{cyt. } c)} = \frac{k_{-1}}{k_1} + \frac{k_{-1}k_2 e}{k_3[AH_2]\,(k_{-1} + k_2)} \tag{13}$$

$$K_{m(\text{cyt. } c)} = \frac{k_{-1}}{k_1} + \frac{k_{-1}k_2 e}{k_3[AH_2]\,(k_{-1} + k_2)\left(1 + \frac{ae}{K_i}\right)} \tag{14}$$

These equations predict for both preparations straight-line relationships for the K_m and the inverse of the ascorbate concentration as shown in Fig. 8A. The point of intersection at the ordinate, representing k_{-1}/k_1, the dissociation constant for the ES complex, is 30 and 43 μM for the isolated and particulate preparation, respectively.

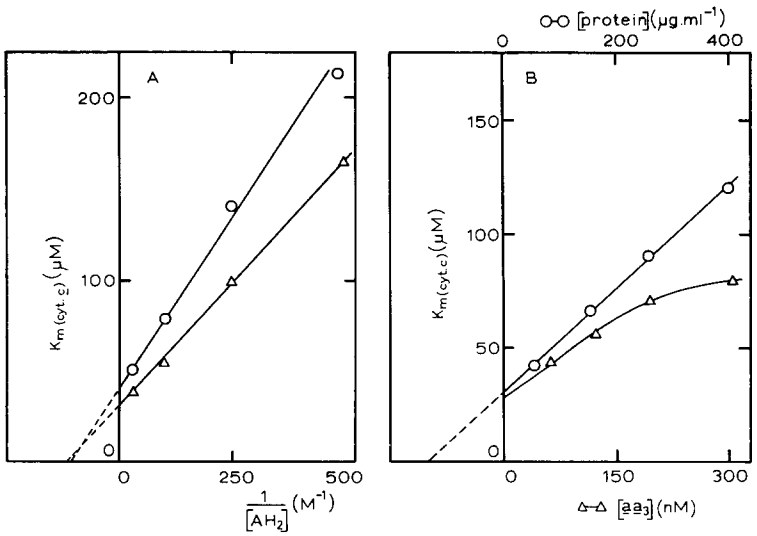

Fig. 8. Effect of enzyme and ascorbate concentration on the apparent $K_{m(\text{cyt. } c)}$ A. Effect of ascorbate concentration. The points are calculated from Fig. 2. B. Effect of enzyme concentration. The points are calculated from Fig. 3. ○—○, heart-muscle preparation; △—△, isolated cytochrome aa_3.

Fig. 8B shows that a straight-line relationship is also found between the K_m and the concentration of the particulate preparation, but not with the isolated preparation, as can be expected from Eqns. 13 and 14. The value of K_m for the particulate preparation at $e = 0$ represents k_{-1}/k_1 and is 31 μM.

The values of the dissociation constant of the ES complex for our isolated and particulate preparations are almost the same, showing that the binding of cytochrome c to cytochrome aa_3 does not change upon purification of cytochrome aa_3.

DISCUSSION

Mechanism of action of cytochrome aa_3

The degree of reduction of cytochrome c in the steady state and the overall enzyme activity can be plotted against the ascorbate, cytochrome c or enzyme concen-

tration. The plots shown in the results and those of $1/(1-\rho)$ *vs.* $[S+P]$, $[AH_2]$ or e and of $1/\rho$ *vs.* ϵ all show straight lines with slopes and points of intersection which fit the rate equation (Eqn. 4). Also when e/v is plotted against $1/[AH_2]$ or $1/v$ against $1/[AH_2]$ or $1/[S+P]$ straight lines appear in agreement with Eqn. 7. For sake of brevity some plots are not shown.

All our data fit a general rate equation for the polarographic/manometric system:

$$v = \frac{C_1 e k[AH_2]\,[S+P]}{C_1 e + k[AH_2]\,\{[S+P]+C_2\}} \qquad (15)$$

in which AH_2 = ascorbate; $S+P$ = total cytochrome c; e = enzyme concentration; k = rate constant for the reduction of ferricytochrome c; C_1 and C_2 are functions of rate constants depending on the mechanism of the oxidation of ferrocytochrome c.

This equation is derived from the general rate equation for the oxidation of ferrocytochrome c for the spectrophotometric system given by MINNAERT[18].

The mechanisms proposed in the literature all fit this general rate equation if certain assumptions are made. MINNAERT[18] has already discussed six mechanisms including the one proposed by SMITH AND CONRAD[26] as extended by SLATER[27] and preferred his Mechanism IV:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} EP \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E + P$$

with the assumption $k_1 = k_6$ and $k_2 = k_5$ *i.e.* the rate constants of the formation and dissociation of ES and EP do not differ much. This may be due to the fact that the only difference between the substrate and the product is the valence state of the iron atom in cytochrome c which is buried deep in the protein[28,29]. Conformational changes of cytochrome c upon oxidation[30] have apparently no influence on the rate constants.

