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ATP binding to cytochrome *c* diminishes electron flow in the mitochondrial respiratory pathway

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Abstract

Eukaryotic cytochrome *c* possesses an ATP-binding site of substantial specificity and high affinity that is conserved between highly divergent species and which includes the invariant residue arginine^{91}. Such evolutionary conservatism strongly suggests a physiological role for ATP binding that demands further investigation. We report the preparation of adducts of the protein and the affinity labels 8-azido adenosine 5′-triphosphate, adenosine 5′-triphosphate-2′,3′-dialdehyde, and 5′-*p*-fluorosulfonylbenzoyladenosine. The two former reagents were seen to react at the arginine^{91}-containing site, yet the reaction of the latter, although specific, occurred elsewhere, suggesting caution is necessary in its use. None of the adducts displayed significant modification of global structure, stability, or physicochemical properties, leading us to believe that the 8-N_3-ATP and oATP adducts are good stabilized models of the noncovalent interaction; yet modification led to significant, and sometimes pronounced, effects on biological activity. We therefore propose that the role of ATP binding to this site, which we have shown to occur when the phosphorylation potential of the system is high under the equivalent of physiological conditions, is to cause a decrease in electron flow through the mitochondrial electron transport chain. Differences in the degree of inhibition produced by differences in adduct chemistry suggest that this putative regulatory role is mediated primarily by electrostatic effects.

Keywords: ATP; 8-azido ATP; cytochrome *c*; FSBA; oATP; regulation; respiration

In eukaryotes operating under aerobic conditions, ATP is predominantly produced by mitochondrial oxidative phosphorylation. The ability of cells to closely match ATP synthesis to ATP hydrolysis, which occurs mostly in the cytosol, has been the subject of much study and is not yet completely understood. It has been proposed that the majority of the mitochondrial respiratory chain is in a state of near equilibrium with the extramitochondrial phosphorylation potential, and overall rate is controlled by the extramitochondrial $[ATP]/[ADP]\cdot[P_i]$ (Wilson et al., 1974). Another view holds that control is exerted through [ATP]/[ADP] at the adenylate translocase (Kuster et al., 1976). Some control has also been shown to occur through passive proton leak across the inner mitochondrial membrane (Groen et al., 1982) and by [NADH]/[NAD^+] (Brown et al., 1990). Using the control strength concept of Kacser and Burns (1973) and Heinrich and Rapoport (1974), it has been suggested that there is no one rate-controlling step in oxidative phosphorylation, control is likely to be shared between several sites, and the degree of control varies with differing respiratory conditions (Groen et al., 1982; Tager et al., 1983) and complexity of the metabolic system (Gellerich et al., 1983). It is likely that in addition to the aforementioned control sites, overall respiration rate is determined by additional mechanisms, including ones that have not yet been characterized.

The present understanding of the control of metabolic flux is that most pathways are regulated by the activities of key enzymes, which are in turn determined by the concentration of allosteric effectors and not by the levels of substrates and products alone. The handling of reducing equivalents can be regarded as akin to any other metabolic pathway, and one might therefore expect that oxidative phosphorylation would be subject to some degree of control by allosteric means. Kadenbach (1986) proposed that respiration and ATP synthesis in higher organisms

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Abbreviations: CHD, 1,2-cyclohexanedione; DHCH arginine, N^7N^8-(1,2-dihydroxycyclohex-1,2-ene)diyl-L-arginine; FSBA, 5′-*p*-fluorosulfonylbenzoyladenosine; oATP, adenosine 5′-triphosphate-2′,3′-dialdehyde.

are controlled by allosteric regulation of key enzymes, which is independent of isosteric control, in addition to substrate and product concentrations. He proposed that one such site is at cytochrome *c* oxidase, an enzyme complex that catalyzes the last step of the electron transport chain. One of these allosteric effectors is suggested to be ATP.

Cytochrome *c* in eukaryotes is a component of the mitochondrial chain of electron carriers, is located in the intermembrane space, and is electrostatically associated with the inner membrane, that shuttles electrons from cytochrome *c* reductase to cytochrome *c* oxidase, the membrane-embedded complexes III and IV. Amino acid sequences of the protein from 96 species (Moore & Pettigrew, 1990), representing the entire range of eukaryotic evolution, have been determined and found to contain several invariant amino acid residues. One such residue, arginine^{91} (Kinemage 1), can be modified (Wallace & Rose, 1983) or replaced (C.J.A. Wallace & I. Clark-Lewis, unpubl.) without any apparent effect upon the ability of the protein to restore oxygen consumption in cytochrome *c*-depleted mitochondria. This immediately provoked the question that if cytochrome *c* can function without it, then why has this residue been absolutely conserved throughout evolution? Corthésy and Wallace (1986, 1988) showed it to be part of an ATP-binding site and proposed that the capacity to bind is the reason for the conservation of arginine^{91}. Craig and Wallace (1991) demonstrated that ATP binds to this site with a dissociation constant within the cellular ATP concentration range at physiological ionic strength and that the occupancy of this site is dependent upon the [ATP]/[ADP] ratio. The affinity of this site was found to be identical for several NTPs, showing binding affinities of PPPi > NTP > PPi > ADP, and we concluded that the strength and specificity of binding is more dependent on the triphosphate moiety than the nucleoside.

