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# ANION AND IONIC STRENGTH EFFECTS UPON THE OXIDATION OF CYTOCHROME *c* BY CYTOCHROME *c* OXIDASE

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**Citrate and other polyanion binding to ferricytochrome *c* partially blocks reduction by ascorbate, but at constant ionic strength the citrate-cytochrome *c* complex remains reducible; reduction by TMPD is unaffected. At a constant high ionic strength citrate inhibits the cytochrome *c* oxidase reaction competitively with respect to cytochrome *c*, indicating that ferrocytochrome *c* also binds citrate, and that the citrate-ferrocytochrome *c* complex is rejected by the binding site at high ionic strength. At lower ionic strengths, citrate and other polyanions change the kinetic pattern of ferrocytochrome *c* oxidation from first-order towards zero-order, indicating preferential binding of the ferric species, followed by its exclusion from the binding site. The turnover at low cytochrome *c* concentrations is diminished by citrate but not the $K_m$ (apparent non-competitive inhibition) or the rate of cytochrome *a* reduction by bound cytochrome *c*. Small effects of anions are seen in direct measurements of binding to the primary site on the enzyme, and larger effects upon secondary site binding. It is concluded that anion-cytochrome *c* complexes may be catalytically competent but that the redox potentials and/or intramolecular behaviour of such complexes may be affected when enzyme-bound. Increasing ionic strength diminishes cytochrome *c* binding not only by decreasing the 'association' rate but also by increasing the 'dissociation' rate for bound cytochrome *c* converting the 'primary' (T) site at high salt concentrations into a site similar kinetically to the 'secondary' (L) site at low ionic strength. A finite $K_m$ of 170 $\mu$M at very high ionic strength indicates a ratio of $K_m^\infty / K_M^0$ of about 5000. It is proposed that anions either modify the $E_0^1$ of cytochrome *c* bound at the primary (T) site or that they perturb an equilibrium between two forms of bound *c* in favour of a less active form.**

Anionic ligands affect the cytochrome *c*-cytochrome *c* oxidase reaction in two ways: by specifically binding the haem iron atoms of cytochromes $a_3$ and *c* [1], and by surface binding to one of the two reacting species, usually cytochrome *c* [2]. Both types of binding, as well as the overall redox reaction itself, are sensitive to ionic strength. Studies with polyanions indicate a decrease in the cytochrome *c* redox potential of 20–30 mV upon anion binding [3] as has also been reported for cytochrome *c* binding to cytochrome $aa_3$ [4]. Anion binding also decreases the rate of ascorbate reduction of cytochrome *c* [3] but not the rates of reduction by uncharged reductants [2,5]. As the redox state of cytochrome *c* affects its conformation only slightly, differential anion binding must reflect either (a) the change in overall charge or (b) close proximity between ligand and haem iron. Osheroff and co-workers [2] suggest

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Abbreviations: TMPD, *N*,*N*,*N'*,*N'*-tetramethyl-*p*-phenylenediamine; Mops, morpholinopropane sulphonate; T site, 'tight' or high-affinity cytochrome *c* binding site on cytochrome $aa_3$; L site, 'loose' or low-affinity cytochrome *c* binding site on cytochrome $aa_3$; TN, turnover number.

that phoshate and citrate bind at the 'top left' of the protein, near lysine-87 (phosphate binding site I).

In addition to specific anion binding, changes in ionic strength affect the reactions of both small and larger (enzyme protein) redox agents with cytochrome *c*. The appropriate theory to use for analysis of such effects is still uncertain [6–8]. Both general (charged sphere, Debye-Hückel) and localized charge interaction seem to be involved. The analysis is complicated in the case of cytochrome *c* oxidase by the existence of two binding sites on the cytochrome $aa_3$, distinguished at low ionic strengths by very different binding constants [9,10]. The primary, or 'tight' (T), site is involved in electron transfer in situ from Complex III to Complex IV [11]; the secondary, or 'loose' (L), site is located elsewhere on cytochrome $aa_3$.

It is uncertain whether each site possesses its own characteristic maximal turnover ($V_{max} = V_T + V_L$) or whether the maximal velocity is identical for the two sites at 100% reduction of bound cytochrome *c* ($V_T = V_L = V_{max}$). In the latter case the usual reaction at the 'T' site may be limited by the dissociation rate for cytochrome *c* from this site [12] or by the degree of reduction of this bound cytochrome *c* [13]. Changes in ionic strength affect both sites. Osheroff and co-workers [10] indicated that citrate was an example of an anion that acts noncompetitively at the 'T' site (decreases $V_T$). Yet they also report [14] that the citrate anion binding site is included in the oxidase binding site. Moreover it is puzzling that small anions can apparently distinguish between the two redox forms of cytochrome *c* while cytochrome *c* oxidase cannot [15].

It was therefore suggested to us (Margoliash, E., personal communication) that a reevaluation of some specific and general ion effects on this system would be useful. One of us [11] had previously analysed all such effects in terms of cation displacement from the oxidase; it is now clear that such effects, if involved at all, play only a small role except in the case of the large polycations [16]. Specific anion effects on cytochrome *c* and general ionic strength effects are both more important. Experiments have therefore been carried out in which specific anion concentrations are varied at a constant ionic strength, which may be expected to discriminate the two kinds of action. But in addition some results have been obtained which may throw some light upon the nature of the interaction between the two redox proteins.

## Materials and Methods

Cytochrome *c* oxidase was prepared from Keilin-Hartree beef heart particles according to Kuboyama et al. [17], and stored at −80°C in 100 mM sodium phosphate (pH 7.4)/0.25% Tween-80. Deoxycholate-treated Keilin-Hartree particles (fully activated oxidase) were prepared essentially according to Smith and Camerino [18] by treating a 10 $mg \cdot ml^{-1}$ suspension with 1% potassium deoxycholate at pH 7.6. Cyanocytochrome $aa_3$ was prepared by incubating 50–100 μM cytochrome $aa_3$ with 2 mM KCN in 100 mM sodium phosphate (pH 7.4) at 4°C for a week (very little autoreduction occurred under these conditions).