NICHOLLS[31] supported the views of MINNAERT[18] and emphasized the importance of an active cytochrome c–hemoprotein complex in the catalytic process of hemoproteins such as cytochrome aa_3 and yeast peroxidase. The mechanism he presented is in fact Mechanism IV of MINNAERT in which intramolecular reactions of the cytochrome c–cytochrome aa_3 complex have been described in more detail.

The mechanism proposed by YONETANI AND RAY[32] is:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P \underset{k_5}{\overset{k_4}{\rightleftharpoons}} EP$$

with the assumption $K_i = K_m$ or

$$\frac{k_5}{k_4} = \frac{k_2 + k_3}{k_1}.$$

Furthermore, they concluded that $k_3 \gg k_2$, so that the assumption becomes $k_5/k_4 = k_3/k_1$. However, it seems unlikely that the dissociation constant for the ferricytochrome c–cytochrome aa_3 complex would be equal to the quotient of the rate constant for the oxidation of the ES complex and the association rate constant of ferrocytochrome c and cytochrome aa_3.

Effect of ascorbate and enzyme

In contrast to observations by MINNAERT[18] our data show that the sole effect of ascorbate in the system is the reduction of ferricytochrome *c*. The discrepancy can be explained by the fact, that the O_2 concentration of the reaction mixture in the manometric system differs considerably in the presence or absence of enzyme. Since the rate of autooxidation is much less at lower O_2 concentration, the activity may be underestimated. In the polarographic system this is not the case as initial rates are taken.

The increase of the molecular activity at lower enzyme concentration was also observed by MASON AND GANAPATHY[33], who suggested that it is due to the formation of an unstable dissociated form of cytochrome aa_3 at low enzyme concentration. Since, however, the dependence of the molecular activity on the enzyme concentration is to be expected if either cytochrome *c* or ascorbate concentrations are not infinite (Eqn. 5), there is no need to assume this dissociation. In accord with our interpretation, when the data presented in Fig. 1 of ref. 33 are replotted as 1/MA *vs.* the enzyme concentration a straight line is obtained, as in the inset of Fig. 3A.

Rate constants

It is clear from our data that the apparent K_m (cytochrome *c*) value is linearly dependent on the inverse of the ascorbate concentration for both preparations. This observation fits the general rate equation (Eqn. 15). Our results are in contrast to those of YONETANI[5], who found an empirical relationship:

$$K_{m(\text{cyt. } c)} \propto \frac{[\text{cytochrome } aa_3]}{\sqrt{\text{ascorbate}}}$$

which does not fit either the general rate equation or the rate equation derived from his mechanism.

In Table I are summarized the measured and calculated rate constants based on MINNAERT's Mechanism IV. Closely agreeing values for k_{-1}/k_1 were obtained from three different plots. Since from the steady-state experimental data only two relationships, k_{-1}/k_1 and $k_{-1}k_2/(k_{-1}+k_2)$, which in the general equation equal C_2 and C_1, respectively, can be extracted the individual rate constants cannot be calculated.

TABLE I

RATE CONSTANTS FOR ISOLATED CYTOCHROME aa_3 BASED ON MINNAERT'S MECHANISM IV

Rate constant	*Measured*	*Calculated*
k_{-1}^*/k_1	30 μM	—
$k_{-1}k_2^*/(k_{-1}+k_2)$	240 sec^{-1}	—
k_1^{**}	$4 \cdot 10^7$ $M^{-1} \cdot sec^{-1}$	—
k_{-1}	—	1200 sec^{-1}
k_2	—	300 sec^{-1}
k_3	23–50 $M^{-1} \cdot sec^{-1}$	—

* In the general equation (Eqn. 15) $k_{-1}/k_1 = C_2$ and $k_{-1}k_2/(k_{-1}+k_2) = C_1$.
** This value is taken from GIBSON *et al.*[34] (*cf.* DISCUSSION).

However, if one assumes that the reduction of ferricytochrome *a* by ferrocytochrome *c* measured by GIBSON *et al.*[34] represents the formation of the *ES* complex (k_1), the k_{-1} and k_2 may be calculated to be 1200 and 300 sec^{-1}, respectively. This value for k_2 agrees well with the value reported[34] (700 sec^{-1}) for the rate of oxidation of reduced cytochrome aa_3 by O_2.

