The effect of complex-formation with polyanions on the redox properties of cytochrome *c*

Lars C. PETERSEN* and Raymond P. COX†

*Department of Clinical Chemistry, Hvidovre Hospital, Kettegårds Alle, DK-2650 Hvidovre, Denmark, and

†Institute of Biochemistry, Odense University, Campusvej 55, DK-5230 Odense M, Denmark

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1. The stable complex formed between mammalian cytochrome *c* and phosvitin at low ionic strength was studied by partition in an aqueous two-phase system. Oxidized cytochrome *c* binds to phosvitin with a higher affinity than reduced cytochrome *c*. The difference was equivalent to a decrease of the redox potential by 22 mV on binding. 2. Complex-formation with phosvitin strongly inhibited the reaction of cytochrome *c* with reagents that react as negatively charged species, such as ascorbate, dithionite, ferricyanide and tetrachlorobenzoquinol. Reaction with uncharged reagents such as *NNN'*-tetramethylphenylenediamine and the reduced form of the *N*-methylphenazonium ion (present as the methylsulphate) was little affected by complex-formation, whereas oxidation of the reduced cytochrome by the positively charged tris-(phenanthroline)cobalt(III) ion was greatly stimulated. 3. A similar pattern of inhibition and stimulation of reaction rates was observed when phosvitin was replaced by other macromolecular polyanions such as dextran sulphate and heparin, indicating that the results were a general property of complex-formation with polyanions. A weaker but qualitatively similar effect was observed on addition of inositol hexaphosphate and ATP. 4. It is suggested that the effects of complex-formation with polyanions on the reactivity of cytochrome *c* with redox reagents are mainly the result of replacing the positive charge on the free cytochrome by a net negative charge. Any steric effects on polyanion binding are small in comparison with such electrostatic effects.

Cytochrome *c* from mammalian mitochondria is a basic protein with a positive charge at neutral pH values. The net charge of oxidized horse heart cytochrome *c* is about +9.5 at pH 7.0 (Barlow & Margoliash, 1966). Many anions, such as chloride, phosphate and citrate, can bind to the cytochrome, influencing its electrophoretic mobility (Barlow & Margoliash, 1966; Margoliash *et al.*, 1970) and other properties, including its redox potential (Schejter & Margalit, 1970; Margalit & Schejter, 1973*a,b*). The formation of complexes with cytochrome *c* oxidase and cytochrome *c* peroxidase (reviewed by Nicholls, 1975) also appears to be largely electrostatic, and negatively charged regions on cytochrome *c*-complexing enzymes seem to be important in the interactions with the cytochrome.

The ionic composition of the medium strongly affects the reaction of cytochrome *c* with small redox reagents such as ascorbate (Minnaert, 1961; Yamazaki, 1962), dithionite (Miller & Cusanovich, 1975), hydroquinone (Williams, 1963) and ferricyanide (Morton *et al.*, 1970; Cassatt & Marini,

1974). The rate of reaction with these negatively charged reagents is decreased as the ionic strength of the medium is increased. In addition, certain complex-forming anions appear to have a more specific inhibitory effect (Peterman & Morton, 1979).

A similar inhibitory effect on cytochrome *c* reduction by ascorbate is produced by complex-formation with cytochrome *c* oxidase and cytochrome *c* peroxidase (Mochan & Nicholls, 1972). The reaction of cytochrome *c* with dithionite is also very much slower when it is complexed to cytochrome *c* oxidase (Petersen & Cox, 1980).

We report here some studies of complex-formation between the cytochrome and macromolecular polyanions and the effects of this on the reaction with small redox reagents, both uncharged and with positive or negative charges.

Materials and methods

Phosvitin, heparin (lithium salt) and cytochrome *c*

(type VI, horse heart) were from Sigma Chemical Co., St. Louis, MO, U.S.A., and were used without further purification. Reduced cytochrome *c* was obtained by treatment with an excess of ascorbate followed by gel filtration through a column of Sephadex G-25 (Pharmacia, Uppsala, Sweden).