AMP, PPPi, PPi, and Pi have been shown to inhibit the interaction between cytochrome *c* oxidase and cytochrome *c* in the millimolar concentration range and ATP and ADP inhibit within their cellular concentration range (Ferguson-Miller et al., 1976; Huther & Kadenbach, 1986; Huther et al., 1988), as well as inducing a conformational change in the oxidase (Reimann et al., 1988). The order of inhibitory effectiveness was found to be PPPi > ATP > PPi > ADP > AMP > Pi (Huther et al., 1988), and different NTPs were found to inhibit this interaction to the same extent as ATP, leading to the conclusion that inhibition is more dependent upon net charge than on the presence of an adenosine moiety (Osheroff et al., 1978). Modification of the oxidase with 8-azido ATP was found to mimic the effects of free ATP, and this modification was inhibited by ATP but not ADP. Despite this, no specific binding site was identified since adduct formation was observed at several points on the oxidase (Huther et al., 1988). These observations were used to support the proposal that cytochrome *c* oxidase acts as a site of regulation of oxidative phosphorylation through feedback inhibition by ATP. The possibility that the effects might be due to interaction with cytochrome *c* as well or rather than the oxidase was not explicitly considered. However, the data we and others had accumulated led us to propose that the binding of ATP to cytochrome *c* is a means by which the electron transport chain is made sensitive to the cell's demand for ATP (Craig & Wallace, 1991). Our proposal is supported by the characterization of an ATP-binding site on cytochrome *c* that is operative in species as diverse as horse and yeast; that is partially saturated at physiological ATP concentrations and whose degree of occupancy is dependent upon the energy state of the cell. In addition, much of the evidence used in supporting the proposal that cytochrome *c* oxidase acts as a site of regulation can be used to support our proposal. The inhibition experiments using free ligand could not distinguish between binding to cytochrome *c* and cytochrome *c* oxidase, both of which are present in the assay. These inhibition parameters are closely paralleled by the binding characteristics of ATP to cytochrome *c* as determined in the absence of any other proteins (Craig & Wallace, 1991). This suggests that the inhibition of biological activity may be at least in part due to the binding of ATP to cytochrome *c*.

The finding that ATP can bind with specificity to a conserved site on cytochrome *c* under physiological conditions is, however, not sufficient to demonstrate that this interaction is of regulatory significance. It must be clearly shown that binding of ATP exclusively to this site modulates the flow through this pathway. The present study was designed to permit a biological assay of the pathway when the binding site is occupied, yet ATP is not free to bind to any other sites on cytochrome *c* or any other protein of this system. This was achieved by covalently attaching ATP analogues to this binding site and assaying in the absence of free ATP. Three analogues were employed: 8-azidoadenosine 5′-triphosphate (8-azido ATP), FSBA, and oATP. It is crucial to demonstrate that these modifications do not seriously affect the overall structural integrity of cytochrome *c* in order to be sure that these adducts are good models of the noncovalent interaction.

Our previous report (Craig & Wallace, 1991) indicated that the reaction of 8-azido ATP with the arginine^{91}-containing binding site of cytochrome *c* yielded an adduct with only 10–20% of the native biological activity but gave few details. Here we elaborate on the characteristics of this adduct and those produced by modification with oATP and FSBA.

Results and discussion

Affinity chromatography

Periplasmic bacterial cytochromes *c* such as cytochrome c_{551} are structurally similar to mitochondrial cytochrome

c. No evolutionary pressure to conserve an ATP-binding site would be expected on these cytochromes since the periplasm presumably contains no ATP. Sequence data show that these cytochromes do not contain any arginine residues in regions equivalent to that of mitochondrial cytochrome *c*'s arginine[91] (Ambler, 1974). Cytochrome c_{551} from *Pseudomonas aeruginosa* was found to elute in the void volume from the ATP affinity column in the presence of 35 mM phosphate. Under these conditions native cytochrome *c* is retained 42 min. Corthésy and Wallace (1988) showed that specific modification of arginine[91] can be obtained with CHD, without causing any detectable changes in the overall structure or activity of the protein, and this modification greatly diminishes the ability of ATP to bind to this site. Cytochrome *c* modified in this manner had a retention time of 2 min on the affinity column under the same conditions. In the absence of borate, native and CHD-modified cytochromes *c* are of identical electric charge, indicating that in the presence of 35 mM phosphate, retention time is a function of ATP affinity and not the ion-exchange properties of the negatively charged resin. These data indicate that the cytochrome c_{551} employed and, by inference, other cytochromes c_{551} do not bind ATP.