Cytochrome *c* (type VI, horse heart), sodium ascorbate, TMPD (dihydrochloride), poly(galacturonic acid) (grade III), Tris (Trizma base), and Mops were Sigma products. Citrate was obtained as the free acid and trisodium salt from J.T. Baker Co. Cytochrome *c* was reduced with a minimal quantity of dithionite and then passed through a Sephadex G-25 column to prepare the ferrous form.

Column chromatography of the enzyme and its complexes was carried out with 2 cm × 25 cm Sephadex G-100 columns in two ways: (a) using 'dissociating' conditions, as described by Hill and Nicholls [5], analysing the results according to Dixon [19]; and (b) using 'associating' conditions, loading the columns with buffer containing cytochrome *c*, and analysing the results according to Hummel and Dryer [20].

Citrate, with p*K* values all lying below 6.5, was assumed to be fully dissociated at pH 7.2; the ionic strength of Mops-Tris buffers is proportional to the Mops added, as the p*K* of Mops is less than that of Tris. To maintain isoionic conditions, therefore, 1 mol citrate added must be substituted for 6 mol Mops or other monovalent anion present, as the ionic strength of a solution of a tribasic salt will be 6-times that of a solution of a monobasic salt equimolar in anion concentration.

Spectrophotometry was carried out with an

Aminco DW-2 instrument (dual wavelength and split beam measurements) or a Gilford 2400 (single beam; steady-state measurements). Stopped-flow studies were done with a Durrum-Gibson D-130 system (light path 2 cm). Polarography employed a Yellow Springs Instrument Co. Clark-type electrode in a temperature-controlled vessel (4.1 ml final volume) coupled to a polarizing box and recorder.

Cytochrome *c* concentration is obtained using $\Delta E_{mM}$ (reduced − oxidized) of 19.7 at 550 nm, or $\Delta\Delta E_{mM}$ (reduced − oxidized, 550–540 nm) of 21.2. Cytochrome $aa_3$ concentration is obtained from $\Delta E_{mM}$ (reduced − oxidized) of 24.8 at 605 nm, or $\Delta\Delta E_{mM}$ (reduced − oxidized, 605–630 nm) of 27.0.

## Results

Increasing salt concentrations slow the reduction of cytochrome *c* by ascorbate [21,22]. This may be attributed either to anion binding by cytochrome *c* or an ionic strength effect on the reduction of the cationic protein by the anionic reductant. To separate these effects, isoionic Tris buffer solutions containing Mops and citrate were prepared at ionic strengths of 12, 30 and 600 mM. The results of stopped-flow spectrophotometry at pH 7.2 and 30°C at constant cytochrome *c* and ascorbate concentrations are summarized in Table I. Three separate trends are apparent. Firstly, as ionic strength increases, the rate constant decreases. Secondly, as citrate concentration increases, the rate constant also decreases; but in this case the constant remains finite at high citrate concentrations. Thirdly, the amount of citrate required for half-maximal effect ($K_d^{obs}$) increases with ionic strength. The binding of the trianion citrate is itself ionic-strength-dependent; and when bound, it only partially blocks electron transfer from ascorbate. Although the reduction by ascorbate is partially inhibited by citrate, the reduction by TMPD is unaffected. Progressive substitution of 30 mM Mops by up to 5 mM citrate (Tris buffer, pH 7.2, 30°C) had no effect on the measured rate constant of $2.2 \cdot 10^4\,M^{-1} \cdot s^{-1}$ for TMPD in the presence of 2 mM ascorbate (stopped-flow measurements; traces not shown). On the other hand, the equilibrium between the TMPD/$TMPD^+$ system and the cytochrome *c* system is citrate-dependent [25]; the reverse reaction involving $TMPD^+$ (the radical cation, Wurster's blue) must therefore be anion-dependent.

Citrate also binds to ferrocytochrome *c* and blocks its oxidation by the enzymatic system. Fig. 1A shows the results of a polarographic assay in the presence of both ascorbate and TMPD at 600 mM ionic strength. Citrate acts competitively towards cytochrome *c*, increasing the $K_m$ from 70 μM to about 250 μM at the highest citrate levels. A plot of $1/K_m$ against citrate concentration (Fig. 1B) indicates that it does not block interaction with the enzyme completely. A similar $K_m$ value is obtained at very high ionic strengths. Fig. 1C shows that the apparent $K_m$ in 3 M KCl is 160 μM; at high ionic strengths, therefore, the $K_m$ no longer increases linearly with ion concentration as reported for lower salt concentrations [11].

At lower ionic strengths, as reported by Fergu-

TABLE I

KINETIC PARAMETERS FOR THE INHIBITION BY CITRATE OF ASCORBATE REDUCTION OF CYTOCHROME *c* AT CONSTANT IONIC STRENGTH

Stopped-flow experiments were carried out with 10 μM cytochrome *c*/10 mM ascorbate at pH 7.2, 30°C. Citrate concentration was varied from 0 to 2 mM (a), 5 mM (b) and 100 mM (c), keeping ionic strength constant with Mops, starting with 12 mM Mops-Tris (a), 30 mM Mops-Tris (b) and 600 mM Mops-Tris (c). $k_0$ = second-order rate constant when [citrate] = 0; $k_\infty$ = second-order rate constant as [citrate] → ∞; $K_d^{obs}$ = [citrate] when $(k_{obs} - k_\infty) = (k_0 - k_\infty)/2$.

| | Ionic strength (mM) | $k_0(M^{-1} \cdot s^{-1})$ | $k_\infty(M^{-1} \cdot s^{-1})$ | $K_d^{obs}$(mM) |
|---|---|---|---|---|
| (a) | 22 | 95 | 48 | 0.6 |
| (b) | 40 | 71 | 45 | 1.0 |
| (c) | 610 | 7.5 | 5.0 | 1.3 |

