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

Implication of an essential arginine residue and the effect of occupancy on the oxidation-reduction potential

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Arg-91 is not part of the active site of cytochrome *c* that mediates binding and electron transfer, yet it is absolutely conserved in eukaryotic cytochromes *c*, indicating a special function. The physicochemical properties of analogues are unaffected by the modification of this residue, so they can be used with confidence to study the role of Arg-91. We have established limiting conditions under which this residue alone is specifically modified by cyclohexane-1,2-dione, and have subsequently shown that ATP, and to a lesser extent ADP or P_i, protects it from the action of the reagent in an oxidation-state-dependent manner. These observations strongly support the idea that this site exerts a controlling influence on cytochrome *c* activity in the electron transport or other cellular redox systems, and we have commenced a study of how that influence might operate. We find that the redox potentials of both cytochrome *c* and analogue are little affected by changing ATP or P_i concentrations.

INTRODUCTION

Respiratory cytochrome *c* from mitochondria is probably the most widely investigated of all electron carriers. Its ease of isolation and purification, its water-solubility, its stability to denaturing conditions and its easy renaturation, together with its relative abundance, have made it an ideal candidate for the examination of the electron-transfer processes of the mitochondrial inner membrane (Dickerson & Timkovitch, 1975).

Structural differences between reduced and oxidized forms of cytochrome *c* have been recognized for some time (Margoliash & Schejter, 1966), the oxidized protein having a more open and flexible conformation than the reduced form (Takano & Dickerson, 1981*a*,*b*). These observations could account for the differences in affinity between oxidation states for cytochrome *c* oxidase (Petersen, 1978; Bill & Azzi, 1984).

We have previously observed that the anion-binding properties of horse heart cytochrome *c* are also dependent on the oxidation state of the protein (Corthésy & Wallace, 1986). By means of chemically modified, chimeric and semisynthetic derivatives, we have demonstrated that specific surface residues are involved in anion recognition by cytochrome *c*. One of them appears to be directly implicated in the oxidation-state-linked modulation of ion binding. The present study reports that the bound anions are able to shield this residue, Arg-91, specifically from a guanidino-group-directed reagent, thus providing direct evidence that this residue, conserved throughout the evolution of mitochondrial cytochrome *c*, participates in the variable affinity site for ATP.

The chemical modification of proteins is a useful, and often specific, way of studying the function of individual amino acid residues in the sequence, particularly when that residue has heightened sensitivity (Lundblad & Noyes, 1984). Much has been learnt about the way in which the guanidino group is employed in proteins by the use of α-dicarbonyl reagents for the modification of arginine residues (Riordan, 1979).

Although several chemical agents are available, we chose cyclohexane-1,2-dione (CHD), a reagent introduced in mild and non-denaturing conditions (Patthy & Smith, 1975*a*,*b*), and which appears suitable for studies in conditions of temperature and pH around physiological values. The adduct formed with arginine, N^7N^8-(1,2-dihydroxycyclohex-1,2-ene)diyl-L-arginine (DHCH-arginine), is stabilized in borate buffer above neutral pH (range 8.0–10.0), or alternatively in acidic conditions (up to 30% acetic acid) even in the absence of borate.

Cytochrome *c* from horse contains only two arginine residues. Arg-38, however, exhibits greatly diminished reactivity, so that we were able to compare the reaction of CHD with Arg-91 alone in the presence and in the absence of physiological anions. Residue 38 is essential to the structural integrity, and hence the functioning, of cytochrome *c* (Wallace & Rose, 1983). Its inertness is probably due to its position and role in the structure of the bottom Ω-loop of the protein (Proudfoot *et al.*, 1986; Proudfoot & Wallace, 1987; Wallace, 1987).

This specificity of reactivity has also been exploited for the preparation of quantities of [DHCH-Arg^{91}]-cytochrome *c* sufficient to permit a study of the influence of the concentration of P_i or ATP in the medium on the redox potentials of modified and unmodified proteins.

EXPERIMENTAL

Materials

Horse heart cytochrome *c* (type III), pepsin, 1-methyl-L-histidine, ATP (grade II) and ADP (grade III) were purchased from Sigma Chemical Co. (Munich,

Abbreviations used: CHD, cyclohexane-1,2-dione; DHCH-arginine, N^7N^8-(1,2-dihydroxycyclohex-1,2-ene)diyl-L-arginine.

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Germany). Cyclohexane-1,2-dione (puriss grade) and *N*-ethylmorpholine (for sequence analysis) were supplied by Fluka A.G., Buchs, Switzerland. Sulphopropyl-Trisacryl gel was bought from LKB Produkter (Bromma, Sweden) and boronate affinity gel (Matrex PBA-30) from Amicon Corp. (Danvers, MA, U.S.A.). Other solvents and reagents came from Merck (Darmstadt, Germany) and were of analytical grade.

