The oxidation-state-dependent ATP-binding site of cytochrome *c*

A possible physiological significance

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Cytochrome *c* binds certain physiological anions that are known to modulate the biological properties of the protein, although it is not known whether this effect is fortuitous or has physiological significance. We have examined the ability of the protein and its semisynthetic analogues to associate with certain of these anions, e.g. ATP, ADP, P_i and citrate. Our results show that specific residues or clusters of residues on the surface of horse heart cytochrome *c* are involved in the recognition sites for these anions. We also observed that binding at one site is linked to the oxidation state of the protein.

INTRODUCTION

Biological electron transport is assured in part by a class of proteins, the cytochromes, which act as electron carriers. Cytochrome *c* is somewhat atypical of the class in being small and highly water-soluble, but for these reasons it is by far the best characterized of this kind of protein (Meyer & Kamen, 1982). Its easy dissociation from the outer face of the mitochondrial inner membrane, where it interacts with respiratory complexes III and IV, and the observed excess of cytochrome *c* over the other components of the respiratory chain (Hackenbrock, 1981) suit it for its putative role as an interface between the membrane-bound electron-transport system and other cellular redox systems (Nicholls *et al.*, 1969; Matlib & O'Brien, 1976; Bernardi & Azzone, 1981), and suggest its candidature for a control point of the electron-transport system itself.

Studies employing gel filtration (Margalit & Schejter, 1973), electrophoresis (Margoliash *et al.*, 1970), ion-exchange chromatography (Brautigan *et al.*, 1978) and n.m.r. spectroscopy (Kayushin & Ajipa, 1973) have shown that cytochrome *c* binds, in a specific manner, certain anions. If this binding were to modulate the interaction of the protein with the other redox carriers, then there would exist the basis for a system whereby the relative concentrations of such anions could have a controlling influence on cellular redox processes.

As yet few data are available on where these small molecules bind on the protein's cationic surface, although this is an essential first step in understanding the physiological significance of the phenomenon. Wallace & Rose (1983) showed that a highly conserved cationic residue of the protein, Arg-91, could be grossly modified without apparent loss of activity in the mitochondrial electron-transport system. The observation poses the question of why this residue should be absolutely conserved in eukaryotic proteins: a possible answer is its participation in an important anion-binding site. We have studied [DMP-Orn⁹¹]cytochrome *c* and other semi-synthetically, or chemically, modified species. Our aim has been to locate such sites, and, ultimately, to understand how anion binding might influence protein function.

EXPERIMENTAL

Materials

Horse heart cytochrome *c* (type III) and 2,4,6-trinitrobenzenesulphonic acid (grade I) were purchased from Sigma Chemical Co., Munich, Germany. Methyl acetimidate was synthesized as described by Hunter & Ludwig (1962). *o*-Methylisourea sulphate (purum grade) was obtained from Serva Feinbiochemica, Heidelberg, Germany. Acetylacetone and citraconic anhydride were supplied by Fluka A.G., Buchs, Switzerland, and were of puriss grade. Methanesulphonyl ethyl succinimidocarbonate was kindly given by Dr. G. I. Tesser (Catholic University, Tuernooveld, Nijmegen, The Netherlands). Trypsin [Worthington type, L-1-chloro-4-phenyl-3-tosylamidobutan-2-one-('TPCK')-treated] was furnished by Cambrian Chemicals, Croydon, Surrey, U.K. Sulphopropyl-Trisacryl gel was bought from LKB, Stockholm, Sweden. ATP (grade II), ADP (grade III) and citrate were from Sigma Chemical Co., and were used as their sodium salts. Other solvents and reagents came from Merck, Darmstadt, Germany, and were of analytical grade.

Acetimidylation of cytochrome *c*

This was performed by method (iv) of Wallace & Harris (1984). The absence of side products was confirmed by ion exchange at neutral pH, as described by these authors, and complete formation of the derivative was checked on the same ion-exchange system with a pH 10.5 phosphate buffer. The difference in p*K* between ϵ -amino groups and ϵ -acetimidyl groups permits separation of incompletely modified forms.