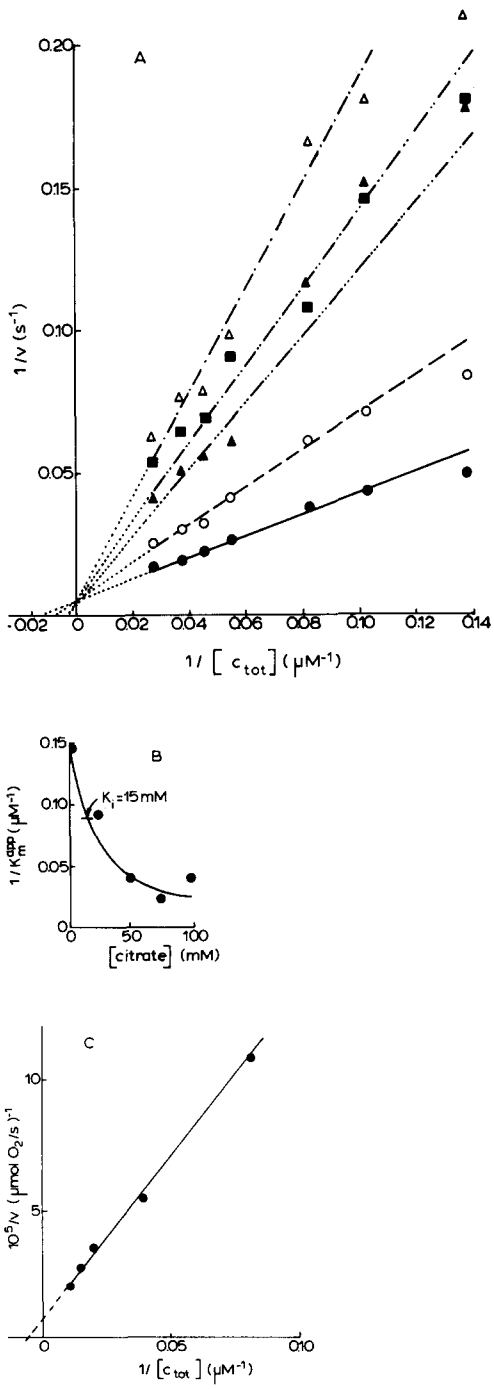



Fig. 1A. Effect of citrate on cytochrome *c* oxidase activity at high ionic strength: polarographic assay. The reciprocal of the turnover ($s^{-1}$) is plotted against the reciprocal of cytochrome *c* concentration ($\mu M$). (B shows a plot of $1/K_m^{app}$ ($\mu M^{-1}$) against citrate concentration.) 36 nM cytochrome $aa_3$ was used in an assay system with 4.8 mM ascorbate/0.18 mM TMPD, at 30°C and pH 7.2. Cytochrome *c* concentration was varied from 7.3 to 36.3 $\mu M$ at a constant ionic strength of 600 mM maintained by appropriate mixtures of Mops-Tris and citrate/Tris buffers as follows: ●———●, 600 mM Mops-Tris; O------O, 450 mM Mops-Tris; 25 mM citrate/Tris; ▲-·-·-▲, 300 mM Mops-Tris; 50 mM citrate/Tris; △-·-△, 150 mM Mops-Tris; 75 mM citrate/Tris; ■-·-·-■, 100 mM citrate/Tris. C. Reciprocal plot of cytochrome *c* oxidase activity against cytochrome *c* concentration at very high ionic strength: polarographic assay. 26 nM cytochrome $aa_3$ was used in an assay system with 4.8 mM ascorbate/0.18 mM TMPD/3.0 M KCl buffered at pH 7.2 with 50 mM sodium phosphate. Cytochrome *c* concentration was varied from 10 to 100 $\mu M$. $T = 30°C$.

son-Miller and co-workers [9,12], the binding of cytochrome *c* is biphasic (Fig. 2). As found by Osheroff et al. [10], the isoionic substitution of Mops by citrate has a noncompetitive action at the higher affinity (T) site. Unlike Osheroff et al. [10], however, we find that citrate also acts competitively at the lower affinity (L) site. Fig. 2B indicates the decline in affinity in the L region with increasing trianion concentration. In this system, Mops behaves as a binding anion compared to cacodylate in the 'T' region (see Discussion). The kinetic constants ($K_m$ and TN values) used to construct the theoretical lines in Fig. 2 are listed in the legend.

The action of citrate on the reduction of cytochrome *c* by ascorbate was the same in the aerobic steady-state system in the presence of both ascorbate and enzyme, as seen directly in the stopped-flow experiments (cf. Tables I and II). The effects on the oxidase reaction under the same conditions are summarized in Table II. As in the polarographic (ascorbate plus TMPD) system, the largest effect at high ionic strengths is seen on the $K_m$ value. With ascorbate as substrate alone, only competitive effects are seen.

The action of citrate on cytochrome *c* oxidase in the absence of reducing agents can be monitored using the direct spectrophotometric assay. At high ionic strength, as reported by Smith and Conrad [23] and by Minnaert [15], the oxidation is first order in ferrocytochrome *c* in both presence and absence of citrate. The rate constant declines with increasing citrate, but again reaches a finite value at high citrate concentrations (at least 100 mM). At lower ionic strengths, substitution of citrate for Mops alters the observed kinetic pat-

