The specificity and K_d at physiological ionic strength of an ATP-binding site on cytochrome *c* suit it to a regulatory role

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Cytochrome *c* binds ATP with marked specificity at a site that contains the evolutionarily invariant residue Arg-91. The binding of ATP to this site was studied using equilibrium gel filtration, equilibrium dialysis and affinity chromatography. At physiological ionic strength the affinity is such that the major change in occupancy coincides with the normal cellular ATP concentration range, and the degree of saturation is proportional to the ratio of [ATP]/[ADP]. The specificity of binding at this site is more a function of the degree of phosphorylation of the nucleotide, than of the nature of the nucleoside moiety. Thus under physiological conditions the degree of occupancy of this site is proportional to the energy state of the cell, providing a means for the regulation of the respiratory chain which is sensitive to cytoplasmic ATP levels.

INTRODUCTION

The primary functional role of mitochondrial cytochrome *c* is to shuttle electrons between respiratory complexes III and IV at the outer surface of the inner mitochondrial membrane. The absolutely conserved amino acid residue Arg-91 of cytochrome *c* can be modified with no apparent loss of the protein's ability to restore oxygen consumption in cytochrome *c*-depleted mitochondria (Wallace & Rose, 1983). A dogma of protein structure-function studies is that residues that have remained invariant throughout evolution must play essential roles in the functioning of the protein. We suggested that this residue must therefore be involved in some previously unrecognized function of cytochrome *c*.

Studies involving gel filtration (Margalit & Schejter, 1973; Corthésy & Wallace, 1986), electrophoresis (Margoliash *et al.*, 1970), ion-exchange chromatography (Brautigan *et al.*, 1978), n.m.r. spectroscopy (Kayushin & Ajipa, 1973; Stellwagen & Schulman, 1973; Andersson *et al.*, 1979; Feng & Englander, 1990) and chemical modification (Taborsky & McCollum, 1979; Corthésy & Wallace, 1988) have shown that cytochrome *c* is capable of specific binding of certain anions, particularly phosphate. Corthésy & Wallace (1986) found that oxidized and reduced horse cytochrome *c* bind an average of 2.47 and 1.64 molecules of ATP per molecule of protein respectively at an ATP concentration of 1 mM in 5 mM-Tris/cacodylate buffer. When the residue Arg-91 was modified to 2,2'-dimethylpyrimidylornithine, the protein was found to bind 1.59 molecules of ATP when in its oxidized form and 1.51 when in the reduced form. These data were interpreted as indicating a specific redox-state-dependent binding site that is nearly saturated under these conditions, and two non-specific sites of lower affinity. We concluded that this high-affinity redox-state-dependent site incorporates Arg-91 and hypothesized that this capacity to bind ATP defines the role for which this residue has been conserved. The most obvious role for an ATP-binding site on the protein surface is a regulatory one, and we suggested that kinetic control of electron-transport rate might result from feedback inhibition by ATP of cytochrome *c* activity (Corthésy & Wallace, 1986).

The present study was undertaken with two objectives in mind. Firstly, to examine the physiological relevance of this binding. Strong and specific binding could be demonstrated under low

ionic strength conditions (Corthésy & Wallace, 1986), but unless it could be shown to occur under conditions equivalent to those of the intermembrane space, and with an affinity within the physiological concentration range, no case for a feedback control mechanism can be made. We have therefore studied binding at physiological ionic strength, and used equilibrium dialysis to calculate the K_d under these conditions.

If indeed ATP is acting as an allosteric effector of cytochrome *c*, then it would be of considerable interest to learn the mechanism by which binding effects a loss of electron-transfer efficiency. Our second objective was therefore to determine which elements of the nucleotide structure were crucial to binding. This knowledge would help to identify those elements of protein structure that constituted the binding site, which in turn might suggest a means by which activity could be regulated.