Guanidination of cytochrome *c*

This was performed by the method of Kimmel (1967). Reaction was stopped at 48 h, since we found this time to be sufficient for complete modification. The product was checked by ion exchange as above.

Alkylation of cytochrome *c*

The (dimethylamino)₁₉- and (isopropylamino)₁₉-cytochromes *c* were prepared by a method similar to that of

Means & Feeney (1968) (C. J. A. Wallace & B. E. Corthésy, unpublished work).

Modified proteins were purified by cation-exchange chromatography on a sulphopropyl-Trisacryl column (1 cm $\times$ 10 cm) at pH 7.0. The derivative, dissolved in distilled water and applied in its oxidized form, was eluted by a linear gradient of 40–200 mM-potassium phosphate buffer.

Preparation of partially acetimidylated [Hse⁶⁵]cytochrome *c*

The acim-(1–65) – (66–104)[Hse⁶⁵]cytochrome *c* and (1–65)–acim-(66–104)[Hse⁶⁵]cytochrome *c* chimerae were prepared by the method of Wallace (1984).

Preparation of [Hse⁶⁵,DMP-Orn⁹¹]cytochrome *c*

Semisynthesis, purification and characterization of this analogue were performed by the method of Wallace & Rose (1983).

Preparation of cytochrome *c*-T (the stable and partially active complex of fragments 1–38 and 39–104)

Native and acetimidylated fragments 1–38 and 39–104 were prepared and purified, and their integrities checked, by the methods described by Proudfoot *et al.* (1984), with the exception of the citraconylated fragments, which were deprotected by the method of Dixon & Perham (1968).

Cytochrome *c*-T, acetimidylated cytochrome *c*-T and the two chimerae were prepared from these fragments as described by Wallace (1984).

Characterization of modified products

Spectrophotometric determinations of typical absorption bands or peaks, used as a proof of cytochrome *c* structure (Dickerson & Timkovich, 1976), were carried out with a Cary model 210 spectrophotometer. In each case, the derivative was dissolved in 50 mM-sodium phosphate buffer, pH 7.0, at a concentration of 0.1 mg/ml for measurements between 200 and 600 nm and at a concentration of 1 mg/ml between 600 and 750 nm. Spectra of the reduced forms were obtained by adding a crystal of ascorbic acid to solutions of the respective ferricytochromes *c*.

Biological activity of the analogues was measured polarographically (Harris & Offord, 1977) in the cytochrome *c*-depleted-mitochondria system of Jacobs & Sanadi (1960).

Redox potentials were determined by the method of mixtures of Davenport & Hill (1952).

Complete modification of lysine residues by reductive alkylation, guanidination and acetimidylation was confirmed by the trinitrobenzenesulphonic acid assay method of Fields (1971). For this purpose, derivatives obtained by freeze-drying from NH₄HCO₃ solutions must be extensively dialysed against water to ensure the complete removal of ammonia.

Analogues were also treated with trypsin, by using the method of Hennig (1975), since failure to cleave is another proof of complete blocking of lysine residues.

Purified [Hse⁶⁵,DMP-Orn⁹¹]cytochrome *c* was checked by a characteristic u.v.-absorption band at approx. 310 nm ($\epsilon = 5600 \text{ M}^{-1} \cdot \text{cm}^{-1}$) in acidic conditions (0.1 M-HCl) resulting from the incorporation of the aromatic group (Vetter-Diechtl *et al.*, 1968).

For amino acid analysis, protein hydrolysis was performed in 6 M-HCl containing 1% (w/v) phenol in evacuated sealed tubes at 108 °C for 24 h. The

hydrolysate was initially dissolved in 50 μ l of ethanol/water/triethylamine (7:1:1, by vol.) and freeze-dried. Phenylthiocarbamoyl derivatives of amino acids were prepared by adding to the hydrolysate (5 nmol) 20 μ l of the initial solvent made 10% (v/v) in phenyl isothiocyanate. After 10 min reaction at room temperature, the sample was dried under high vacuum, then redissolved in 0.2 M-sodium acetate buffer, pH 6.25, containing 10% (v/v) methanol and applied to a reverse-phase h.p.l.c. system with a stepwise acetonitrile gradient. We used a Brownlee RP-18 Spheri-5 column driven by a Waters Associates model 510 system.