Modification of cytochrome *c* with CHD

Boric acid (0.496 g) was dissolved in distilled water, and the pH was adjusted to 8.0 with 2 M-NaOH solution; the final volume was 20 ml (0.4 M). A 0.1 M solution of CHD (0.224 g) in this buffer was prepared, and the pH was immediately re-adjusted to 8.0 with 2 M-NaOH. A 12.4 mg portion of cytochrome *c* was dissolved in 0.5 ml of distilled water, then an equal volume of buffered CHD solution was added and the mixture left for 30 min at 37 °C. The reaction was stopped with 1 ml of 30% (v/v) acetic acid. Dialysis (18/32 Visking tubing boiled in 1% $NaHCO_3$ for 15 min) against three changes of cold 1% acetic acid removed excess reagent, and the product was freeze-dried.

The addition of buffered CHD solution to the protein results in its rapid reduction. For studies with ferricytochrome *c*, 1 mM-$K_3Fe(CN)_6$ was included to maintain the oxidized state. We checked that the oxidant did not interfere with the reaction of CHD and cytochrome *c*, nor competed with the anions under study for the same binding site, causing an artifactual shielding effect. In experiments with ferrocytochrome *c*, the protein was reduced with $Na_2S_2O_4$ before reaction. In tests of the shielding effect of physiological anions, ATP, ADP and P_i were added as their sodium salts to the protein solution and the pH was adjusted before CHD addition.

U.v.–visible spectra were recorded on a Cary model 210 spectrophotometer.

Modification of cytochrome *c* with CHD under the conditions described above was quantified by separation of modified and unmodified protein, either by cation-exchange chromatography or by affinity chromatography, under conditions stabilizing the DHCH adduct.

Purification and characterization of modified cytochrome *c*

The DHCH group forms, in neutral and mild alkaline conditions, a stable complex with borate ion; the net charge on the protein is decreased by 1, so that modified and unmodified species are separable by cation-exchange chromatography: we used an SP-Trisacryl column (1 cm × 10 cm), and a linear gradient of KCl, pH 8.0, containing 0.1 M-borate, made from 60 ml of 0.1 M and 60 ml of 0.4 M solutions. Fractions corresponding to each peak were pooled, desalted on a Sephadex G-25 (fine grade) column in 1% acetic acid and finally freeze-dried.

In order to avoid the undesirable presence of material denatured by freeze-drying from acidic solutions (Boon *et al.*, 1978), we treated each freeze-dried product with phosphate/urea buffer followed by gel filtration according to the method of Boon *et al.* (1979). We adjusted the buffer to pH 8.0 and included 0.1 M-borate to stabilize the DHCH adduct.

Alternatively modified and unmodified forms can be separated on Matrex boronate affinity gel (Wallace & Rose, 1983).

In order to prove that the early peak from cation-exchange separations, or the bound fraction from the affinity column, is the modified protein, native and CHD-treated cytochrome *c* were digested with trypsin and with pepsin. The digests were compared by analytical paper electrophoresis with the use of specific staining methods for guanidino groups and CHD adducts. Amino acid compositions were determined for modified or arginine-containing peptides recovered from preparative electrophoreses. Tryptic digestion was performed by using the method of Proudfoot *et al.* (1986). For peptic hydrolysis 3 mg of protein was dissolved in 100 μl of 1% acetic acid (resulting pH 2.6), then 20 μl of pepsin