EXPERIMENTAL

Materials

Horse heart (type III) and tuna heart cytochrome *c*, cyclohexane-1,2-dione (CHD), disodium pyrophosphate (PP₂) and pentasodium tripolyphosphate (PPP₅) were obtained from Sigma. Nucleotides were purchased from Boehringer Mannheim. Column chromatography resins were supplied by Pharmacia LKB. Citric acid, NaHCO₃, KH₂PO₄ and KCl were obtained from Fisher Scientific. Sodium ferricyanide and NaOH were purchased from Baker. Boric acid, acetic acid, NaCl, MgCl₂, Na₂SO₄, H₂SO₄ and MgSO₄ were from BDH. CaCl₂ and urea were purchased from Anachemia and Na₂S₂O₄ from Merck.

Methods

Equilibrium gel filtration. The proposal of Corthésy & Wallace (1986) that there are two equivalent weak and one strong ATP-binding site on cytochrome *c* under low ionic-strength conditions was tested by the generation of an ATP-binding curve. The binding of ATP to cytochrome *c* was studied using equilibrium gel filtration by the method initially developed by Hummel & Dreyer (1962). This method has the advantage of requiring only a small amount of protein per run, but is not capable of accurately quantifying weak associations. This problem can be overcome by increasing the protein loading, although there remain limitations, namely the availability of protein and the

Abbreviation used: CHD, cyclohexane-1,2-dione.

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inability of high loadings to flow properly down the column without loss of resolution.

A 1.0×60 cm column of Sephadex G-25 (fine grade) was equilibrated with 3.5 column vol. of a given concentration of ATP dissolved in 10 mM-Tris/cacodylate (pH 6.95). Tris/cacodylate does not bind to cytochrome *c* (Barlow & Margoliash, 1966). In order to prevent bacterial contamination and resulting ATP hydrolysis, fresh buffer was prepared for each experiment. A sample of cytochrome *c* (1–5 mg) was dissolved in 0.5 ml of the equilibration buffer and loaded on to the column. The protein was then eluted with the same buffer. Five successive runs were performed per equilibration. Fractions of volume 1 ml were collected and the concentration of ATP and cytochrome *c* were measured by their absorbances at 260 nm ($\epsilon = 15400 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at pH 7) and 410 nm ($\epsilon = 106100 \text{ M}^{-1} \cdot \text{cm}^{-1}$) respectively. An experimentally determined A_{260}/A_{410} ratio of 0.199 for cytochrome *c* was used in the calculation to determine the degree of binding. The data were analysed by the method of Scatchard (1949).

Equilibrium dialysis. Equilibrium dialysis was performed to determine the ability of horse cytochrome *c* to bind ATP at high ionic strength. Solutions of ATP ranging in concentration from 0.5 mM to 8 mM were made in Ringer's physiological buffer (120 mM-NaCl, 4.7 mM-KCl, 2.5 mM- CaCl_2 , 1.2 mM- KH_2PO_4 , 1.2 mM- Na_2SO_4 , 25 mM- NaHCO_3) and were adjusted to pH 6.95. Ferricytochrome *c* (200 μl ; 25–100 mg/ml) dissolved in the buffer was dialysed against 50 ml of buffer for 5 h while being constantly stirred. ATP and cytochrome *c* concentrations inside and outside the dialysis sac were determined spectrophotometrically at 260 nm ($\epsilon = 15400 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at pH 7) and 410 nm ($\epsilon = 106100 \text{ M}^{-1} \cdot \text{cm}^{-1}$) respectively.

The Ringer's buffer was altered in additional experiments to determine the effects of different ions on binding. A proportion of the NaCl was replaced with MgCl_2 to determine the effects of Mg^{2+} at a variety of concentrations. A binding curve was also produced where the CaCl_2 and the NaCl in the buffer were replaced with KCl.