Tris(phenanthroline)cobalt(III) perchlorate was synthesized and assayed as described by McArdle *et al.* (1974). Dextran sulphate (17% sulphur, mol.wt. 500000) and dextran T-500 were from Pharmacia. Other chemicals were from E. Merck, Darmstadt, Germany, or Sigma Chemical Co., St. Louis, MO, U.S.A.

Stopped-flow spectrophotometry was carried out with a commercial apparatus (Applied Photophysics, London W1X 3HA, U.K.) modified by replacing the stopping syringe by an electromagnetic valve to allow computer control of the measuring cycle as described by Petersen & Cox (1980) and in more detail by Cox *et al.* (1979). Slower reactions and spectra were measured by using Cary 118C or Beckman Acta M VI spectrophotometers.

Aqueous two-phase partition was carried out with a system containing 7% (w/w) dextran T500 and 4.4% (w/w) poly(ethylene glycol) 6000 as described by Petersen (1978).

All kinetic measurements were made at room temperature (20–22°C). In some cases the media used contained the non-ionic detergent Tween 20 to provide conditions identical with those used in experiments with cytochrome *c* oxidase. Tween appeared to have no significant effects on the reaction rates.

Results

Measurements of complex-formation between cytochrome c and phosvitin by aqueous two-phase partition

Phosvitin is a storage protein from egg yolk with a mol.wt. of about 40000. More than half of the amino acid residues are *O*-phosphoserine (Allerton & Perlmann, 1965). This produces a very high negative charge density along the chain, and the physico-chemical properties of the molecule are essentially those of a polyanion (Giancotti *et al.*, 1973). Taborsky (1970) and Yoshimura *et al.* (1979) showed that phosvitin formed a stable complex with cytochrome *c* that could be detected by ultracentrifugation. Up to 20 molecules of cytochrome could be bound per molecule of phosvitin.

We have previously (Petersen, 1978) demonstrated that the aqueous two-phase partition methods developed by Albertsson and his colleagues [see Albertsson (1978) for a review] can be used to study complex-formation between cytochrome *c* and

cytochrome *c* oxidase. This system can also be used to investigate the interaction between cytochrome *c* and phosvitin. In Fig. 1 the partition coefficient between the two phases in a dextran/poly(ethylene glycol) system is plotted as a function of the concentration of added phosvitin. The results show that both oxidized and reduced cytochrome *c* bind to phosvitin, with the oxidized form having a higher affinity. From a knowledge of the partition coefficients of free cytochrome *c* and free phosvitin, the

Fig. 1. Effect of phosvitin on the partition coefficient of cytochrome *c* in an aqueous two-phase system

The two-phase system contained 4.4% (w/w) poly(ethylene glycol) and 7% (w/w) dextran in 20 mM-NaCl/10 mM-Tris/HCl, pH 7.0. The concentrations of reduced cytochrome *c* (O) and oxidized cytochrome *c* (Δ) were calculated from the absorbances in the two phases at 406 and 415 nm respectively. The partition coefficient is the ratio between the concentrations in the top and bottom phases. The total cytochrome *c* concentration was 2 µM in both cases. The curves represent the calculated partition coefficients for free plus complex-bound cytochrome for dissociation constants of 0.75 µM for oxidized cytochrome *c* and 1.8 µM for reduced cytochrome *c*. They were calculated from experimental values of the partition coefficients of free cytochrome *c* (0.78), free phosvitin (0.12) and the cytochrome *c*-phosvitin complex (0.084). The value for the complex was estimated from the partition coefficient of the cytochrome at saturating phosvitin concentrations. One phosvitin molecule (mol.wt. 40000) is assumed to bind 20 cytochrome molecules with equal affinity.

dissociation constants for oxidized and reduced cytochrome *c* can be estimated. The best fit of a theoretical curve to the results (Fig. 1) is obtained by assuming a dissociation constant of $0.75\mu\text{M}$ for oxidized and $1.8\mu\text{M}$ for reduced cytochrome *c*. In the calculation we used a value of 20 cytochrome *c* sites per phosvitin molecule (Taborsky, 1970). Yoshimura *et al.* (1979) provided evidence for different classes of binding sites during complex-formation between cytochrome *c* and phosvitin. We have not taken this extra factor into account in our estimates, which correspond to a decrease in the redox potential of 22 mV on binding to phosvitin. M. Erecińska [unpublished work, cited by Vanderkooi & Erecińska (1976)] reported a decrease of redox potential from 0.290 to 0.255 V on phosvitin binding without giving details of experimental conditions.