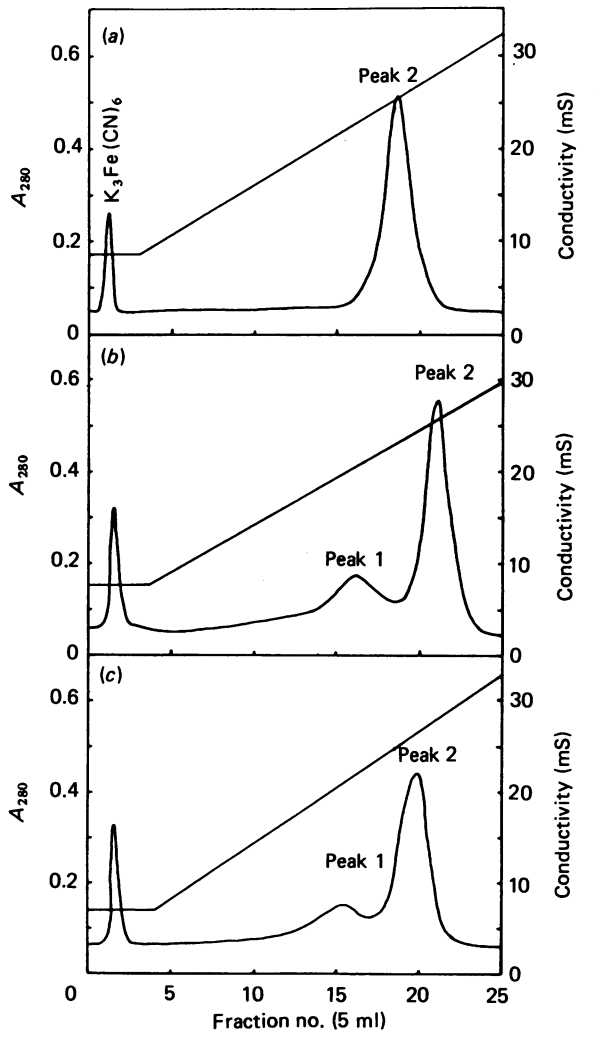

Fig. 1. Ion-exchange chromatography of cytochrome *c* and derivatives

The chromatography conditions are given in the Experimental section. The straight line indicates the elution gradient. (*a*) Native cytochrome *c*. (*b*) CHD-treated cytochrome *c*. (*c*) Cytochrome *c* treated with CHD in the presence of $K_3Fe(CN)_6$. The product appearing in the void volume of the column is $K_3Fe(CN)_6$ added to oxidize the protein quantitatively and ensure reproducible chromatographic behaviour.

solution (15 mg in 100 μl of 1% acetic acid) was added to each sample. After incubation overnight at 37 °C, digestion was stopped by freezing the samples in an ethanol/solid CO_2 bath, then drying. They were redissolved in 1% acetic acid before paper electrophoresis.

DHCH-arginine, prepared by the method of Patthy & Smith (1975*a*,*b*), was subjected to the conditions of the enzymic digestions described above. The modified amino acid and 5 μl portions of peptic and tryptic digests were electrophoresed on Whatman no. 1 paper at pH 1.9 (water/acetic acid/formic acid, 45:4:1, by vol.) for 25 min at 100 V/cm. The sheets were cut and separately stained with cadmium/ninhydrin reagent, with the Sakaguchi reagent, which is specific for free guanidino groups, and with, in sequence, 0.5 M-hydroxylamine/HCl, pH 7.0, and aq. 0.5 M-$NiCl_2$ (Patthy & Smith, 1975*a*,*b*) for the DHCH-arginine adduct.

Arginine- and DHCH-arginine-containing peptides from native cytochrome *c* and derivatives were prepared by high-voltage paper electrophoresis on Whatman 3MM paper at pH 1.9 for 30 min at 75 V/cm. Such peptides were located by staining two tracks cut from the paper, either with hydroxylamine/$NiCl_2$ or Sakaguchi reagent. The appropriate areas were then cut out of the bulk of the sheet, rolled up and placed in a pipette tip in a glass tube. The paper was wetted with about 500 μl of 1% acetic acid, then, after 5 min at room temperature, the liquid was eluted from the tip by centrifugation at 1000 *g* for 5 min and freeze-dried, then repurified by h.p.l.c.

Determination of oxidation–reduction potentials

In order to study the variation of redox potential with the concentration of physiologically important anions in the medium, cytochrome *c* and [DHCH-Arg^{91}]-cytochrome *c* were dissolved at about 1 mg/ml in 2 mM-borate/5 mM-cacodylate buffer, adjusted to pH 8.2 with Tris base. To 900 μl of this solution were added 100 μl of 100 mM-$K_4Fe(CN)_6$ and 1 μl of 100 mM-$K_3Fe(CN)_6$, thus setting the redox potential of the buffer at 310 mV (Wallace *et al.*, 1986). At this potential cytochrome *c* (E_m = 260 mV in 0.05 M-phosphate buffer, pH 7.0) is partly reduced and the height of the characteristic band at 550 nm indicates the proportion of the reduced form. Small volumes of 2 M-ATP (sodium salt) or P_i (sodium salt) pH 8.2, were added and A_{550} was measured after each addition. After the addition of 100 μl of ligand solution, hence a final concentration of 200 mM of the anion being studied, the solution was titrated with ferricyanide to determine midpoint redox potential by the method of Wallace *et al.* (1986). The variation of A_{550} was then used to calculate the change in redox potential during the anion titration.

RESULTS AND DISCUSSION

When cytochrome *c* was treated with CHD, ion-exchange chromatography showed a peak (peak 1) of modified material preceding the normal position of elution of the native protein (Fig. 1). The low yield