Procedure for equilibrium gel-filtration experiments

The association between cytochrome *c* and its analogues and physiological anions, such as ATP, ADP, P_i and citrate, was studied by the gel-filtration method initially developed by Hummel & Dreyer (1962). Speed, convenience and economy of sample are some of the advantages offered by this method compared with equilibrium dialysis and ultracentrifugation (Wood & Cooper, 1970).

A Sephadex G-25 (fine grade) column (1.0 cm $\times$ 60 cm) was equilibrated at room temperature with 3.5 column volumes of a solution of the chosen ligand (1 mM) in 5 mM-Tris/cacodylate buffer, pH 6.95. A sample of cytochrome *c* or one of its derivatives (about 5 mg) was dissolved in 0.5 ml of the equilibration solution. After 15 min at room temperature, the protein solution was applied to the top of the Sephadex column and eluted with the equilibration solution.

In order to prevent artifact ion-exchange effects of the gel at low ionic strength, binding experiments were performed in 5 mM-Tris/cacodylate buffer, which does not itself bind to cytochrome *c* (Barlow & Margoliash, 1966), nor does it perturb measurements.

Protein concentrations were measured by absorption at 410 nm ($\epsilon = 106100 \text{ M}^{-1} \cdot \text{cm}^{-1}$); ATP and ADP were determined by their absorbance at 260 nm ($\epsilon = 15400 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at pH 7.0), P_i by the method of Taussky & Shorr (1953) and citrate by the colorimetric assay method of Saffran & Densted (1948).

Protein solutions were oxidized or reduced, as required, by addition of solid K₃[Fe(CN)₆] or Na₂S₂O₄, then desalted on a column of Sephadex G-25F in 50 mM-NH₄HCO₃ and immediately freeze-dried.

RESULTS AND DISCUSSION

Our results will only be interpretable if our protein analogues are pure, are well characterized, and have not been denatured during the preparation.

The major peak obtained by ion-exchange chromatography at pH 7.0 of the products of the reductive alkylations, or at pH 10.5 of the products of acetimidylation and guanidination of cytochrome *c*, contain the pure fully modified protein: we find that tryptic cleavage is restricted to the labile arginine residue (Harris & Offord, 1977); lysine is not found in amino acid analyses, though dimethyl-lysine, isopropyl-lysine, acetimidyl-lysine and homoarginine are present; and the products lack free ϵ -amino groups in the trinitrobenzenesulphonic acid assay method.

Sometimes peaks preceding that of 19-*N*- ϵ -acetimidylcytochrome *c* were observed on ion exchange at pH 10.5. These are likely to be less than fully modified materials:

Table 1. Biological and physicochemical properties of chemically modified and semisynthetic ferricytochromes *c*

	Biological activity relative to native cytochrome <i>c</i>	Redox potential (V)	Characteristic adsorption maxima (nm)
Native cytochrome <i>c</i>	100	0.260	410, 528, 695
[<i>N</i> ^ε - <i>N</i> ^ε -Dimethylamino] ₁₉ -cytochrome <i>c</i>	85–95	0.255	408, 528, 695
[<i>N</i> ^ε -Isopropylamino] ₁₉ -cytochrome <i>c</i>	100	0.256	407, 528, 695
[<i>N</i> ^ε -Guanidyl] ₁₉ -cytochrome <i>c</i>	80–85	0.248	406, 527, 695
[<i>N</i> ^ε -Acetimidyl] ₁₉ -cytochrome <i>c</i>	82–90	0.245	408, 528, 695
(1–65)-Acim-(66–104)-[Hse ⁶⁵]- cytochrome <i>c</i>	100	0.253	408, 528, 695
Acim-(1–65)-(66–104)-[Hse ⁶⁵]- cytochrome <i>c</i>	80–85	0.238	406, 527.5, 695
[Hse ⁶⁵ ,DMP-Orn ⁹¹]cytochrome <i>c</i>	100	0.255	305, 409, 528, 695
Cytochrome <i>c</i> -T	30	0.115	408, 528, 695
[<i>N</i> ^ε -Acetimidyl] ₁₉ -cytochrome <i>c</i> -T	20–30	0.120	407, 528, 695
(1–38)-Acim-(39–104)-cytochrome <i>c</i> -T	20–25	N.D.	407, 527, 695
Acim-(1–38)-(39–104)-cytochrome <i>c</i> -T	25–30	N.D.	407, 528, 695