For experiments with ferrocycytochrome *c*, the protein was reduced with $\text{Na}_2\text{S}_2\text{O}_4$ and dialysed to remove the excess reducing agent. The protein was removed from the dialysis sac, the concentration adjusted and the protein placed into new dialysis sacs in the Ringer's ATP solution. After dialysis to equilibrium the protein was reoxidized by reducing the pH to below 2 where cytochrome *c* is in its state II form which autoxidizes. After 10 min the pH was brought back to neutrality and the ATP and cytochrome *c* concentrations were determined. The method was also used to study the binding of ATP and ADP to ferricytochrome *c* in solutions containing a constant total nucleotide concentration of 4 mM with varying [ATP]/[ADP] ratios.

Modification of cytochrome *c* with CHD. Horse cytochrome *c* was modified with CHD to yield N^7N^8 -(1,2-dihydroxycyclohex-1,2-ene)diyl-L-arginine-91 cytochrome *c* (DHCH-Arg-91 cytochrome *c*) as described by Corthésy & Wallace (1988). Cytochrome *c* (1 mM) was incubated at 37 °C in 0.2 M-borate (pH 8) with a 50-fold molar excess of CHD for 30 min. The reaction was stopped by acidification with an equal volume of 30% (v/v) acetic acid and the sample was dialysed against three changes of cold 1% (v/v) acetic acid and freeze-dried. The protein was dissolved in 8 M-urea containing 0.1 M-borate (pH 8) and buffer exchanged into 0.1 M-borate (pH 8) containing 0.1 M-KCl to allow any denatured material to refold into its native conformation. The sample was subjected to ion-exchange chromatography on Trisacryl SP using 120 ml of 0.1 M-borate (pH 8) containing 0.1–0.4 M-KCl.

Affinity chromatography. Cytochrome *c* binds strongly to ATP immobilized on agarose. One can thus compare the relative

affinities of other nucleotides and anions by their capacity to elute the protein from an agarose-hexane ATP affinity column. Ligand (5 mM) in 30 mM-phosphate buffer (pH 6.95) was used to elute horse ferricytochrome *c* from the column. The relative affinities of the ligands for cytochrome *c* were measured as retention times. The nucleotide concentrations were determined spectrophotometrically using their molar absorption coefficients ($\epsilon_{260 \text{ nm}} = 15400 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for ATP, dATP and ADP at pH 7; $\epsilon_{248 \text{ nm}} = 12200 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for ITP at pH 6; $\epsilon_{271 \text{ nm}} = 9000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for CTP at pH 7; $\epsilon_{262 \text{ nm}} = 10000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for UTP at pH 7; $\epsilon_{253 \text{ nm}} = 13700 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for GTP at pH 7). In addition the retention times of protein eluted with solutions of 15 mM-ATP, -ADP, -PPP_i and -PP_i at pH 6.95 were determined in the absence of additional phosphate.

The retention times of native ferricytochrome *c* and DHCH-Arg-91 ferricytochrome *c* on the ATP affinity column were compared in the presence of 30 mM-phosphate (pH 7).

In order to determine the effects of pH on binding, the retention times of tuna ferricytochrome *c* on the ATP affinity column were measured in solutions of 30 mM- H_2SO_4 adjusted with NaOH to a range of pH values.

RESULTS AND DISCUSSION

Affinity of cytochrome *c* for ATP

Cytochrome *c* contains at least three phosphate-binding sites although only two have been as yet well characterized. One of these sites is in the immediate vicinity of Thr-89 and Lys-87 and the other is near Lys-25-His-26-Lys-27 (Stellwagen & Schulman, 1973; Brautigan *et al.*, 1978; Ferguson-Miller *et al.*, 1978; Osheroff *et al.*, 1978; Taborsky & McCollum, 1979). ATP and ADP have been found to bind at both these sites (Taborsky & McCollum, 1979; Stellwagen & Schulman, 1973). A pronounced difference between the two redox states with respect to anion binding has been observed (Margoliash *et al.*, 1970; Margalit & Schejter, 1973; Corthésy & Wallace, 1986, 1988). A third phosphate-binding site may be at the haem crevice near Lys-13 (Andersson *et al.*, 1979; Arean *et al.*, 1988).