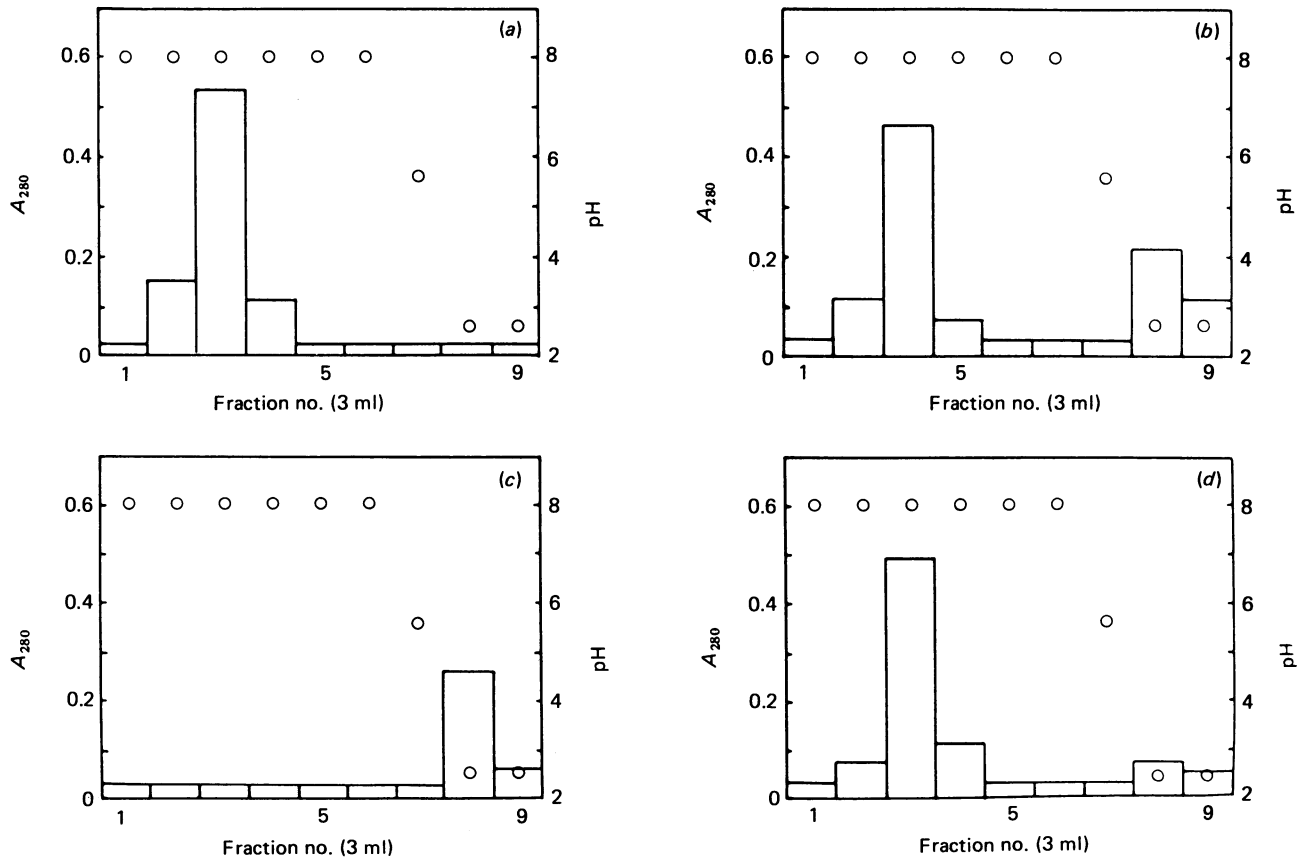

Fig. 2. Chromatographic behaviour of cytochrome *c* and CHD-modified material on boronate gel

The ○ symbols indicate pH. (*a*) Native cytochrome *c*. (*b*) CHD-treated cytochrome *c*. (*c*) Ion-exchange-purified CHD-modified cytochrome *c* (peak 1). (*d*) CHD-treated ferricytochrome *c* shielded by 50 mM-ATP.

observed (approx. 15%) is probably due to the low molar excess of reagent that we have employed (CHD/protein ratio 50:1), and to steric hindrance, for the addition of larger amount of CHD or the use of urea at a final concentration of 8 M (Rose *et al.*, 1981) increased arginine modification in the polypeptide (B. E. Corthésy, unpublished work).

Superimposable elution profiles resulted from cation-exchange separation of cytochrome *c* that has been treated with CHD in the presence and in the absence of $K_3Fe(CN)_6$. This oxidant does not inhibit reaction.

The borate column separated two species, one bound, the other not (Fig. 2). The bound species, thus containing a *cis*-diol moiety, was released on decreasing the eluent pH. This material was eluted at the position of peak 1 when run on the ion-exchange system described above. Conversely, peak 1 material is completely retained by the Matrex PBA-30 resin, so there can be little doubt that the bound fraction and peak 1 are identical, and contain DHCH-arginine residue(s).

Thus CHD-modified protein may be conveniently resolved from unmodified cytochrome *c* either by affinity chromatography on boronate gel or by cation-exchange separation under conditions that stabilize the adduct.

Like the Arg-91-modified analogues described by Wallace & Rose (1983), this product has a fully developed 695 nm band indicative of an unmodified haem environment, and has values for biological activity with cytochrome *c* reductase and for redox potential very close to those of the native protein, when measured under standard conditions (Wallace *et al.*, 1986; Wallace & Proudfoot, 1987).