The strength of the complex between cytochrome *c* and phosvitin depends on the ionic strength of the medium (Fig. 2). Above a concentration of 0.1 M-KCl, the complex is essentially dissociated, in agreement with the conclusions of Taborsky (1970) from ultracentrifugation studies.

Reduction of free and complex-bound cytochrome *c* by ascorbate

Fig. 3 shows the effect of phosvitin on the rate of reduction of cytochrome *c* by ascorbate. Al-Ayash

& Wilson (1978) and Kihara *et al.* (1978) showed that the main active species in the reaction of ascorbate with cytochrome *c* at pH 7.4 was the ascorbate $^{2-}$ ion. In 10 mM-potassium phosphate, the rate of ascorbate reduction is decreased by increasing phosvitin concentrations until a limiting value is reached. The phosvitin concentration required to produce the maximum effect depends on the amount of cytochrome *c* present.

In 100 mM-potassium phosphate, there was no effect of phosvitin on the rate of reduction by ascorbate even at concentrations of the polyanion 100-fold higher than those producing an effect in the 10 mM-buffer. The rate of reduction of the free cytochrome was, however, less at the higher ionic strength, as expected for a reaction between molecules of opposite charge.

Fig. 4 shows a more detailed investigation of the effect of the ionic strength of the medium on the rate of reduction of cytochrome *c* by ascorbate in the presence and absence of phosvitin. In these experiments we used Tris buffer with the pH adjusted with Mes (4-morpholine-ethanesulphonic acid) and varied the ionic strength with KCl. There were no significant differences between this buffer and Tris/cacodylate buffer, which seems not to bind to cytochrome *c* (Barlow & Margoliash, 1966). At the lowest ionic strengths the kinetics of reduction showed a small slow phase as reported by Goldkorn & Schejter (1977) and the rate constant for the faster phase was used in this case.

Fig. 2. Effect of ionic strength on the partition of cytochrome *c* in the presence and absence of phosvitin. The two-phase system contained 4.4% (w/w) poly(ethylene glycol), 7.0% (w/w) dextran, 10 mM-Tris base adjusted to pH 7.4 with Mes (4-morpholine-ethanesulphonic acid), $8.3\mu\text{M}$ -cytochrome *c* and various concentrations of KCl. The partition coefficient is plotted as a function of the ionic strength for the free cytochrome (O) and for the cytochrome in the presence of $44\mu\text{g}$ of phosvitin/ml (●).

Fig. 3. Effect of phosvitin on the reduction of cytochrome *c* by ascorbate

Cytochrome *c* reduction was measured at 550 nm or 415 nm in potassium phosphate buffer, pH 7.4, containing 0.5% (v/v) Tween 20. The reaction was started by injecting ascorbate to a final concentration of 2 mM from a microsyringe into a rapidly stirred cuvette containing the other reagents. O, 10 mM-buffer/ $5\mu\text{M}$ -cytochrome *c*. □, 100 mM-buffer/ $5\mu\text{M}$ -cytochrome *c*. ●, 10 mM-buffer/ $0.5\mu\text{M}$ -cytochrome *c*. The points with arrows represent the rates of reduction of the cytochrome in the absence of phosvitin.

Fig. 4. Effect of ionic strength on the reaction between ascorbate and cytochrome *c* in the presence and absence of phosvitin

The reduction of cytochrome *c* (10 μ M) by ascorbate (0.5 mM) was monitored at 550 nm. The medium contained 10 mM-Tris adjusted to pH 7.4 with Mes and various concentrations of KCl. The dependence of the logarithm of the second-order rate constant on the square root of the ionic strength is given for free cytochrome *c* (O) and for the cytochrome in the presence of 67 μ g of phosvitin/ml (Δ).