We propose that the Arg-91-containing ATP-binding site is the same site as that which binds phosphate near Lys-87 because of the close proximity of these residues and since reporter groups for this site, Met-65 and Thr-89, are close to the guanido group of this residue (Arean *et al.*, 1988). This proposal is further supported by the finding that modification of Arg-91 eliminates the high-affinity phosphate binding to horse cytochrome *c* (Corthésy & Wallace, 1986).

From the data of Corthésy & Wallace (1986) the dissociation constants for the binding of ATP to cytochrome *c* under low ionic-strength conditions can be calculated if one assumes that DMP-Orn-91 ferrocycytochrome *c* does not bind any ATP at the Arg-91-containing site (in fact this analogue binds 0.96 mol of ATP less than native cytochrome *c*) and the two remaining distal sites are unaffected by this modification and are of equal affinity. Under these conditions these constants are found to be 3.3×10^{-4} M for the two weaker sites and 4×10^{-5} M for the Arg-91-containing site of the oxidized form of the protein. Data determined by equilibrium gel filtration for mol of ATP bound per mol of cytochrome *c* over a wide range of concentrations were plotted by the method of Scatchard (1949) in Fig. 1. The fit to theoretical curves generated from the above values is quite good, although the nature of the experimental system does not permit the determination of values at lower ATP concentrations than 0.1 mM. Our observations with the native protein are thus in agreement with the proposal of two equivalent weaker binding sites and one high-affinity site developed from the comparison of native and modified cytochromes.

Fig. 1. Scatchard plot of the binding of ATP to ferricytochrome *c* under low ionic-strength conditions

A Scatchard plot of the binding of ATP to ferricytochrome *c* in 10 mM-Tris-cacodylate is shown. The solid lines represent the theoretical curves for the binding of ATP to two weaker identical sites with a K_d of 0.33 mM and one stronger site with a K_d of 4×10^{-2} mM.

Fig. 2. Binding of ATP to cytochrome *c* under physiological ionic-strength conditions

The relationship between the concentrations of ATP and its degree of binding to ferricytochrome *c* (●) and ferrocyanochrome *c* (○) in Ringer's solution is shown.

Analogous binding curves for the interaction of ATP and the reduced form of the protein were not completed because it was found that the protein undergoes partial autoxidation on the gel-filtration column. This oxidation occurred at room temperature within 45 min in the absence of any metal ions other than Na^+ . Reduced cytochrome *c* does not normally autoxidize at neutral pH (Dickerson & Timkovitch, 1975), so that this result suggests a change in protein structure induced by the binding of ATP.

Because of the danger of irrelevance to the physiological reality, further experiments at low ionic strength were not pursued. The choice of an alternative buffer is not a simple one, since, because of general intracellular compartmentalization, the small volume of the intermembrane space and the highly polar surfaces of the closely opposed membranes confining it, it is clearly impossible to know the precise ionic composition of the milieu in which cytochrome *c* normally operates. We do have a better idea of the ionic strength, and for both these reasons we chose to employ Ringer's physiological buffer, as well as for its general acceptance in related experimental systems.

The expected decreased affinity for ATP under these conditions meant that such high protein loadings would be needed that equilibrium gel-filtration experiments are made unreliable by poor flow characteristics. This necessitated the use of equilibrium

dialysis in which a large protein loading could be used. Cytochrome *c* concentration was determined at the end of the experiment so that any change in this value due to the flow of water into the sac was avoided. The degree of saturation of the binding site was found to be independent of the protein concentration over the range employed.