Patthy & Smith (1975*a*,*b*) and Austen & Smith (1976) reported that tryptic digestion of CHD-modified proteins in 0.1 M-borate buffer, pH 8.0, or in 0.05 M-phosphate buffer, pH 6.8, occurred without loss of DHCH-arginine, and was limited to peptide bonds *C*-terminal to lysine residues. We were unable to reproduce their results for cytochrome *c*, and trial experiments showed that DHCH-arginine was stable neither in 0.1 M-borate buffer, pH 8.0, nor in 0.05 M-phosphate buffer, pH 6.8, under

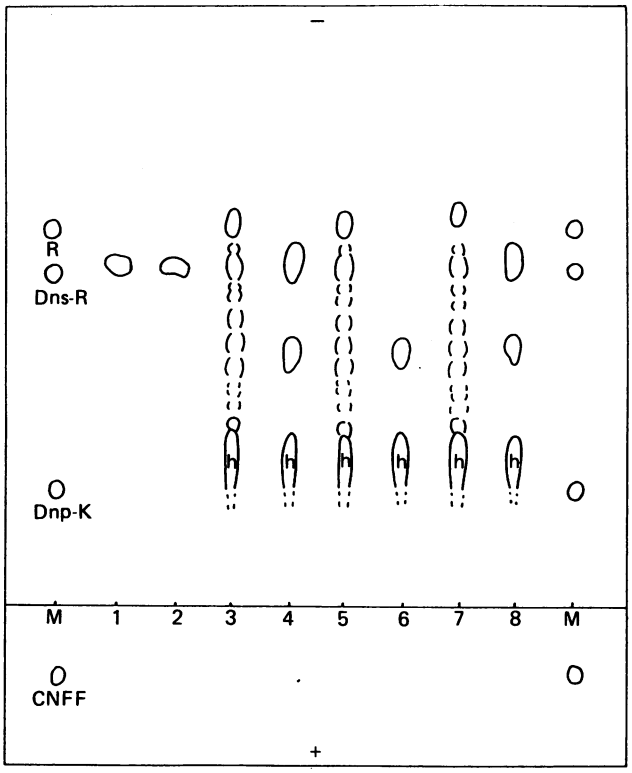

Fig. 3. Paper electrophoresis of cytochrome *c* and [DHCH-Arg^{91}]cytochrome *c* at pH 1.9

Lane 1, DHCH-arginine, before exposure to 1% acetic acid. Lane 2, DHCH-arginine, after exposure to 1% acetic acid for 12 h; no free arginine is recovered; spots appear after staining with ninhydrin/Cd^{2+}. Lanes 3 and 4, peptic digest of cytochrome *c* stained with ninhydrin/Cd^{2+} (3) and Sakaguchi reagent (4). Lanes 5–8, peptic digest of peaks 1 (5 and 6) and 2 (7 and 8) obtained with ninhydrin/Cd^{2+} (5 and 7) and Sakaguchi reagent (6 and 8). Abbreviations: M, marker mixture, CNFF: Cyanol FF; Dnp-K, dinitrophenyl-lysine; Dns-R, dansylarginine; h, haem peptide.

Table 1. Amino acid analyses of arginine-containing peptic peptides of native and CHD-modified cytochrome *c*

Peptides were obtained by elution from paper electrophoretograms of peptic digests made as described in the Experimental section: a general consequence of this method is elevated glycine contents. The sequences of peptides 37–46 and 83–93 of native horse cytochrome *c* are GRKTGQAPGF and AGIKKKTERED respectively (Dickerson & Timkovitch, 1975). Proline is not detected by the *o*-phthalaldehyde method. Abbreviations: PBA, Matrex PBA-30 resin; N.O., not observed.

		Amino acid composition (mol of residue/mol)							
	Cytochrome *c* ...	Native		CHD-treated ion-exchange peak 1		CHD-treated ion-exchange peak 2		CHD-treated PBA bound	
Amino acid	Peptide ...	37–46	83–93	37–46	83–93	37–46	83–93	37–46	83–93
Asp			1.85 (2)		1.58 (2)		1.80 (2)		1.55 (2)
Thr		0.82 (1)		0.90 (1)		0.96 (1)		0.86 (1)	
Glu		1.18 (1)	2.23 (2)	0.98 (1)	1.98 (2)	1.20 (1)	2.24 (2)	1.09 (1)	1.94 (2)
Gly		4.03 (3)	2.31 (1)	3.71 (3)	1.50 (1)	3.62 (3)	1.36 (1)	3.45 (3)	1.69 (1)
Ala		1.00 (1)	0.90 (1)	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)	1.00 (1)
Ile			1.00 (1)		0.98 (1)		0.98 (1)		0.92 (1)
Phe		0.95 (1)		0.92 (1)		0.89 (1)		0.91 (1)	
Lys		0.98 (1)	2.83 (3)	1.00 (1)	2.68 (3)	1.17 (1)	2.93 (3)	1.02 (1)	2.51 (3)
Arg		1.00 (1)	1.03 (1)	0.98 (1)	N.O.	0.88 (1)	1.00 (1)	1.00 (1)	N.O.