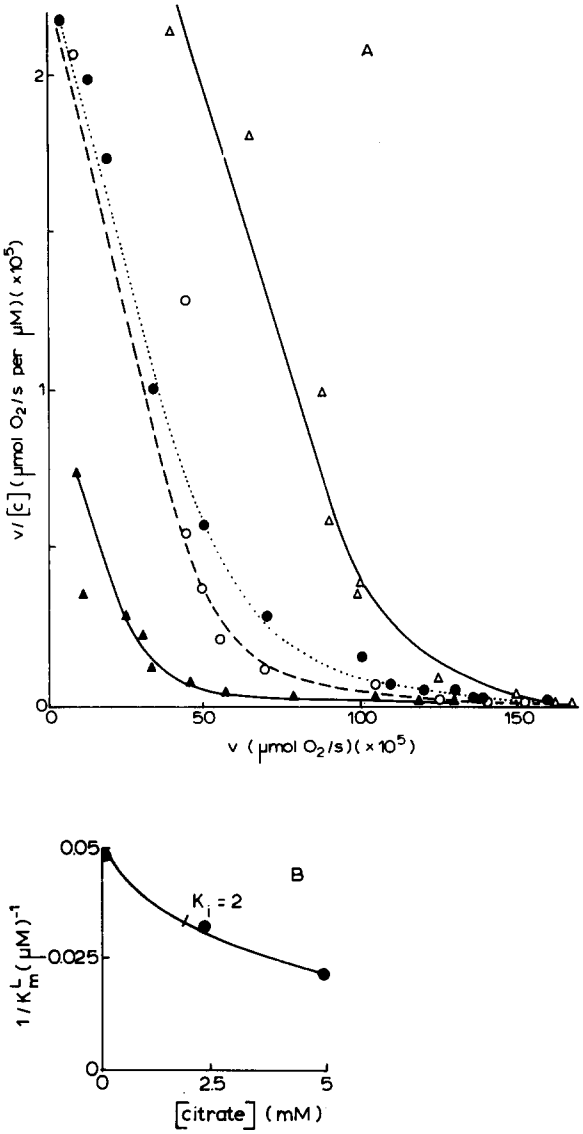



Fig. 2. Effect of citrate on cytochrome *c* oxidase activity at low ionic strengths: polarographic assay. 0.026 $\mu$M cytochrome $aa_3$ was used with 4.8 mM ascorbate and 180 $\mu$M TMPD in 4.1 ml final volume, pH 7.2, 30°C in the presence of cytochrome *c*. Eadie-Hofstee plots show the effect of varying cytochrome *c* concentration from 0.024 to 97 $\mu$M in the presence and absence of citrate, $\mu$=30 mM. ●·····●, 30 mM Mops-Tris buffer; ○------○, 2.5 mM citrate, 15 mM Mops-Tris buffer; ▲———▲, 5 mM citrate/Tris buffer; △———△, 30 mM cacodylate/Tris buffer; Theoretical curves are obtained using $V_{max}$ = 190 nequiv.·s$^{-1}$, $K_m^T$=0.25 $\mu$M, and the following $V_T$ and $K_m^L$ values: ●·····●, $K_m^L$=20 $\mu$M, $V_T$=32% $V_{max}$; ○- - -○, $K_m^L$=31 $\mu$M, $V_T$=28% $V_{max}$; ▲———▲, $K_m^L$=47 $\mu$M, $V_T$=16% $V_{max}$; △———△, $K_m^L$=18 $\mu$M, $V_T$=53% $V_{max}$. B. Plot of $1/K_m^L$ against citrate concentration.

tern. The logarithmic time courses now curve downwards, indicating a reaction order less than 1. Calculation of the resulting $K_m$ (ferrocytochrome *c*) and $K_p$ (ferricytochrome *c*) values [24] gives the results in Fig. 3. Here the ratio $K_m/K_p$ is plotted against citrate concentration. Unlike the situation at higher ionic strengths [15,23], $K_m/K_p$ is not always equal to 1.0. As citrate concentration increases, the ratio $K_m^L/K_p^L$ decreases (see Discussion).

Differential effects of citrate on complex formation between the enzyme and the two redox forms of cytochrome *c* were also monitored by column chromatography. Cytochrome *c*/cytochrome $aa_3$ mixtures were chromatographed on columns loaded with cytochrome *c* at medium and low ionic strengths in the presence and absence of

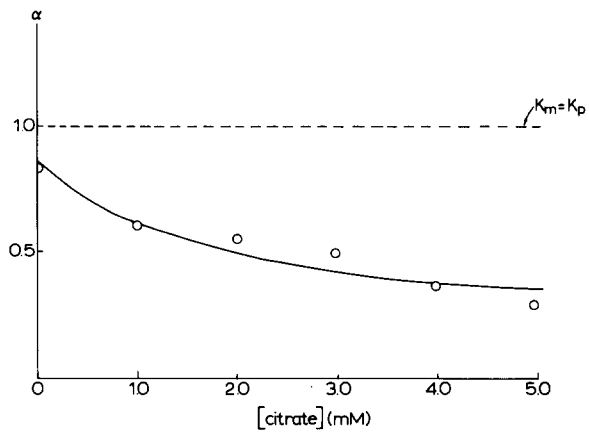



Fig. 3. Effect of citrate on the $K_m/K_p$ ratio observed in the spectrophotometric assay at low ionic strengths. 1.2 nM cytochrome $aa_3$ was used to oxidize ferrocytochrome *c* at pH 7.2, 30°C and $\mu$=30 mM. The indicated concentrations of citrate were progressively substituted for Mops in a Mops-Tris buffer. $K_m/K_p$ values were obtained from Jennings-Niemann plots as described by Cornish-Bowden [24]. Progress curves are plotted in the form $t/\ln(S_0/S)$ vs. $(S_0-S)/\ln(S_0/S)$, where $t$ is time, $S_0$ the initial substrate concentration and $S$ the substrate concentration at time $t$. Each progress curve then extrapolates to a value of $(S_0-S)/\ln(S_0/S)$ equal to $S_0$ when $t$=0; the line joining these extrapolated points for several $S_0$ values corresponds to a Hanes plot of $S_0/v$ against $S_0$. The ratio of the slope of a given progress curve potted in this way to the slope of the Hanes plot itself is equal to $(1-K_m/K_p)$; in the case of Smith-Conrad kinetics the progress curves plotted in this way are horizontal and $(1-K_m/K_p)$=0. In the absence of product inhibition, the progress and Hanes plots are superimposed and $(1-K_m/K_p)$=1.