Adduct formation

Azido photoaffinity probes have been used to form covalent bonds with proteins in the presence of UV light via nitrene intermediates (Knowles, 1972). 8-Azido ATP has been used as such for the elucidation of nucleotide binding sites and kinetic effects of binding (Huther & Kadenbach, 1986; Huther et al., 1988). This probe is structurally very similar to ATP (Fig. 1). Upon binding and activation the intermediate is very reactive and can form a covalent bond with any amino acid R group in or adjacent to the binding site (Haley, 1977), and if unbound the probe will probably react with the solvent rather than attaching nonspecifically to the protein (Czarnecki et al., 1979).

Modification of cytochrome *c* with 8-azido ATP was carried out under low ionic strength conditions where cytochrome *c* has a high affinity for ATP (Craig & Wallace, 1991). Under these conditions the protein is capable of binding up to three molecules of ATP, but because the arginine[91]-containing binding site has a higher affinity than the other two nonspecific binding sites, careful selection of the concentration of the ligand allows modification at this site only.

Modification with 8-azido ATP causes a change in net charge of the protein due to the triphosphate moiety, thus ion exchange is the method of choice for separation of the reaction mixture. Any doubly or triply modified material produced will either not bind to the resin or elute early due to the additional negative charge. Some multiply modified material is found to elute in the void volume. Two major and three minor products were formed and

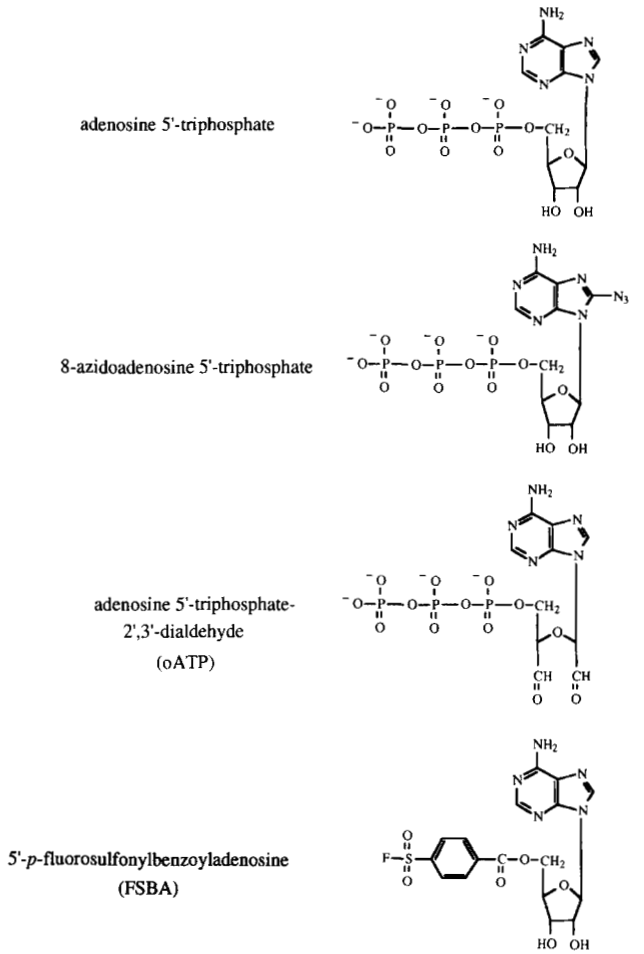

Fig. 1. Structures of the ATP analogs. The chemical structures of ATP, 8-azido ATP, oATP, and FSBA are shown.

eluted in a cluster far removed from the unmodified protein at a buffer conductivity of 6–7 mS for the main peaks, whereas native elutes at 16–18 mS (Fig. 2). The products showed identical spectra. Difference spectra drawn versus native cytochrome *c* showed the addition of an absorbance peak with a λ_{max} at 258 nm with an absorbance coefficient of 6,300 M^{-1} cm^{-1} (Fig. 3). The marked difference in the magnitude of the absorbance of this peak from that of ATP, which has an absorbance coefficient of 15,400 M^{-1} cm^{-1} at this wavelength, indicates that it is likely that the purine ring is modified in the course of the reaction. The spectrum of 8-azido cAMP shows a similar change in spectral properties upon irradiation (Haley, 1977).