radioactivity is covalently incorporated into them upon lysine-specific reductive alkylation with NaB³H₄ (C. J. A. Wallace, unpublished work). They are produced either when the methyl acetimidate employed is old, or if the pH of the prepared reagent solution was allowed to rise much above 11.0, where hydrolysis of methyl acetimidate is rapid. In either case the concentration of active reagent is lowered, and modification is incomplete as a consequence. Although warning of the dangers of low pH of the reagent solution, Wallace & Harris (1984) did not draw attention to these additional problems.

The chimeric [Hse⁶⁵] analogues (Wallace, 1984) were prepared from fragments derived from cytochrome *c* and acetimidyl-cytochrome *c* that were homogeneous on ion-exchange chromatography. The fragments themselves also proved to be homogeneous.

[Hse⁶⁵,DMP-Orn⁹¹]cytochrome *c*, when analysed for its amino acid composition, has lost an arginine residue but gained an ornithine residue. The increase in *A*₃₁₀ corresponds to an incorporation of one DMP group per protein molecule.

Table 1 presents physicochemical characteristics of derivatives compared with native cytochrome *c*. Three parameters were studied, all of which depend on the structurally sensitive haem environment: the spectrum in the u.v. and visible regions, ability to restore electron transport in cytochrome *c*-depleted mitochondria, and oxidation-reduction potentials. The absorption spectra depend on the side-chain groups packing the porphyrin ring and the way that they are stabilized by covalent and non-covalent binding in the polypeptide chain. The biological activity measures the reactivity between cytochrome *c* and its physiological substrates, cytochrome *c* reductase and cytochrome *c* oxidase: changes in electron-transfer rate could be a consequence of alteration of the binding-site conformation. A normal redox potential is a reflection of a haem group located in a low-dielectric hydrophobic environment (Kassner, 1972), constructed of a highly conserved set of side chains (Takano & Dickerson, 1981), and is similarly sensitive to the disruption of the tertiary structure.

All the analogues employed in the present study have values for these parameters that are very close to those of native cytochrome *c*, with the exception of the cytochromes *c*-T, which are deficient in electron-transfer

ability and exhibit a lower redox potential. However, the complex of complementary unprotected tryptic fragments in 50 mM-phosphate buffer or in 5 mM-Tris/cacodylate buffer exhibits an absorption band at 695 nm, whether it was prepared from 19-*N*-ε-citraconyl- or 19-*N*-ε-methanesulphonylethylloxycarbonyl-cytochrome *c*. This band is a sensitive signal of the ligation of the haem iron atom with the sulphur atom of Met-80 (Schechter & Saludjian, 1967). Partially or fully acetimidylated cytochromes *c*-T also show this typical band and behave identically in those buffers. The appearance of the band in complexes is indicative of a well-conserved three-dimensional structure, even at low ionic strength in cacodylate buffer. In consequence and in view of the results obtained by D. E. Harris, R. E. Offord, C. J. A. Wallace & A. E. I. Proudfoot (unpublished work), we believe that the cytochromes *c*-T may also give informative results on anion-binding properties.

Fig. 1(a) shows an elution profile typical of the chromatographic procedure devised by Hummel & Dreyer (1962). The leading peak, which emerges from the column at the elution volume of the protein, contains the ligand associated with protein and is followed by a trough, which appears at the elution volume of the ligand. It is apparent from Fig. 1(b) that the excess of ligand over the basal level in the peak is equal to the deficit in the trough. This criterion, as well as the observation of a short region between the peak and the trough in which the ligand concentration returns to its baseline value, is indicative of satisfactory performance of the column used.