TABLE II

EFFECT OF CITRATE ON THE OXIDATION OF CYTOCHROME *c* BY CYTOCHROME $aa_3$: STEADY-STATE ASSAY

Citrate concentration was 2 mM (a), 5 mM (b) and 100 mM (c). Tris buffer (pH 7.2), 30°C, with cytochrome *c* concentration from 1.1 to 18.0 μM; cytochrome $aa_3$ was 0.17 μM (a, b), 0.34 μM (c); 14 mM sodium ascorbate. The time taken to anaerobiosis was monitored as well as the steady-state reduction of cytochrome *c* ($[c]_{red}/[c]_{tot}$). The observed velocity was converted to the presumed velocity at 100% reduction of cytochrome *c*, *v*, according to $v = v_{obs}[c]_{tot}/[c]_{red}$. $TN_{max} = TN_{obs} \cdot [c]_{tot}/[c^{2+}]$ (μequiv./s per μmol cytochrome $aa_3$). $k_{obs}$ = second-order rate constant for the reduction of cytochrome *c* by ascorbate (cf. Table I). $K_m$ = concentration of cytochrome *c* giving half-maximal turnover ($K_m^L$ for at least (a) and (b)).

| Medium | | μ (mM) | $K_m$ (μM) | $TN_{max}$ ($s^{-1}$) | $k_{obs}/2$ ($M^{-1} \cdot s^{-1}$) |
|---|---|---|---|---|---|
| (a) | Mops-Tris | 12 | 3 | 71 | 70 |
| | citrate/Tris | 12 | 5.6 | 71 | 36 |
| (b) | Mops-Tris | 30 | 9 | 147 | 35 |
| | citrate/Tris | 30 | 13.3 | 147 | 24 |
| (c) | Mops-Tris | 600 | 58 | 118 | 10 |
| | citrate/Tris | 600 | 250 | 118 | 6.5 |

TABLE III

COLUMN CHROMATOGRAPHY OF CYTOCHROME *c*/CYTOCHROME $aa_3$ MIXTURES UNDER VARIOUS CONDITIONS: EFFECT OF CITRATE

| Column type | Cytochrome *c* | Medium (pH 7.2, 4°C) (mM) | Cytochrome $c/aa_3$ | $K_d$ (μM) |
|---|---|---|---|---|
| Dissociating (see Materials and Methods) | ferri- | 5 Mops-Tris | 0.82 [a] | 0.03 [c] |
| | | 0.86 citrate/Tris | 0.73 | 0.09 |
| | | 30 Mops-Tris | 0.63 | 0.19 |
| | | 5 citrate/Tris | 0.45 | 0.50 |
| Associating [e] (see Materials and Methods) | ferri- | 5 Mops-Tris | 1.65 [b] | 16.2 [d] |
| | | 0.86 citrate/Tris | 1.22 | ≥100 |
| | | 30 Mops-Tris | 1.21 | ≥100 |
| | | 5 citrate/Tris | 0.98 | large |
| Associating | ferro- | 5 Mops-Tris | 1.53 [b] | 22.2 [d] |
| | | 0.86 citrate/Tris | 1.35 | 50.0 |
| | | 30 Mops-Tris | 1.10 | ≥200 |
| | | 5 citrate/Tris | 1.01 | large |

[a] Measured directly directly from spectra.

[b] Measured from area of trough divided by area of $aa_3$ peak.

[c] Calculated using Eqn. i (cf. Ref. 19):

$$[c/aa_3] - \ln[c/aa_3] - 1 = \frac{K_d V}{[aa_3]_{tot}} \quad \text{(i)}$$

where *V* is the column volume and $[aa_3]_{tot}$ the total amount of enzyme present on the column.

[d] Calculated using Eqn. ii:

$$K_d = (2 - [c/aa_3])[c]/([c/aa_3] - 1) \quad \text{(ii)}$$

where $[c/aa_3]$ is the ratio of cytochrome *c* bound to cytochrome *c* oxidase.

[e] 2 ml 28 μM cytochrome *c* plus 28 μM cytochrome $aa_3$ placed on Sephadex G-100 columns equilibrated with the indicated buffers plus 28 μM cytochrome *c*.

citrate (Table III). In each case more than 1 mol cytochrome *c* is bound per equivalent of cytochrome $aa_3$; that is, the 'tight' (T) site is always occupied and the indicated variability is at the 'loose' (L) site. Table III lists the calculated equilibrium constants for the latter site, according to the equation in the legend, for both fully oxidized and fully reduced systems.

To estimate $K_d$ values at the T site, a 'dissociating' column was employed, as described in Materials and Methods. Such columns give $[c]:[aa_3]$ ratios less than 1 (Table III), and the binding presumably reflects that seen kinetically at low cytochrome *c* concentrations and low ionic strength (Fig. 2). The ratios obtained are not greatly affected by the presence of citrate but the strong dependence of $K_d$ on the ratio (Eqn. i in Table III) suggests that the $K_d$ values may be rather different.

Although citrate binding does not change the rate of reduction of cytochrome *c* in solution by TMPD, if citrate-cytochrome *c* does bind to cytochrome $aa_3$ (see Discussion), then either the reducibility of bound *c* by TMPD, or the reduction of cytochrome *a* by bound *c*, might be affected. Stopped-flow experiments at low ionic strength showed no sign of a specific citrate effect on the rate constant for TMPD reduction of cytochrome *c* bound to cytochrome $aa_3$ at site 'T' (i.e., in 1:1 mixtures). Fig. 4 illustrates some typical results obtained upon mixing equimolar cytochrome *c*/cyanocytochrome $aa_3$ solutions with ascorbate (Fig. 4A) and with ascorbate plus TMPD (Fig.4B). The reduction of cytochrome *c*, and of cytochrome *a* in mixtures, by ascorbate, is decreased in the presence of citrate (cf. Fig. 4A trace sets (i) and (ii)). In each case the rate-limiting step is the reduction of free cytochrome *c* by ascorbate; the subsequent events represent rapid equilibration between free cytochrome *c*, bound cytochrome *c*,