Using equilibrium dialysis, ATP binding was seen to increase from zero to 1 mol/mol of cytochrome *c* over the concentration range 0.5–8 mM under physiological ionic-strength conditions (Fig. 2). Dissociation constants of 3.3 and 4 mM can be calculated for the oxidized and reduced forms of the protein. Intuitively, one would expect that the single binding site apparent at high ionic strength corresponds to the site of high affinity seen under hypo-osmotic conditions. Cytochrome *c* can be specifically modified at Arg-91 alone with CHD as described in the Experimental section (Corthésy & Wallace, 1988). In the presence of 30 mM- P_i (pH 7) this analogue was found to have a retention time of 7.5 min on an ATP affinity column whereas the native protein is retained 46 min. The marked difference in retention times confirms that this residue is part of an ATP-binding site and the rapidity with which the modified protein passes through the column indicates that it is the Arg-91-containing site that is operative while the two other sites have nearly negligible affinity for column-bound ATP under the increased ionic-strength conditions. This view is further supported by the observation that the covalent attachment to cytochrome *c* of the affinity label 8- N_3 ATP, which is abolished by the prior modification of Arg-91, occurs exclusively at a single site at both high and low ionic strengths at low reagent concentration (D. B. Craig & C. J. A. Wallace, unpublished work).

Low concentrations of Mg^{2+} have no effect on the dissociation constant. At stoichiometric concentrations of Mg^{2+} to ATP a modest decrease in site occupancy occurs, whereas 30 mM- Mg^{2+} causes a substantial decrease in affinity. Thus the Mg^{2+} concentration of the intermembrane space could act as a modulator of ATP binding. The site occupancy was not affected by the replacement of Ca^{2+} and the majority of the Na^+ in the buffer with K^+ .

Calculations based on the binding data of Corthésy & Wallace (1986) indicate that there is a difference of two orders of magnitude between the dissociation constants of this site in oxidized and reduced cytochrome *c* under the low ionic-strength conditions they employed. Little difference in the K_d of the reduced form of the protein is observed when the low-ionic-strength buffer is replaced with Ringer's solution. However, when the oxidized form is used, the K_d increases with the increase in ionic strength to a value similar to that of the reduced form (Table 1). Using n.m.r. spectroscopy, salt-dependent conformational changes have been found to occur, especially in the oxidized form of the protein, in the vicinity of residues 83–89 (Feng & Englander, 1990). In 5 mM-phosphate buffer, ferri-cytochrome *c* has a larger radius of gyration and maximum linear dimension than ferrocyanochrome and this difference is suppressed by the addition of 200 mM-NaCl (Trehwella *et al.*, 1988). The convergence of the affinities of the two redox states with an increase in ionic strength is likely to be due to the adoption of more similar conformations.

In the cell, a decrease in the concentration of ATP is accompanied by an increase in the concentration of ADP. If the binding of ATP is a physiologically significant signal its binding must be distinguishable from that of ADP. Thus nucleotide binding to ferricytochrome *c* was determined under conditions in which the $[\text{ATP}]/[\text{ADP}]$ ratio was varied but the total nucleotide concentration kept constant. It was found that total nucleotide binding was dependent on the $[\text{ATP}]/[\text{ADP}]$ ratio, showing an increase in binding after an initial plateau (Fig. 3). Although

Table 1. Dissociation constants of the binding of ATP to the Arg-91-containing site

The dissociation constants for the binding of ATP to the Arg-91-containing binding site of ferricytochrome *c* and ferrocytochrome *c* are given for the low and physiological ionic-strength conditions.

	K_d (mM)	
	5 mM-Tris/cacodylate	Ringer's solution
Ferricytochrome <i>c</i>	0.04	3.3
Ferrocyclochrome <i>c</i>	7	4.0

Fig. 3. Binding of nucleotide to ferricytochrome *c* in solutions containing a variable [ATP]/[ADP] ratio

The binding of nucleotide to ferricytochrome *c* is shown when the total nucleotide concentration is constant at 4 mM and the [ATP]/[ADP] ratio is variable.

ADP is capable of binding to ferricytochrome *c* under these conditions, its dissociation constant is between two and three times greater than that of ATP.