A plot of the square root of ionic strength against the logarithm of the second-order rate constant should be linear if the limiting Debye-Hückel theory is valid (Atkins, 1978). This is clearly not the case in Fig. 4. Although linear relationships between rate constants and the square root of the ionic strength are sometimes observed for reactions of redox proteins with small molecules at relatively high ionic strengths, the limiting theory does not apply under these conditions, and, even if linear plots are obtained, the slopes do not appear to give correct values of the charge on the molecule (Goldkorn & Schejter, 1979).

In the presence of phosvitin a pronounced inhibition of the reaction with ascorbate is observed at ionic strengths of less than 0.1. The positive slope of the curve at low ionic strengths suggests that, under these conditions, both reactants have a negative charge.

Effect of phosvitin on the reaction of cytochrome *c* with other redox reagents

The reduction of cytochrome *c* by tetrachloro-

Table 1. Effect of phosvitin on the reaction of cytochrome *c* with redox reagents

Rate constants were determined from the slopes of plots of $k_{+1}^{obs.}$ against reactant concentration under pseudo-first-order conditions. The medium was 10 mM-potassium phosphate buffer, pH 7.4, containing 0.5% (v/v) Tween 20, except for the reactions with tris(phenanthroline)cobalt [Co(phen)₃]³⁺, when 20 mM-Tris/HCl, pH 7.4, was used. The cytochrome *c* concentration was 5 μ M, and its reduction or oxidation was measured at 550 nm. Phosvitin, when present, was at a saturating concentration of 100 or 167 μ g/ml. For *NNN'*-tetramethylphenylenediamine and the *N*-methylphenazonium ion (present as the methylsulphate) the reductants were maintained in the reduced state by the addition of 10 mM ascorbate, and the rate due to ascorbate alone was allowed for in calculation of the rate constants.

Reactant	Rate constant (M ⁻¹ · s ⁻¹)		Ratio
	+Phosvitin	Control	
Ascorbate	6.9	95	0.07
Tetrachlorobenzoquinol	1.2 × 10 ⁴	4.2 × 10 ⁵	0.03
Dithionite	—	—	0.2
<i>NNN'</i> -Tetramethyl-phenylenediamine	4.4 × 10 ⁴	2.6 × 10 ⁴	1.7
Reduced <i>N</i> -methylphenazonium ion	8.0 × 10 ⁵	6.0 × 10 ⁵	1.3
Fe(CN) ₆ ³⁻	—	—	<0.05
Co(phen) ₃ ³⁺	1.2 × 10 ⁵	440	270

benzoquinol was also inhibited by phosvitin. The results of Rich & Bendall (1979) suggest that the quinol monoanion QH⁻ is often the active species in the reactions of quinols with cytochrome *c*. The rate constant observed with tetrachlorobenzoquinol was much higher than the value of 45 M⁻¹ · s⁻¹ reported for benzoquinol under similar conditions (Castro *et al.*, 1977). This difference can probably be attributed to the lower pK of the tetrachlorobenzoquinol, resulting in a higher concentration of the active species.

In the case of the complex with phosvitin, plots of $k_{+1}^{obs.}$ against reductant concentration did not pass through the origin, and the amplitudes of the reaction declined as the concentration of reductant was lowered, suggesting that, when the redox potential of the cytochrome is lowered by complex-formation, an equilibrium position is reached in which not all of the cytochrome is reduced. The second-order rate constants given in Table 1 are calculated from the slopes of plots of $k_{+1}^{obs.}$ against reductant concentration.

Complex-formation with phosvitin was also found to inhibit the reduction of cytochrome *c* by dithionite and its oxidation by ferricyanide (Table 1). No investigation of the concentration-dependence of the reactions was made with these reagents, and only a

comparison of the relative rates is given. In the case of ferricyanide the oxidation of the free cytochrome at low ionic strength is so rapid that it was not possible to make an accurate measurement under pseudo-first-order conditions, and an estimate of the minimum value for the inhibition is given.