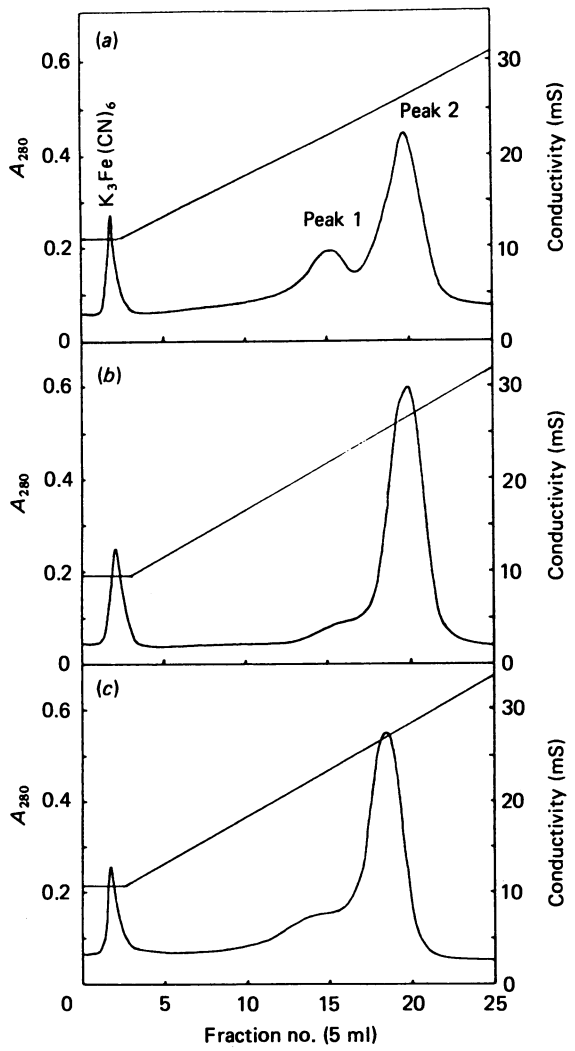

Fig. 4. Shielding by anions of cytochrome *c* from CHD attack

(*a*) Ferrocytochrome *c* treated with CHD in the presence of 20 mM-ATP. (*b*) Ferricytochrome *c* treated with CHD in the presence of 20 mM-ATP; note the absence of peak 1. (*c*) Partial shielding of ferricytochrome *c* against CHD by 20 mM-phosphate.

the conditions of time and temperature given by these authors.

Since the use of trypsin was precluded, we turned to peptic digestion in 1% acetic acid. In these acidic conditions (pH 2.6), DHCH-arginine was perfectly stable, even after exposure for 12 h (Fig. 3, lanes 1 and 2), and the enzymic cleavage, monitored by time-course paper electrophoresis, went to completion in this period (Fig. 3, lanes 3 and 4), even though the chosen pH was a little higher than in standard digestion conditions found in the literature (pH 1.9–2.0; Allen, 1981).

Peptic digests showed that modification occurred specifically at Arg-91. Peaks 1 and 2 of the CHD-treated protein were digested and the digests were compared after paper electrophoresis and staining (Fig. 3, lanes 5–8). When stained with the Sakaguchi reagent, specific for arginine-containing peptides, the digest of untreated cytochrome *c* showed two orange spots (lane 4). Peak 2 exhibited the same pattern (lane 8). In the digest of peak 1, however, only one of these two peptides is revealed: the other is no longer arginine-positive. From the electrophoretic mobilities, by using the method of Offord (1977) we can calculate that the quantitatively modified peptide in peak 1 is that containing Arg-91, whereas that which remains arginine-positive contains Arg-38. At high loadings (80–100 nmol), paper electrophoretograms can be stained specifically for DHCH-containing peptides by using 0.5 M-hydroxylamine/HCl solution at pH 7.0 and aq. 0.5 M-$NiCl_2$ (Patthy & Smith, 1975*a*,*b*), forming a pink Ni^{2+}–dioxime complex. One such peptide was observed in the digest of modified material. The peptic peptides containing arginine residues, 37–46 and 83–93, were eluted from paper electrophoretograms and analysed for amino acid composition. The results are shown in Table 1 and demonstrate conclusively that residue 91, but not residue 38, is modified in anion-exchange fraction I or in boronate-gel-bound material, but not in ion-exchange fraction II.

Thus reaction of CHD with cytochrome *c* under mild conditions results in the partial modification of Arg-91, and no other residues. The guanidino group of Arg-38 is probably protected from attack by ion-pairing to the inner haem propionate group (Takano & Dickerson, 1981*a*,*b*; Moore *et al.*, 1984; Proudfoot *et al.*, 1986; Proudfoot & Wallace, 1987).

ATP, ADP and P_i specifically protect ferricytochrome *c*, but not ferrocytochrome *c*, against the action of CHD on Arg-91. ADP at 20 mM partially shields the oxidized protein to the same degree as P_i. However, significantly greater protection is provided by 50 mM-P_i than by 50 mM-ADP, and ATP at either 20 or 50 mM affords an almost complete shielding of Arg-91 (Figs. 2 and 4). Of the three physiological anions tested, ATP has a distinctly more potent inhibitory effect on reaction between cytochrome *c* and CHD.