The concentration of ATP in cells varies from species to species and between different tissues within a single species. The cellular ATP concentration in rat tissue has been found to be 2.6, 3.4 and 8.1 mM in brain, liver and muscle respectively. Human erythrocytes and *Escherichia coli* have been found to have cellular ATP concentrations of 3.4 and 7.9 mM respectively (Lehninger, 1982). The equilibrium-dialysis data show that the steepest part of the binding curve of ATP to cytochrome *c* corresponds to the cellular concentration levels and that the amount of binding is proportionate to the [ATP]/[ADP] ratio. Hence, occupancy of this site will be very responsive to the energy state of the cell. As phosphate potential increases the degree of occupancy increases, and if this could be shown to modulate the electron-transfer properties of the protein, a functional feedback inhibition system would exist. We have observed that if this site is occupied by affinity labelling with an ATP analogue, then the activity of the cytochrome in a reductase assay is decreased to 10–20% of the normal value (D. B. Craig & C. J. A. Wallace, unpublished work).

Characteristics of the binding site

An examination of the ability of different nucleotides and other anions to bind to cytochrome *c* would be expected to give insight into the structural requirements for binding at this site. However, the large amounts of nucleotide required for equilibrium-dialysis experiments made it necessary to use a more economical method. Affinity chromatography is the method of choice. The ease with which different nucleotides elute cyto-

Fig. 4. Ability of different ligands to elute ferricytochrome *c* from an ATP affinity column

The time required for citrate (Δ), PP_i ($\blacksquare$), ADP ($\blacktriangle$), PPP_i ($\square$), NTPs ($\circ$) and CTP ($\diamond$) to elute ferricytochrome *c* from an ATP affinity column are given (a). The data for NTPs ($\bullet$) and CTP ($\blacklozenge$) were repeated on a second column of the same resin. The data for the NTPs [ATP ($\bullet$), dATP ($\circ$), ITP ($\blacklozenge$), GTP ($\diamond$) and UTP ($\blacktriangle$)] are shown in more detail for the first column (b) and the second column (c).

chrome *c* from an ATP affinity column is a measure of their affinity for cytochrome *c*. A shorter retention time indicates a higher affinity. Although this method does not allow quantification of the binding, it allows comparison of the affinity of different ligands and requires the use of only small amounts of materials.

In the presence of 30 mM-phosphate buffer, pH 6.95, the NTPs, with the exception of CTP, eluted cytochrome *c* from an ATP-affinity column with retention times that did not differ significantly from one another (Fig. 4). The inability of cytochrome *c* to distinguish between ATP, dATP, ITP, GTP and UTP indicates that the nucleoside moiety of the NTPs is probably not a determinant of the specificity of binding, although the stronger affinity for CTP is intriguing.

The retention time with ADP was greater than those for the NTPs. The difference between ATP and ADP clearly indicates the importance of the additional phosphate group. In fact, total charge is of considerable significance, as is illustrated by the values for the pyrophosphate and triphosphate series. The similarity in affinities of PPP_i^{5-} and ATP^{4-} and of PP_i^{4-} and ADP^{3-} suggests that the nucleoside element is equivalent to one additional charge to the strength of binding. Retention times determined with buffers containing ATP, ADP, PPP_i and PP_i at

Table 2. Retention time of ferricytochrome *c* on an ATP affinity column

The retention time of ferricytochrome *c* with elution by solutions of 15 mM-ATP, -ADP, -PPP_i and -PP_i (pH 6.95) on an ATP affinity column are shown.

Ligand	Retention time (min)
ATP	1.5
ADP	> 20
PPP _i	1
PP _i	14

Fig. 5. Retention time of ferricytochrome *c* on an ATP affinity column versus pH

The effect of pH on the ability of immobilized ATP to retain ferricytochrome *c* in the presence of 30 mM-SO₄²⁻ is shown.

a concentration of 15 mM in the absence of additional phosphate were in agreement with these data (Table 2). Although cytochrome *c* appears to be capable of binding other NTPs with equal or stronger affinity, one would expect that at normal cellular concentrations the binding of these nucleotides by cytochrome *c* would not be significant in relation to that of ATP.