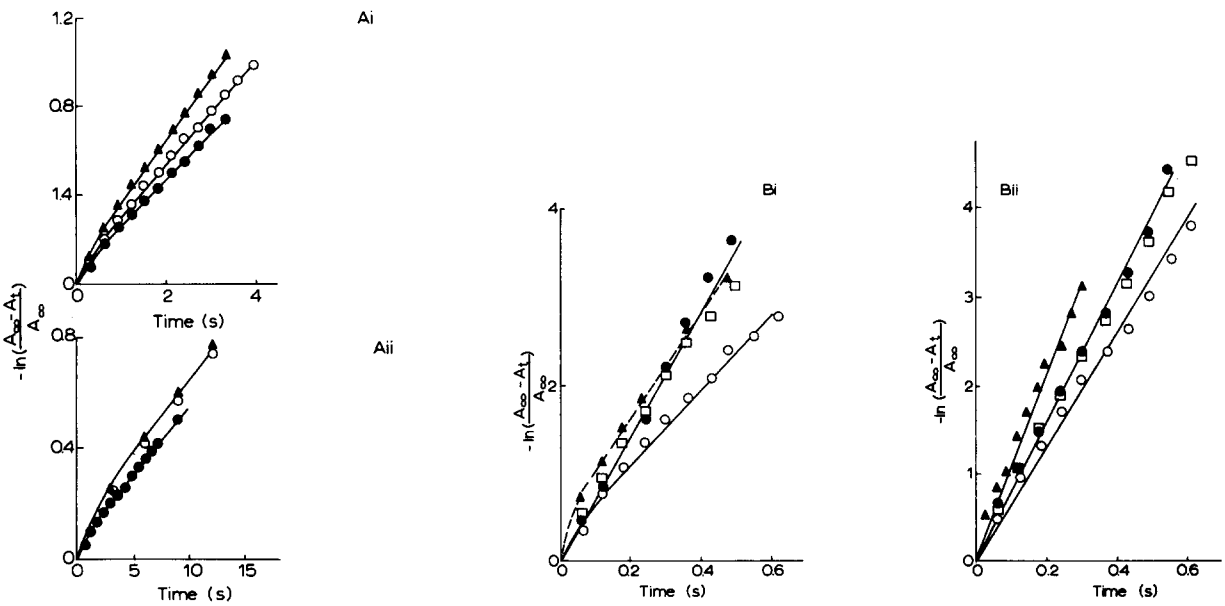



Fig. 4. Reduction of cytochromes *c* and *a* in the presence and absence of anions. A. Reduction by ascorbate at low ionic strength. 2.5 $\mu$M ferricytochrome *c* plus 2.5 $\mu$M cyanocytochrome $aa_3$ (final $[CN^-]=200$ $\mu$M, see Materials and Methods) from one syringe were reduced with 6 mM ascorbate (final concentration) from another, and the reduction monitored at 550, 605 and 445 nm. pH 7.2, $\mu=5$ mM, 30°C. (i) Reaction in 5 mM Mops-Tris buffer; (ii) reaction in 0.86 mM citrate/Tris buffer; ●, 550 nm; ○, 445 nm; ▲, 605 nm. B. Reduction by ascorbate and TMPD at higher ionic strength. 4 $\mu$M ferricytochrome *c* plus 4 $\mu$M cyanocytochrome $aa_3$ (final $[CN^-]=100$ $\mu$M, see Materials and Methods) from one syringe were reduced with 2 mM ascorbate plus 0.3 mM TMPD from another, and the reaction was monitored at 550, 605 and 445 nm. pH 7.2, $\mu=30$ mM, 30°C. (i) Reaction in 30 mM Mops-Tris buffer, (ii) reaction in 5 mM citrate/Tris buffer; ●, 550 nm; □, 550 nm (absence of $aa_3$); ▲, 605 nm; ○, 445 nm. Experiments carried out with the Durrum-Gibson stopped-flow instrument; light path 2 cm.

and acceptor centres on the cyanocytochrome $aa_3$. Reduction of *c*, both free and bound, by TMPD is unaffected by substituting citrate for Mops (Fig. 4B). The reduction of cytochrome *a* seemed to be slightly faster in the presence than in the absence of citrate (cf. Fig. 4B, trace sets (i) and (ii)), probably reflecting a decrease in the effective $E_h$ of bound cytochrome *c*.

## Discussion

Osheroff and co-workers [2,10] distinguish several types of anion binding to cytochrome *c*. How typical is citrate? It is clear that total charge is important; polyanions are more effective than monoanions. Polygalacturonate [3] is one of the most effective species for inducing the kinetic change from first-order (Smith-Conrad) to near zero-order in the spectrophotometric assay. ATP (tetraanion) is even more effective than citrate in blocking ascorbate reduction [25]. Total charge alone, however, is not the sole factor. The calcium-EDTA complex is much more effective than EDTA itself at pH 7 as an inhibitor of ascorbate-cytochrome *c* interaction, despite their presumed equality of charge [26]. And although cacodylate buffer allows a higher turnover at the 'T' site than does Mops (Fig. 2), ascorbate reduction of cytochrome *c* is more rapid in Mops than in cacodylate media [25]. Neither monoanion can be treated as an ideal nonbinding buffer ion.

The classical buffer anion used in cytochrome *c* oxidase studies is phosphate [11], for which two binding sites on cytochrome *c* have been reported [2]. Smith and co-workers [27] have shown that at low ionic strength there are marked differences between the results obtained by the spectrophotometric and polarographic techniques. In higher ionic strength phosphate buffer, those differences disappear [11,12]. Moreover, unlike the observations in presence of citrate, in phosphate buffer of 20 mM or higher, $K_m \approx K_p$, and the reactions obey first-order (Smith-Conrad) kinetics uniformly. Kinetic parameters obtained in this laboratory from analogous experiments are listed in Table IV (cf. Ref. 11).