Anion-binding capacities of cytochrome *c* and its analogues are presented in Table 2. ATP, ADP and P_i are bound by cytochrome *c* at the ligand concentration (1 mM) used for equilibration and running of the column. Osheroff *et al.* (1978) reported that 20 mM-citrate competes with P_i and must therefore be bound on the protein, but we did not observe significant binding at a citrate concentration of 1 mM.

There appear to be three binding sites for P_i, of which one is lost upon generalized lysine modification despite the charge retention in the analogues. The exception is the guanidinated protein, in which the capacity to bind P_i is increased. This may be a direct consequence of the introduction of 19 guanidyl groups, with a non-specific

Fig. 1. Equilibrium gel-filtration methods for the quantification of ion binding, as applied to cytochrome *c* and its analogues

(a) Elution profile of the passage of 5 mg of (1-65)-acim-(66-104) [Hse⁶⁵]ferricytochrome *c* through a Sephadex G-25 (fine grade) column. The column was equilibrated with 1 mM-ATP in 5 mM-Tris/cacodylate buffer, pH 6.95. Effluent was monitored at 280 nm. (b) Elution diagram obtained by analysis of individual fractions (1.5–1.7 ml) of effluent from the same run. Cytochrome *c* was determined by its absorbance at 410 nm, ATP by the decrease in absorbance at 260 nm of the trough, and the increase, after deproteinization, of the peak. —, Concentration of ATP; ----, concentration of cytochrome *c*.

Table 2. Anion-binding capacity of cytochrome *c* and its analogues

The degree of protein binding was calculated from the anion content of individual fractions after gel filtration at an anion concentration of 1 mM (see the Experimental section for further details). The differences in binding observed thus represent both differences in the numbers of sites and of their affinities. Abbreviations: N.D., not determined; *, not determined because of the precipitation of the protein during equilibration.

	Oxidation state	Anions bound (mol/mol of protein)			
		P _i	ATP	ADP	Citrate
Native cytochrome <i>c</i>	Fe ³⁺	2.58	2.47	1.85	0.0
	Fe ²⁺	2.51	1.64	1.78	0.1
[N ^ε -N ^ε -Dimethylamino] ₁₉ -cytochrome <i>c</i>	Fe ³⁺	1.70	2.04	1.84	N.D.
	Fe ²⁺	1.82	1.39	1.88	N.D.
[N ^ε -Isopropylamino] ₁₉ -cytochrome <i>c</i>	Fe ³⁺	1.66	1.95	1.91	N.D.
	Fe ²⁺	1.68	1.28	2.00	N.D.
[N ^ε -Guanidyl] ₁₉ -cytochrome <i>c</i>	Fe ³⁺	3.14	*	*	N.D.
	Fe ²⁺	3.41			N.D.
[N ^ε -Acetimidyl] ₁₉ -cytochrome <i>c</i>	Fe ³⁺	1.71	1.97	2.08	0.0
	Fe ²⁺	1.62	1.60	1.91	0.0
(1-65)-Acim-(66-104)-[Hse ⁶⁵]cytochrome <i>c</i>	Fe ³⁺	2.56	1.54	1.60	N.D.
	Fe ²⁺	2.44	1.51	1.71	N.D.
Acim-(1-65)-(66-104)-[Hse ⁶⁵]cytochrome <i>c</i>	Fe ³⁺	1.71	1.84	1.89	N.D.
	Fe ²⁺	1.65	1.59	1.88	N.D.
[Hse ⁶⁵ ,DMP-Orn ⁹¹]cytochrome <i>c</i>	Fe ³⁺	1.50	1.59	1.59	N.D.
	Fe ²⁺	1.42	1.51	1.63	N.D.
Cytochrome <i>c</i> -T	Fe ³⁺	1.93	1.91		N.D.
	Fe ²⁺	1.96	1.55	N.D.	N.D.
[N ^ε -Acetimidyl] ₁₉ -cytochrome <i>c</i> -T	Fe ³⁺	1.68	1.89		N.D.
	Fe ²⁺	1.50	1.44	N.D.	N.D.
(1-38)-Acim-(39-104)-cytochrome <i>c</i> -T	Fe ³⁺	1.99	1.95		N.D.
	Fe ²⁺	1.73	1.62	N.D.	N.D.
Acim-(1-38)-(39-104)-cytochrome <i>c</i> -T	Fe ³⁺	1.39	1.91		N.D.
	Fe ²⁺	1.50	1.54	N.D.	N.D.