The extinction coefficient is calculated on the basis of the Soret band absorbance, assumed to be identical to that of native cytochrome *c*. It is of course conceivable that the modification has affected the A_{410} of the heme. We consider this possibility less likely for several reasons. The reagent is attached at the protein surface (because internal structure is not disrupted): on theoretical grounds,

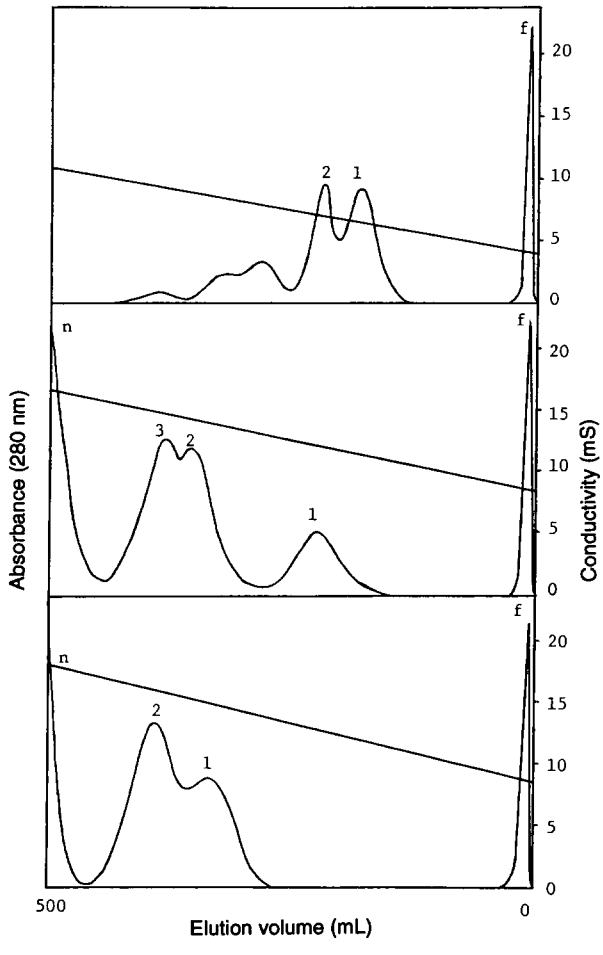

Fig. 2. Elution profiles of the products of modification of cytochrome *c* with the ATP analogs. The elution profiles from cation-exchange chromatography of the products of modification of cytochrome *c* with 8-azido ATP (top), oATP (middle), and FSBA (bottom) are shown. Peaks are labeled as native cytochrome *c* (n), ferricyanide (f), and major modified cytochrome *c* peaks are numbered. Details of the chromatographic conditions are given in the text.

only changes in the coordination sphere of the heme iron would be expected to produce change. Examination of the spectral properties of semisynthetic analogues confirms that although internal modifications close to the heme do perturb spectra, surface changes do not (Wallace et al., 1989; Wallace & Clark-Lewis, 1992). Further, it is extremely unlikely, were changes to occur, that complete superimposability of those spectra on cytochrome *c* over the entire visible range would be observed. However, this is the case with all our adducts. A final point is that in the case of the oATP adducts described below, the absorbance increase in the UV, based on constant A_{410}, are entirely consistent with the addition of one or two adenosine moieties.

Cytochrome *c* modified at arginine^{91} with CHD was found to yield no detectable product upon reaction with 8-azido ATP. This demonstrates that blocking of the arginine^{91}-containing binding site prevents further modification, indicating that it is this site to which the label is attached. The clustering of the five peaks in the elution profile far removed from the unmodified cytochrome *c*, their identical spectra, and the loss of all five peaks by prior modification with CHD indicate that the five peaks represent isomers of the attachment of a single label to the arginine^{91}-containing binding site. The clustering of the five peaks together also indicates that it is unlikely that they result from a differing degree of phosphorylation of the label. Both of the two high-yield isomers were subjected to further study.

The production of five isomers upon reaction of 8-azido ATP at the arginine^{91}-containing site may indicate that the purine moiety is loosely held, thus allowing the nitrene group attached to carbon-8 to react at different locations. Loose binding of the purine is consistent with the proposal that interactions with the nucleoside moiety account for only a small fraction of the binding strength (Craig & Wallace, 1991).

As ionic strength increases to isoionic, cytochrome *c* no longer binds ATP at its two nonspecific sites (Craig & Wallace, 1991). ATP was found to decrease the modification yield with 8-azido ATP at physiological ionic strength; this effective competition provides additional evidence that modification occurs exclusively at the arginine^{91}-containing site.