Citrate binds to a single site on cytochrome *c* [2,10]. The citrate-cytochrome *c* complex shows a modified reactivity with ascorbate (Table I) and with cytochrome c oxidase (Table II). The ferrous form is slightly less sensitive to citrate than the ferric form, as summarized in Table V.

It may be important to note that the kinetic evidence presented does not require that the two effects represent interaction at the same site. Two non-haem-linked sites with different citrate affinities, one involved in ascorbate reduction and the other in cytochrome $aa_3$ oxidation, would lead to

TABLE IV

COMPARISON OF KINETIC CONSTANTS OBTAINED IN SPECTROPHOTOMETRIC AND POLAROGRAPHIC ASSAYS OF CYTOCHROME *c* OXIDASE

Deoxycholate-treated particles (maximally activated cytochrome $aa_3$ between 1 nM [d] and 25 nM [e] were used in the presence or absence of 5–20 mM ascorbate, 0.18 mM TMPD and varying quantities of cytochrome *c* at pH 7.4 and 30°C. T='tight' site (primary); L='loose' site (secondary).

| Medium (mM) | Site | Maximal turnover ($s^{-1}$) | | Michaelis constant ($\mu$M) | |
|---|---|---|---|---|---|
| | | Spectrophotometric [d] | Polarographic [e] | Spectrophotometric [d] | Polarographic [e] |
| (a) 100 phosphate [a] | — | 310 | 375 | 22.5 | 25 |
| (b) 20 phosphate [b] | T | 75 | 107 | 1.3 | 1.2 |
| | L (max) | 180 | 210 | 8.5 | 10 |
| (c) 5 Mops-Tris [c] | T | 14 | 60 | ≈0.1 | ≈0.1 |
| | L (max) | 67 | 165 | 2.8 | 2.0 |

[a] $\mu$=230 mM. [b] $\mu$=46 mM. [c] $\mu$=5 mM. [d] Smith-Conrad assay. [e] Oxygen electrode.

TABLE V

COMPARISON OF EQUILIBRIUM CONSTANTS FOR COMPLEX FORMATION BETWEEN CITRATE AND CYTOCHROME *c* OBTAINED IN ASCORBATE REDUCTION AND CYTOCHROME *c* OXIDASE SYSTEMS

| Experimental conditions | Redox state of cytochrome *c* | $\mu$ (mM) | $K_d$ (mM) |
|---|---|---|---|
| Ascorbate reduction of cytochrome *c* | ferric | 19 | 0.8 |
| | | 30 | 1.2 |
| | | 600 | 13 |
| Cytochrome *c*-cytochrome oxidase assay | ferrous | 19 | [a] |
| | | 30 | 2.0 |
| | | 600 | 15–30 |

[a] Citrate effect no longer competitive (see Fig. 2 and Discussion). pH 7.2, 30°C.

the same result as one haem-linked site. Redox titrations, however, indicated that one haem-linked site exists with $K_d$ of 2 mM (ferric) and 8 mM (ferrous) at $\mu = 30$ mM [25]. It seems plausible to propose that this site is also the major one monitored kinetically.

The results of Margalit and Schejter [28] suggested that anion binding to cytochrome *c* decreased its redox potential at low ionic strength but increased this potential at high ionic strength [11]. That is, the electrostatic component of anion binding is greater when cytochrome *c* is in its ferric form, while the nonelectrostatic (steric) contribution is greater in the ferrous state. The apparent increase in effectiveness of citrate at higher ionic strengths (Table II) in modifying $K_m$ may be due to the latter phenomenon.

The binding of cytochrome *c* to cytochrome *c* oxidase also has electrostatic (Tables II and III) and nonelectrostatic (Fig. 1C) components. In this case, the binding of the ferrous and ferric forms of cytochrome *c* can be monitored both chromatographically (Table III) and kinetically (Tables II and IV). At low ionic strength in the absence of binding ions, and at high ionic strength in the presence of binding ions such as citrate (Figs. 1A and 2) or phosphate (Table IV; [15,23]) the $K_m$ ($\approx K_d$ for ferrocytochrome *c*) and $K_p$ ($\approx K_d$ for ferricytochrome *c*) values are closely similar, giving rise to the classical Smith-Conrad kinetic behaviour. But at lower ionic strength with binding ions, $K_m < K_p$ (Fig. 3) and the enzymatic oxidation takes on near zero-order characteristics. This is an inevitable consequence of the presence of species binding ferricytochrome *c* preferentially.

At low ionic strengths, two binding sites for cytochrome *c* are evident in cytochrome *c* oxidase (Refs. 9 and 11; Fig. 2). The T ('tight') site has different turnovers in the polarographic and spectrophotometric assays (Table IV; [27]), indicating that the latter assay is increasing the rate of cytochrome *c* dissociation, a conclusion tentatively advanced previously [29] and supported by Wilms and co-workers in stopped-flow experiments [30].

What site is active at high ionic strengths? If the effect of ionic strength were solely upon the association reaction, then the observed spectrophotometric turnovers would eliminate the possibility that the T site was functional (Table IV). Ions, including polycations, do, however, seem to increase the dissociation constant (cf. Ref. 30). If the major effect of increasing $\mu$ is on the 'off' constant for cytochrome *c*, then the T site at high ionic strengths will act like the L site. Eqn. 1 gives the simplest model:

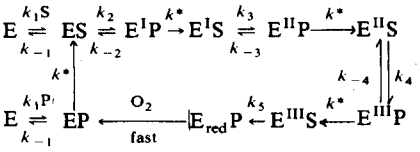
$$c + aa_3 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} c(aa_3) \underset{k'_{-1}}{\overset{k'_1}{\rightleftharpoons}} caa_3 \qquad (1)$$

where $k_{on} = k_1 k'_1/(k_{-1} + k'_1)$ and $k_{off} = k'_1 k_{-1}/(k_{-1} + k'_1)$. If the $c$-$(aa_3)$ complex is inactive and involves only some of the electrostatic bonds required for the fully active complex [31], then increasing $\mu$ may be effective in reducing the value of $k'_1$. When $k'_1 \gg k_{-1}$, $k_{on}(= k_1)$ and $k_{off}(= k'_{-1} k_{-1}/k'_1)$ will both be affected by increasing ionic strength. Citrate, which acts 'competitively' towards the functional site at high ionic strength (Fig. 1A), does tend to promote dissociation at lower ionic strength (Table III), indicating some chemical similarity between the two processes.