but weak affinity for P_i . Such an effect would also explain the precipitation of this analogue in solutions containing nucleotides. The results with the partially acetimidylated analogues unambiguously place the site in the 1–65 region. The affinity for P_i is generally decreased in the non-covalent complex, but is further diminished by acetimidylation. The results with the hybrid complexes imply that the lost site is in the 1–38 segment.

The consequences of modification of Arg-91 imply that this residue constitutes, or is part of, a P_i -binding site. These results are in general agreement with n.m.r. studies (Stellwagen & Schulman, 1973) and chemical-modification experiments (Ferguson-Miller *et al.*, 1978), which suggested two sites of P_i binding, one in the proximity of His-26 and Lys-27, and one close to Lys-87.

ADP is apparently bound at two sites, and its binding is insensitive to lysine modifications; in contrast, affinity for ATP is decreased by such modifications. One observation that differentiates the binding of ATP from that of other anions is the difference between oxidation states; ferricytochrome *c* binds one more ATP molecule than does ferrocyclochrome *c*. This differentiation is retained in the lysine-modified forms, and in cytochrome *c*-T and derivatives, but is abolished by the conversion of Arg-91 into DMP-Orn-91, thus implicating this residue as a constituent of the oxidation-state-dependent ATP-binding site. The failure of this site to recognize ADP implies that it is to the terminal phosphate moiety that Arg-91 binds.

It is known that the anions tested can alter the affinity of cytochrome *c* for its oxidase. Ferguson-Miller and co-workers have suggested that this is a result of binding to oxidase (Ferguson-Miller *et al.*, 1976), or, in the case of P_i , to cytochrome *c* itself (Ferguson-Miller *et al.*, 1978). We propose that the effect is not simply fortuitous, but that the association of cytochrome *c* and its substrates is regulated by ion binding to cytochrome *c*.

The binding of ATP at the Arg-91-containing site is likely to be the most physiologically significant. Wallace & Rose (1983) noted that gross modification of this residue, despite its invariance among eukaryotic cytochromes *c*, did not alter electron-transfer ability. They therefore proposed an indirect functional role for the residue. The oxidation-state-dependent association of ATP is unique among the anions studied. Binding occurs at the perimeter of the domain of interaction with redox partners, and the presence of the nucleoside moiety could have substantial effects on the protein. Finally, such an association represents a feedback between the consumers of metabolic energy and the system producing it.

If ATP, or indeed any other anion, is acting as an allosteric effector, how might it function? Neutralization of one or more of the positive charges that constitute the binding site for other proteins (Capaldi *et al.*, 1982) will diminish affinity, and anion binding anywhere on the highly cationic surface of cytochrome *c* will alter the dipole moment of the protein, which is proposed to influence reactivity by steering the approach of cytochrome *c* to substrates (Koppenol & Margoliash, 1982).

The presence of a nucleoside moiety could affect contacts between proteins directly, by steric effects on the interaction domain, or indirectly, by inducing conformational change in cytochrome *c*. Redox potential and thus relative reactivity can be modulated by either the electrostatic potential at, or the hydrophobic shielding of, the redox centre. Nucleotide binding will alter both

parameters, and could be influential on others suggested to have a role in interaction between respiratory-chain components, flexibility of cytochrome *c* and desolvation of the colliding surfaces (Dethmers *et al.*, 1979).

Whatever the precise mechanism involved, effects on relative affinities of cytochrome *c* with other redox proteins could have a wider role than simple regulation of oxidative phosphorylation rate. By such means, the metabolic state of the cell could govern the availability of cytochrome *c* to subsidiary redox systems in its role as electron shuttle (Matlib & O'Brien, 1976; Bernardi & Azzone, 1981).

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