ACKNOWLEDGEMENTS

We wish to thank Prof. E. C. Slater for his interest and stimulating criticism and Mr. A. O. Muijsers for his advice and critical reading of the manuscript, and Messrs. R. Le Clercq and J. Kaper for expert technical assistance. This work was supported (in part) by grants from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.) under the auspices of the Netherlands Foundation for Chemical Research (S.O.N.).

REFERENCES

1 E. STOTZ, A. M. ALTSCHUL AND T. R. HOGNESS, *J. Biol. Chem.*, 124 (1938) 745.
2 H. BOREI, *Arkiv Kemie Mineral. Geol.*, 20A (1945) No. 8.
3 E. C. SLATER, *Biochem. J.*, 44 (1949) 305.
4 K. MINNAERT, *Biochim. Biophys. Acta*, 54 (1961) 26.
5 T. YONETANI, *J. Biol. Chem.*, 237 (1962) 550.
6 L. SMITH AND P. W. CAMERINO, *Biochemistry*, 2 (1963) 1428.
7 D. C. WHARTON AND D. E. GRIFFITHS, *Arch. Biochem. Biophys.*, 96 (1962) 103.
8 D. KEILIN AND E. F. HARTREE, *Biochem. J.*, 41 (1947) 500; *cf.* E. C. SLATER, *Biochem. J.*, 45 (1949) 1.
9 L. R. FOWLER, S. H. RICHARDSON AND Y. HATEFI, *Biochim. Biophys. Acta*, 64 (1962) 170.
10 D. H. MACLENNAN AND A. TZAGOLOFF, *Biochim. Biophys. Acta*, 96 (1965) 166.
11 B. F. VAN GELDER AND E. C. SLATER, *Biochim. Biophys. Acta*, 73 (1963) 663.
12 E. MARGOLIASH AND O. F. WALASEK, in R. W. ESTABROOK AND M. E. PULLMANN, *Methods in Enzymology*, Vol. X, Academic Press, New York, 1967, p. 339.
13 K. SKOV AND G. R. WILLIAMS, in K. OKUNUKI, M. D. KAMEN AND I. SEKUZU, *Structure and Function of Cytochromes*, Univ. of Tokyo Press, Tokyo, 1968, p. 349.
14 B. F. VAN GELDER AND E. C. SLATER, *Biochim. Biophys. Acta*, 58 (1962) 593.
15 A. G. GORNALL, C. J. BARDAWILL AND M. M. DAVID, *J. Biol. Chem.*, 177 (1949) 751.
16 T. YONETANI, *J. Biol. Chem.*, 236 (1961) 1680.
17 T. YONETANI, *J. Biol. Chem.*, 235 (1960) 3138.
18 K. MINNAERT, *Biochim. Biophys. Acta*, 50 (1961) 23.
19 H. LINEWEAVER AND D. BURK, *J. Am. Chem. Soc.*, 56 (1934) 658.
20 M. DIXON AND E. C. WEBB, *Enzymes*, Longmans, London, 1964, p. 329.
21 L. SMITH, *J. Biol. Chem.*, 215 (1955) 833.
22 T. YONETANI, *J. Biochem. Tokyo*, 46 (1959) 917.
23 L. T. KREMZNER AND W. W. WAINIO, *Biochim. Biophys. Acta*, 52 (1961) 209.
24 S. J. COOPERSTEIN, *J. Biol. Chem.*, 238 (1963) 3750.
25 Y. ORII AND K. OKUNUKI, *J. Biochem. Tokyo*, 58 (1965) 561.
26 L. SMITH AND H. CONRAD, *Arch. Biochem. Biophys.*, 63 (1956) 403.
27 E. C. SLATER, *Biochem. Soc. Symp. Cambridge, Engl.*, 15 (1958) 76.
28 R. E. DICKERSON, M. L. KOPKA, C. L. BORDERS, JR., J. VARNUM, J. E. WEINZIERL AND E. MARGOLIASH, *J. Mol. Biol.*, 29 (1967) 77.
29 R. E. DICKERSON, D. EISENBERG, T. TAKANO, O. BATTFAY AND L. SAMSON, *Abstr. 8th Intern. Congr. Biochem., Switzerland*, 1970, p. 13.
30 D. W. URRY AND P. DOTY, *J. Am. Chem. Soc.*, 87 (1965) 2756.
31 P. NICHOLLS, *Arch. Biochem. Biophys.*, 106 (1964) 25.
32 T. YONETANI AND G. S. RAY, *J. Biol. Chem.*, 240 (1965) 3392.
33 H. S. MASON AND K. GANAPATHY, *J. Biol. Chem.*, 245 (1970) 230.
34 Q. H. GIBSON, C. GREENWOOD, D. C. WHARTON AND G. PALMER, *J. Biol. Chem.*, 240 (1965) 888.