In contrast, the reduction of cytochrome *c* by *NNN'**N'*-tetramethylphenylenediamine or the reduced form of the *N*-methylphenazonium ion (present as the methylsulphate), both of which react as uncharged species, occurred at similar rates in the presence of phosvitin and in its absence (Table 1). Both these reductants were kept in the reduced state by the addition of an excess of ascorbate. The apparent rate constants for the reduction increased linearly with the mediator concentration over the range studied.

A third type of behaviour was observed for the oxidation of reduced cytochrome *c* by the tris(phenanthroline)cobalt(III) ion. The rate of reaction was greatly enhanced by complex-formation with phosvitin. At low ionic strengths the oxidation of both free and complex-bound cytochrome followed pseudo-first-order kinetics, but the graph of observed rate constant against oxidant concentration did not pass through the origin, although full oxidation of cytochrome *c* was observed even at the lowest oxidant concentration used. At higher ionic strengths the plot for the oxidation of free cytochrome passed through the origin and the calculated second-order rate constant was in excellent agreement with those of McArdle *et al.* (1974). The second-order rate constants for the reaction of free and phosvitin-bound cytochrome *c* at low ionic strengths were estimated from the slopes of the plots of oxidant concentration against k_{+1}^{obs} and are shown in Table 1.

Complex-formation between cytochrome *c* and other polyanions

Interaction between cytochrome *c* and phosvitin is unlikely to have any direct physiological significance and is presumably the result of the polyanionic character of the phosphoprotein. Dextran sulphate is a polysaccharide with polyanionic character resulting from covalently linked sulphate groups. The reduction of cytochrome *c* by ascorbate was inhibited by dextran sulphate at low ionic strength (Table 2), whereas reduction by *NNN'**N'*-tetramethylphenylenediamine was slightly stimulated. Oxidation by tris(phenanthroline)cobalt(III) was enhanced more than 200-fold in a buffer containing 10 mM-Tris base with the pH adjusted to 7.4 with Mes. The formation of turbid suspensions in the presence of the cobalt complex as the dextran sulphate concentration was increased prevented measurements at saturating polyanion concentrations.

Table 2. *Effect of polyanions on the rate of reaction of cytochrome c with ascorbate*

The reaction mixture contained 10 μ M-cytochrome *c* and 0.4 M-sodium ascorbate in 10 mM-Tris buffer adjusted to pH 7.4 by addition of Mes. The anions were added as sodium salts, except in the case of heparin, where the lithium salt was used.

Anion	Concentration	Rate constant $M^{-1} \cdot s^{-1}$
Control	—	200
ATP	1 mM	44
Inositol hexaphosphate	1 mM	21
Phosvitin	40 μ g/ml	6
Heparin	40 μ g/ml	1
Dextran sulphate	40 μ g/ml	0.3

The results with dextran sulphate were thus very similar to those with phosvitin, and it seems reasonable to suppose they are a general result of the binding of macromolecular polyanions to the cytochrome.

The effects of negatively charged species on the reactivity of cytochrome *c* is not confined to macromolecular polyanions. Table 2 shows that inositol hexaphosphate and adenosine triphosphate also decreased the rate of reduction of the cytochrome by ascorbate, though the quantitative effects were smaller than with dextran sulphate or heparin (another polysaccharide with polyanionic character resulting from glucuronic acid residues and covalently-linked sulphate groups).

For both ATP and inositol hexaphosphate the rate of the reaction with ascorbate was independent of ionic strength at KCl concentrations lower than about 0.1 M, suggesting the formation of a complex with an effective charge of zero under these conditions.

Inositol hexaphosphate and ATP were also observed to cause a severalfold increase in the rate of oxidation of cytochrome *c* by tris(phenanthroline)cobalt(III) under conditions similar to those used for the experiments described in Table 2 (results not shown).