Protection by adenine nucleotide and P_i of Arg-91 in the oxidized protein from CHD modification provides direct evidence for the involvement of that residue in an oxidation-state-dependent nucleotide-binding site. This strongly supports the conclusions drawn from studies of semisynthetically prepared Arg-91-modified cytochromes *c* (Wallace & Rose, 1983; Corthésy & Wallace, 1986). The observation that both this residue and the binding phenomenon (*Saccharomyces* cytochrome *c* shows the same binding characteristics for ATP as the horse protein; B. E. Corthésy, unpublished work) are invariant throughout eukaryote evolution suggests that the binding is not fortuitous, but functional in the ways proposed by Corthésy & Wallace (1986).

The difference in ability to protect manifested by the anions tested (ATP > P_i > ADP) strongly suggest that it is the terminal phosphate group of ATP that binds to Arg-91. Because of the shape and the charge distribution of the guanidinium side chain, it is able to make strong and specific bonds with phosphate or carboxylate groups (Salunke & Vijayan, 1981). It has been proposed that arginine residues, which occur relatively infrequently in protein sequences, are employed specifically where such a binding role is demanded (Riordan, 1979). In the case of horse cytochrome *c*, the two arginine residues seem to have such a role, Arg-38 binding to the carboxylate group of the haem propionate (Takano & Dickerson, 1981*a*,*b*; Moore *et al.*, 1984; Proudfoot *et al.*, 1986; Proudfoot & Wallace, 1987) and Arg-91 to the terminal phosphate group of an ATP molecule bound specifically in the oxidized state.

The binding, albeit weaker, of ADP implies that the

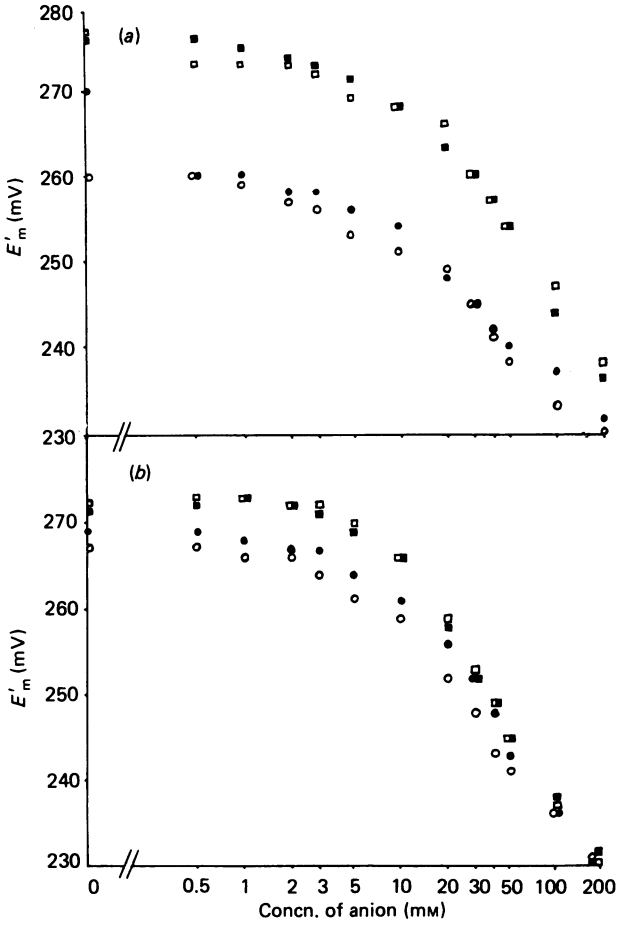

Fig. 5. Variation of redox potential with anion concentration for native and CHD-modified cytochrome *c*

(*a*) ATP. (*b*) P_i. ● and ○, [DHCH-Arg^{91}]cytochrome *c*; □ and ■, native protein.

nucleotide element is also recognized by the protein: indeed, our results obtained with a semisynthetic analogue modified at Glu-66 (Wallace & Corthésy, 1986) show that ATP is considerably less strongly bound than in the native protein and suggests this role for a well-conserved but mysterious feature of the general cytochrome *c* structure (Dickerson, 1972). The helix 62–70 has a glutamate residue at the outside of each turn, forming a stripe of negative charge in a generally very cationic surface. Such dispositions of acidic residues are generally employed in hydrogen-bonding with the hydroxy groups of the ribose moiety of nucleotides (Rossman *et al.*, 1975).

We thus propose that there exists on the surface of ferricytochrome *c* a highly structured binding site with a strong affinity for ATP, but which can also bind its components, ADP and P_i. In ferrocytochrome *c* this site is lost, presumably as a result of the small conformation differences that exist between the two oxidation states (Petersen, 1978; Takano & Dickerson, 1981*a*,*b*). A very precise association between ATP and the site is also implied by the insensitivity of ADP and P_i binding to a change in oxidation state (Corthésy & Wallace, 1986).

This specificity of association, the proximity of the binding site to the active site of cytochrome *c*, the absolute conservation of Arg-91 and the known influence on electron-transfer rates of the presence of some of the anions we have examined (Ferguson-Miller *et al.*, 1976, 1978) have led us to propose that such binding might play a regulatory role in the interaction of cytochrome *c* with other components of mitochondrial redox enzyme systems.