Treatment of cytochrome *c* with CNBr in 70% formic acid causes cleavage of the 65–66 and 80–81 peptide bonds, yielding fragments 1–65, 66–80, and 81–104. Although the majority of the attached label is removed under the conditions employed, all of the remaining label was found to be attached to fragment 81–104. [$N^{\varepsilon}N^{\varepsilon}$-dimethylamino]$_{19}$-lysine, [$N^{\varepsilon}$-isopropylamino]$_{19}$-lysine, and [$N^{\varepsilon}$-acetimidyl]$_{19}$-lysine cytochromes *c*, all of which have had all their lysine residues modified, show a decreased modification yield upon incubation with 8-azido ATP that cannot be accounted for by a difference in binding site occupancy alone (Table 1). This implies that lysine residues are involved in the mechanism of modification. Lysines 86, 87, and 88 are adjacent to position 91 and represent possible candidates as attachment sites.

oATP is structurally similar to ATP (Fig. 1) and has been found to react with lysine and ornithine residues. The reactive groups on this analogue are the two aldehydes at the 2′ and 3′ positions on the disrupted ribose ring. The reaction was assumed to occur via a Schiff's base (Easterbrook-Smith et al., 1976), but further evidence indicates that this reaction probably does not occur via this mechanism. In order for a stable product to be formed, reduction of the Schiff's base is required. The presence of $NaBH_4$ has been found to have no effect on the labeling of protein nor did the use of NaB^3H_4 lead to the incorporation of radioactivity (King & Colman, 1983). [^{14}C]oATP was found to radiolabel protein, whereas [^{32}P]oATP did not (Lowe et al., 1979). Modification with

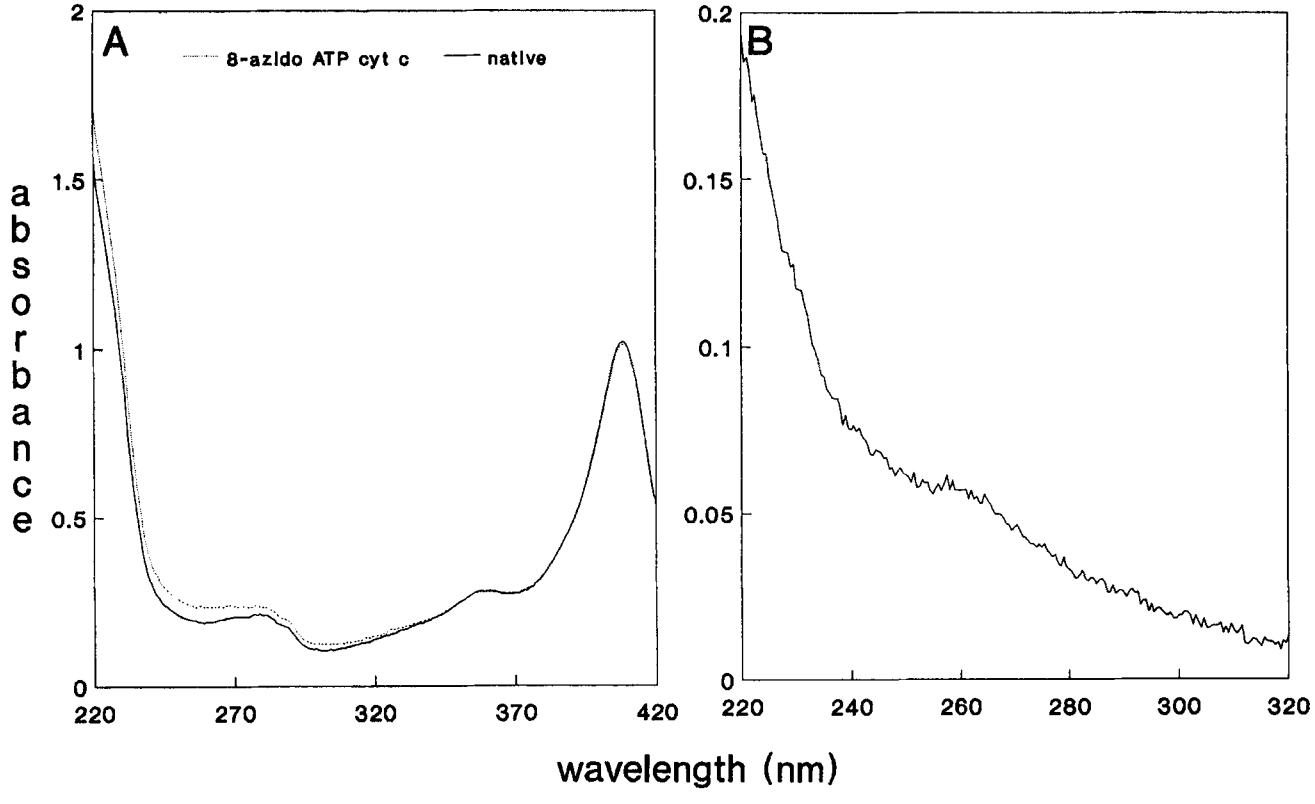

Fig. 3. Spectra of 8-azido ATP-modified cytochrome *c*. The spectra of native and 8-azido ATP-modified cytochrome *c* (**A**) and the difference spectra of 8-azido ATP-modified minus native cytochrome *c* (**B**) are shown.

oATP has been found to be stabilized by the presence of borate, indicating the presence of a diol (Gregory & Kaiser, 1979). A second mechanism for modification by oATP has been proposed that involves both aldehyde groups of oATP and removal of the triphosphate moiety producing a dihydroxymorpholino derivative (Fig. 4; Gregory & Kaiser, 1979; King & Colman, 1983).