Osheroff *et al.* (1978) found that ATP has an inhibitory effect on the association of cytochrome *c* and cytochrome *c* oxidase which was presumably due to specific binding to one of the two proteins or their complex. They note that several NTPs have identical inhibitory effects on the association of cytochrome *c* and cytochrome *c* oxidase and proposed that the triphosphate moiety rather than the nitrogenous base is responsible for the inhibition. Using n.m.r. spectroscopy Kayushin & Ajipa (1973) found that the nucleoside moiety as well as the phosphates of ATP interact with cytochrome *c*. Our data indicate that the presence of a triphosphate tail is the prime determinant of the high-affinity binding, but that some contribution is made by the nucleoside moiety.

Wallace & Corthésy (1986) showed that Glu-66 is important in the stabilization of the active conformation of cytochrome *c* and that its replacement by lysine diminished the affinity of the Arg-91-containing ATP-binding site. We suggested that in analogy to other nucleotide-binding sites it might form hydrogen bonds with a ribosyl hydroxy group of ATP. The inability of the affinity-column experiments to differentiate between ATP and 2-deoxy ATP indicates, however, that, if such an interaction is occurring, it does not involve the hydroxy group in the 2' position.

The importance of the negative charge on the triphosphate moiety of ATP for binding can be further assessed by determining the effect of pH on the retention time of cytochrome *c* on an ATP

affinity column. The competing anion was 30 mM-sulphate, chosen because it can effectively displace immobilized ATP from the binding site and because its ionization state does not change in the pH range used. We also found it necessary to avoid cations with pK values in the pH range used, therefore the titration was performed with NaOH.

Horse cytochrome *c* has a histidine residue at position 33 with a pK of about 6.5 which will titrate concurrently with the titration of the terminal phosphate of ATP on the affinity resin, so tuna cytochrome *c*, with tryptophan at this position, was used for this experiment.

The binding of ferricytochrome *c* to the ATP affinity column was found to be pH-dependent (Fig. 5). An increase in pH from 6 to 7.5 causes an increase in retention time which coincides with the empirical pK of the fourth ionization of ATP, thus confirming the relationship of total negative charge and strength of binding. The large increase in retention caused by the deprotonation is consistent with our view that it is the triphosphate moiety that is the prime determinant of the high degree of selectivity of this binding site for ATP compared with other anions.

The retention time of cytochrome *c* on the affinity column rapidly decreases between pH 9 and 10. This range corresponds to the transition of the protein from its state III form to state IV, in which Met-80 co-ordination is lost (Dickerson & Timkovitch, 1975). These data suggest that the conformational change associated with this transition disrupts the binding site.

In order to ensure that this drop in affinity in the alkaline region was not due to an increase in ionic strength associated with the adjustment of the pH, a back titration was performed. No hysteresis was observed.

The shape and charge distribution of the guanidinium group of the side chain of arginine residues allow it to make strong and specific bonds to phosphate and carboxylate groups (Salunke & Vijayan, 1981). Riordan (1979) proposed that arginine residues are present in proteins where such a role is required. Our data indicate that Arg-91 is one element of the ATP-binding site, possibly interacting with the γ -phosphate. The dependence of affinity on total negative charge of the polyanionic species tested implies that other positively charged groups on the protein surface are also important, and indeed lysine-modified cytochromes *c* bind ATP less well (Corthésy & Wallace, 1986). Obvious candidates for this role are the lysine residues 86, 87 and 88. Since the nucleoside moiety makes some contribution to binding strength, it is possible that the previously implicated Glu-66 (Wallace & Corthésy, 1986) is involved with binding either the ribose 3-hydroxy or the polar groups of the base. Further experiments are required to map the binding site, as well as to confirm the physiological significance of binding by demonstrating an appropriate biological response.

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