We have therefore looked at one of the ways in which such a system could act, through a direct effect on the redox potential of the protein. It is known that modifications of surface charge can affect potential (Rees, 1980; Wallace & Corthésy, 1986) and that there is a direct relationship between redox potential and electron-transfer rate between the reductase and cytochrome *c* (Wallace & Proudfoot, 1987). The results of determination of potential at different P_i and ATP concentrations for cytochrome *c* and [DHCH-Arg^{91}]-cytochrome *c* are summarized in Fig. 5. For cytochrome *c* with a concentration of ATP (1 mM) sufficient for nearly full occupancy of the oxidation-state-dependent binding site (Corthésy & Wallace, 1986) redox potential is unchanged from that in the absence of nucleotide. Most significantly, the behaviour of the analogue is essentially identical. Only at high concentrations of ATP and P_i do the potentials decline in parallel, presumably as a consequence of the change in ionic strength of the buffer. We conclude, therefore, that any regulatory effect of ATP on the protein does not act through modifying oxidation–reduction potential, and that effects on binding to physiological partners or on the thermodynamic barrier to electron transfer should be examined. It has been shown that the presence of these anions does affect the rate of cytochrome *c* oxidation by the oxidase, though it has been assumed that the effect of the anion is entirely at the level of the oxidase (Kadenbach, 1986).

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REFERENCES

Allen, G. (1981) in Laboratory Techniques in Biochemistry and Molecular Biology, vol. 9 (Work, T. S. & Burdon, R. H., eds.), pp. 59–60, North-Holland, Amsterdam

Austen, B. M. & Smith, E. L. (1976) J. Biol. Chem. **251**, 5835–5837

Bill, K. & Azzi, A. (1984) Biochem. Biophys. Res. Commun. **120**, 124–130

Boon, P. J., Tesser, G. I. & Nivard, R. J. F. (1978) in Semisynthetic Peptides and Proteins (Offord, R. E. & Di Bello, C., eds.), pp. 115–126, Academic Press, London

Boon, P. J., Tesser, G. I. & Nivard, R. J. F. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 61–65

Corthésy, B. E. & Wallace, C. J. A. (1986) Biochem. J. **236**, 359–364

Dickerson, R. E. (1972) Sci. Am. **226**, 58–72

Dickerson, R. E. & Timkovich, R. (1975) Enzymes 3rd Ed. **11**, 397–547

Ferguson-Miller, S., Brautigan, D. L. & Margoliash, E. (1976) J. Biol. Chem. **251**, 1104–1115

Ferguson-Miller, S., Brautigan, D. L. & Margoliash, E. (1978) J. Biol. Chem. **253**, 149–159

Kadenbach, B. (1986) J. Bioenerg. Biomembr. **18**, 39–54
Lundblad, R. L. & Noyes, C. M. (1984) Chemical Reagents for Protein Modification, vol. 2, pp. 1–45, CRC Press, Boca Raton
Margoliash, E. & Schejter, A. (1966) Adv. Protein Chem. **21**, 113–286
Moore, G. R., Harris, D. E., Leitch, F. A. & Pettigrew, G. W. (1984) Biochim. Biophys. Acta **764**, 331–342
Offord, R. E. (1977) Methods Enzymol. **47**, 51–69
Patthy, L. & Smith, E. L. (1975*a*) J. Biol. Chem. **250**, 557–564
Patthy, L. & Smith, E. L. (1975*b*) J. Biol. Chem. **250**, 565–569
Petersen, L. C. (1978) FEBS Lett. **94**, 105–108
Proudfoot, A. E. I. & Wallace, C. J. A. (1987) Biochem. J. **248**, 965–967
Proudfoot, A. E. I., Wallace, C. J. A., Harris, D. E. & Offord, R. E. (1986) Biochem. J. **239**, 333–337
Rees, D. C. (1980) J. Mol. Biol. **141**, 323–326
Riordan, J. F. (1979) Mol. Cell. Biochem. **26**, 71–92
Rose, K., Priddle, J. D. & Offord, R. E. (1981) J. Chromatogr. **210**, 301–309
Rossman, M. G., Liljas, A., Brändén, C.-I. & Banaszak, L. J. (1975) Enzymes 3rd Ed. **11**, 61–102
Salunke, D. M. & Vijayan, M. (1981) Int. J. Pept. Protein Res. **18**, 348–351
Takano, T. & Dickerson, R. E. (1981*a*) J. Mol. Biol. **153**, 79–94
Takano, T. & Dickerson, R. E. (1981*b*) J. Mol. Biol. **153**, 95–115
Wallace, C. J. A. (1987) J. Biol. Chem. **262**, 16767–16770
Wallace, C. J. A. & Corthésy, B. E. (1986) Protein Eng. **1**, 23–27
Wallace, C. J. A. & Proudfoot, A. E. I. (1987) Biochem. J. **245**, 773–779
Wallace, C. J. A. & Rose, K. (1983) Biochem. J. **215**, 651–658
Wallace, C. J. A., Corradin, G., Marchiori, F. & Borin, G. (1986) Biopolymers **25**, 2121–2132

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