The L site is seen only at low ionic strength, presumably because its $K_d$ becomes very large as $\mu$ increases and due to the increase in $k_{off}$ (Eqn. 1) it is no longer required for maximal activity. Its binding may be in fact be 'simple', resembling the first step of Eqn. 1.

There seem to be at least two ways of explaining the apparent 'noncompetitive' behaviour of citrate at the 'T' site (Ref. 10; Fig. 2). Both alternatives require that the T site be capable of binding citrate-cytochrome *c*, at least at low ionic strengths.

Calculations based on column chromatography suggest that the anion complex is indeed bound with a decreased affinity (if this were not so, the measured $K_d$ values given in Table III for the dissociating column in presence of citrate would be expected to be larger). The first alternative involves a consideration of (i) the redox potential of bound cytochrome *c*, and (ii) the multiple electron transfer sequence necessary for reduction of one molecule of oxygen in the complete catalytic cycle. Although citrate-cytochrome *c* probably has a lower redox potential than that of free cytochrome *c* at low ionic strength, at high ionic strength (or whenever the electrostatic effects are screened) it may have a higher potential [11,28]. A similar increase may occur in the cytochrome *c*-enzyme complex. Although the reduction of high potential centres on the oxidase may still be possible, the reduction of low potential centres may now be disfavoured. Eqn. 2 gives a scheme for the turnover at site T under conditions of saturating concentrations of cytochrome *c*.



(2)

where, $k^* \rightarrow (k_{red}[\text{TMPD}] + k_{-1})$ as ($[S] \rightarrow \infty$), S = cytochrome $c^{2+}$, P = cytochrome $c^{3+}$, and $E^I$, $E^{II}$, $E^{III}$ and $E_{red}$ are progressively more reduced forms of the enzyme. When $k^*$ is small (i.e., when most of the bound *c* is in the ferric form), and when [S] is large, the turnover given by the mechanism of Eqn. 2 is that of Eqn. 3:

$$TN_T = v/e = k^*/\left[\left(\frac{K_3 + K_4 + K_5}{4}\right) + 1\right] \qquad (3)$$

where $K_3 = k_{-3}/k_3$, $K_4 = k_{-4}/k_4$ and $K_5 = k_{-5}/k_5$. Because reversible steps occur in the complete cycle, the maximal turnover at finite TMPD levels is not (contrary to Ref. 13) given by $k^*$ alone but by a smaller number, depending on the value of the equilibria $K_3$, $K_4$ and $K_5$. A cytochrome *c* with a higher redox potential when bound (e.g., citrate-bound cytochrome *c*, and perhaps primate cytochrome *c*, cf. Refs. 9 and 12) will thus have a lower turnover, even though its rate of reduction by TMPD (stopped-flow studies) is unchanged. Citrate will then behave non-competitively towards the T site at low ionic strengths.

However the increased percentage reduction of cytochrome *a* under stopped-flow conditions in the presence of citrate suggests that the redox potential of bound citrate-cytochrome *c* may be lower, rather than higher, than that of bound anion-free cytochrome *c*. An alternative mechanism based upon Eqn. 1 may therefore be considered, as shown in Eqn. 4:

$$c^{2+}(aa_3) \underset{k^1_{-1}}{\overset{k^1_1}{\rightleftharpoons}} c^{2+}aa_3 \underset{k^*}{\overset{k_2}{\rightleftharpoons}} c^{3+}aa_3 \qquad (4)$$

where $k^* = k_{red}$ [TMPD], essentially as in Eqn. 2. The turnover given by the mechanism of Eqn 4 is that of Eqn. 5:

$$TN_T = v/e = k_2 k^*/[k^*(K_1 + 1) + k_2] \qquad (5)$$

where $K_1 = k^1_{-1}/k_1$. An increase in $K_1$ induced by citrate (as ionic strength was presumed to decrease $k^1_1$ in Eqn. 1 above) would thus decrease $TN_T$ and show up kinetically as noncompetitive behaviour. A decision between the models of Eqns. 4 and 2 will require futher experimentation.

If the T site is functional at both high and low ionic strengths in the polarographic assay, and at high ionic strength in the spectrophotometric assay [13], what is the role of the L site? At low ionic strengths it may act as the 'shuttle' system creating the 'oxidase excess' situation seen under the usual physiological conditions [29] as well as providing a mechanism for intermembrane electron exchanges [32,33]. The equality of maximal turnover at high and low ionic strengths [34] supports the previous arguments [13,29] that the L site alone is not catalytically competent, but serves to funnel electrons to an active cytochrome *c* molecule bound at the T site, perhaps via cytochrome *a* or one of the copper centres in the molecule. In a model system such as that of Eqn 2, the availability of reduced cytochrome *c* molecules bound at the L site can shortcircuit some of the steps, for example by converting EP into $E^IP$ or ES into $E^IS$. When such shortcircuiting is rapid, while the direct steps ($k^*$) are slow, as in the spectrophotometric assay at low ionic strength, the formation of bound S (cyto-

chrome $c^{2+}$ at the T site), required for those steps in Eqn. 2 which cannot be shortcircuited, will be via a reversal of electron transfer between, say, cytochrome $a^{2+}$ and the bound *c* cytochrome. The apparent equilibrium between tightly bound cytochrome *c* and cytochrome *a* (Fig. 4; [5]) may be essential for the functioning of the enzyme under all catalytic conditions.

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