Two major oATP-modified cytochrome *c* peaks eluted at a buffer conductivity of 13–15 mS, which is much closer to the native protein (16–18 mS) than the 8-azido ATP adducts (6–7 mS, Fig. 2) indicating that oATP modification did not cause the extensive charge change seen in the latter. Furthermore, we found the ion-exchange elution profile to be identical whether borohydride was included or not, demonstrating that the reduction step is unnecessary. This is consistent with the proposed mechanism of modification, which does not involve a Schiff's base and whereby the triphosphate moiety is cleaved.

Spectra of these two adducts were identical, and difference spectra showed the addition of a peak with a λ_{max} at 254 nm with an absorbance coefficient of 16,800 $M^{-1}\,cm^{-1}$ (Fig. 5), consistent with the addition of a single oATP residue. In addition to the two major peaks, a minor peak with a higher mobility on the column was

Table 1. *Modification yields of cytochrome c with 8-azido ATP*[a]

Sample	[8-N_3 ATP] (mM)	% Site occupancy	% Modification
Native	0.8	81–86	30
[$N^{\varepsilon}N^{\varepsilon}$-dimethylamino]$_{19}$-lysine	3	85	16
[N^{ε}-isopropylamino]$_{19}$-lysine	3	87	14
[N^{ε}-acetimidyl]$_{19}$-lysine	3	67	8

[a] The site occupancy, calculated from the data of Corthésy and Wallace (1986), and modification yields of native, [$N^{\varepsilon}N^{\varepsilon}$-dimethylamino]$_{19}$-lysine, [$N^{\varepsilon}$-isopropylamino]$_{19}$-lysine, and [$N^{\varepsilon}$-acetimidyl]$_{19}$-lysine cytochromes *c* with 8-azido ATP are shown.

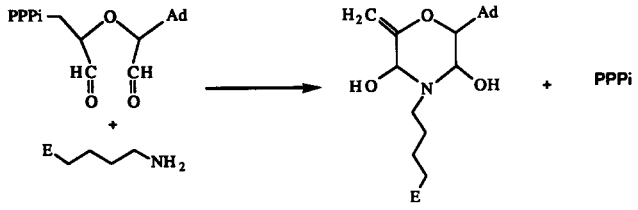

Fig. 4. Reaction of oATP with lysine residues. The reaction of a lysine residue with oATP, as proposed by Gregory and Kaiser (1979) and King and Colman (1983), is shown.

observed. This peak showed a λ_{max} at 254 nm with an absorbance coefficient of 29,900 M^{-1} cm^{-1}. This product represents double modified cytochrome *c*. Quantities of all three products were sufficient for further study.

DHCH-arginine[91] cytochrome *c* yielded no detectable product when incubated with oATP indicating that the covalent attachment occurs at the site containing arginine[91], and that the doubly modified material results from a double attachment at this site. We suggest that the triphosphate moiety of oATP is oriented by the positive charge of arginine[91] and adjacent lysine residues (86, 87, and 88). Subsequent reaction covalently attaches the aldehyde groups of oATP to an adjacent residue, and the phosphates are cleaved in the process. This frees the arginine[91] and adjacent lysines and allows them to bind another molecule of oATP for a second reaction cycle. Free ATP was found to decrease adduct yields with oATP at physiological ionic strength.

FSBA has also been used as an ATP affinity label. It contains adenosine and the ribose moieties, but with the electronegative sulfonylfluoride element in place of negatively charged phosphate groups (Fig. 1). The sulfonylfluoride group can react with many nucleophilic amino acid residues including serine, tyrosine, lysine, and histidine (Colman, 1990).

Modification with FSBA produced two products in good yield, which eluted on the ion-exchange column before, but close to, the unmodified material, in a similar position to the oATP adducts (Fig. 2). Difference spectra showed a major peak with a λ_{max} of 243 nm with an absorbance coefficient of 23,700 M^{-1} cm^{-1} and a shoulder at 259 nm with an absorbance coefficient of 17,900 M^{-1} cm^{-1} (Fig. 6). Unreacted FSBA in ethanol has two λ_{max} at 232 nm and 259 nm with absorbance coefficients of 21,700 M^{-1} cm^{-1} and 15,800 M^{-1} cm^{-1}, respectively (Pal et al., 1975), so our results indicate that only one FSBA residue was attached per cytochrome *c* molecule.