Discussion

The interaction of cytochrome *c* with its natural reaction partners including the cytochrome *bc*₁ complex and cytochrome *c* oxidase involves the lysine residues surrounding the exposed 'edge' of the haem moiety (Weiss & Juchs, 1978; Reider & Bosshard, 1978).

This positively charged region is also likely to be the site of interaction of the macromolecular polyanions used here. The measurements of cytochrome *c* binding to phosvitin (Figs. 1 and 2) indicate that the interaction is electrostatic and that

oxidized cytochrome *c* binds with a higher affinity than does reduced cytochrome *c*. These properties of the phosvitin complex are similar to those of other cytochrome *c* complexes; electrostatic interaction and differential binding of the oxidized and reduced forms have been suggested from direct binding studies with the cytochrome *bc₁* complex (Weiss & Juchs, 1978), cytochrome *c* oxidase (Petersen, 1978) and mitochondrial membranes (Vanderkooi *et al.*, 1973), and also from observations of a decrease in the redox potential of cytochrome *c* on complex-formation with phosphate, liposomes, cytochrome *c* oxidase and peroxidase, and mitochondrial membranes [reviewed by Nicholls (1975)]. These properties are apparently a general effect of cytochrome *c* binding to negatively charged sites.

Cytochrome *c* complexed to polyanions differs markedly from the free cytochrome in its reactivity towards some, but not all, redox reagents. The rate of reaction with negatively charged reagents is greatly decreased, the reaction of uncharged reagents is little affected and is even slightly stimulated, whereas the reaction with the positively charged cobalt complex was strongly enhanced. The most obvious explanation of this is that the effects of polyanions result from their neutralizing the positive charges on the cytochrome *c* molecule and producing a complex that is more readily approached by positively charged species but repels a negatively charged one. A complex with a macromolecular polyanion will have an overall negative charge, and the ionic-strength-dependence of the ascorbate reduction of the cytochrome *c*-phosvitin complex is that expected for the reaction with a negatively charged cytochrome complex.

The general consensus of opinion appears to be that the 'haem-edge' area on the cytochrome *c* molecule, which is involved in the exchange of electrons with natural redox partners, is also the site of the reactions with small molecules (Dickerson & Timkovich, 1975; Vanderkooi & Erecinska, 1976; Ferguson-Miller *et al.*, 1979; Timkovich, 1979). It is noteworthy that the lack of inhibition by complex-formation of the reaction with uncharged reagents and, even more, the stimulation observed with the positively charged cobalt complex, suggest that any effects of steric hindrance at this site are small in comparison with the electrostatic effects resulting from a change in the sign of the overall charge on the complex.

A decreased reactivity with negatively charged reagents seems to be a common property of complexes of cytochrome *c*. A lower rate of reduction by ascorbate is found on complex-formation with phosphate, liposomes, submitochondrial particles and cytochrome *c* oxidase and peroxidase [reviewed by Nicholls (1975)]. A complex between oxidized cytochrome *c* and cytochrome *c* oxidase

with a decreased reactivity towards dithionite was observed during the reduction of 'oxygen-pulsed' oxidase (Petersen & Cox, 1980).

It is worthwhile to consider whether complex-formation between cytochrome *c* and its natural reaction partners might produce effects on the rates of reaction with uncharged and positively charged reagents similar to those we have observed with macromolecular polyanions. This possibility is related to the differences observed by several investigators of the cytochrome *c* oxidase reaction between the kinetics of oxidation of added reduced cytochrome *c* (the 'spectrophotometric assay') and the kinetics of oxygen uptake in the presence of the cytochrome, ascorbate and *NNN'**N'*-tetramethylphenylenediamine (the 'polarographic assay'). Margoliash *et al.* (1977) and Smith *et al.* (1979) have made a detailed study of these differences and have suggested that *NNN'**N'*-tetramethylphenylenediamine was able to react with cytochrome *c* while it was still bound to the oxidase. The results obtained here with phosvitin are compatible with this suggestion. Further comparisons of the properties of the complexes studied here with those between cytochrome *c* and its natural reaction partners (such as cytochrome *c* oxidase) might allow separation of the effects of binding to polyanions and those of binding to a specific site.

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