DHCH-arginine[91] cytochrome *c* was found to be susceptible to modification by FSBA, indicating that blocking

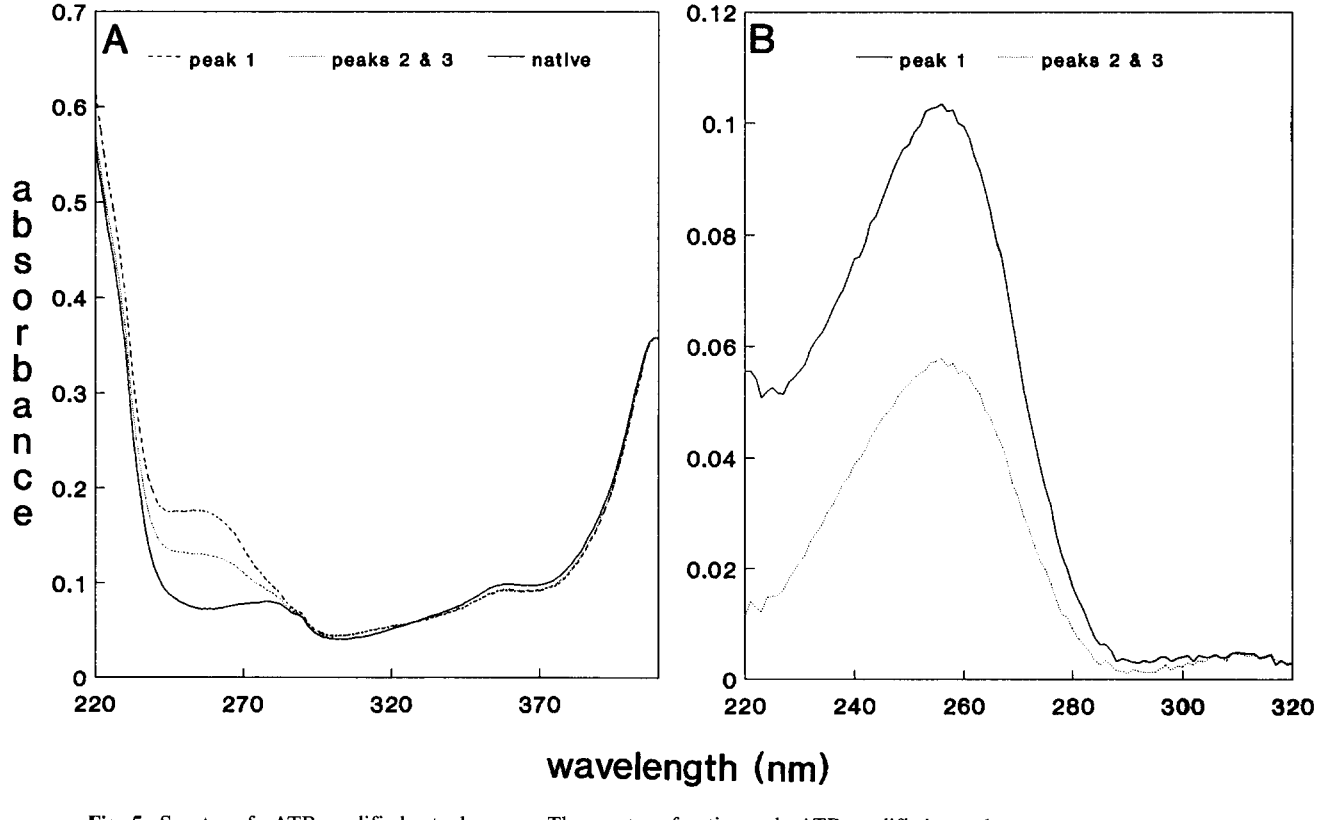

Fig. 5. Spectra of oATP-modified cytochrome *c*. The spectra of native and oATP-modified cytochrome *c* (**A**) and the difference spectra of oATP-modified minus native cytochrome *c* (**B**) are shown.

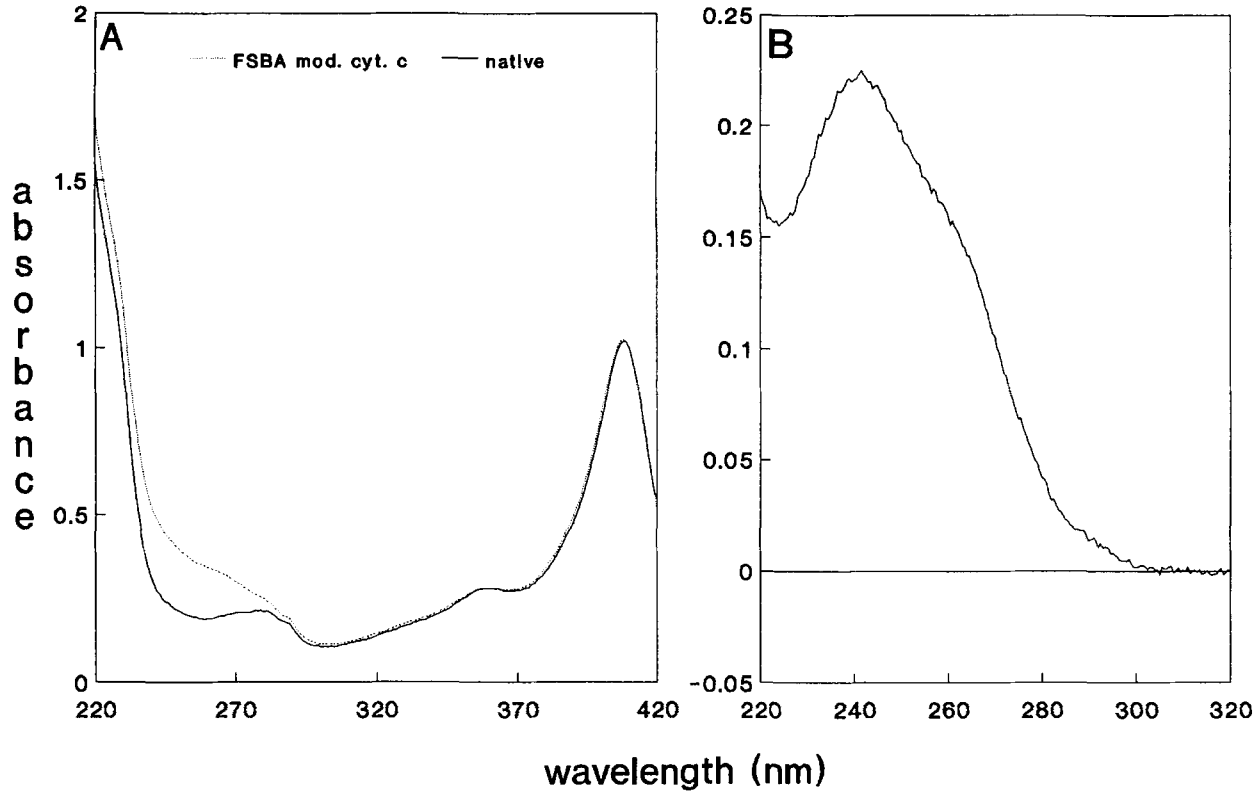

Fig. 6. Spectra of FSBA-modified cytochrome *c*. The spectra of native and FSBA-modified cytochrome *c* (**A**) and the difference spectra of FSBA-modified minus native cytochrome *c* (**B**) are shown.

of the arginine residue does not eliminate modification. Incubation of cytochrome *c* with FSBA in the presence of ATP did not affect the yield of modified protein. Thus although specific modification is occurring, as shown by reproducible ion-exchange elution profiles, the location of modification is unclear. This result suggests that the widely used FSBA may not always be a useful affinity label for studies of nucleotide binding, especially in a system where the binding of nucleotides has a large electrostatic component.

Properties of the adducts

Modified cytochrome *c* samples were collected from the elution profile by conservative cuts sacrificing yield for purity. Although these modifications were found to be stable for several months at −20 °C, fresh samples were prepared for each analysis.

The presence of the 695-nm absorbance band of cytochrome *c*, which signals coordination of methionine[80] sulfur to the heme iron, is considered to be a sensitive indicator of the internal structural integrity of the protein. Modifications that alter structural stability can cause large changes in the *pK* of the loss of this absorbance with increasing pH (Dickerson & Timkovitch, 1975) or eliminate it altogether. None of the adducts had a pK_{695} that deviated greatly from that of the native protein, showing values that ranged from 8.85 ± 0.16 to 9.16 ± 0.08 versus native's value of 9.25, indicating that these surface modifications did not affect the internal structure of the protein. Similarly, the redox potentials of these adducts were found to range from 253 ± 2 mV to 273 ± 1 mV, which is very close to native values that range in the literature from 256 mV to 266 mV. This parameter is also most sensitive to disruption of the protein's conformation (Wallace & Proudfoot, 1987). Finally, visible spectra of the adducts and the native protein were identical in both absorbance maxima and intensity. Thus, these modifications do nothing to disrupt the basic cytochrome *c* fold.

The cytochrome *c*-ATP analog adducts were tested for their ability to promote oxygen consumption in cytochrome *c*-depleted mitochondria relative to that of native cytochrome *c*. The assay buffer contained succinate, ADP, glucose, and hexokinase. Although the outer mitochondrial membrane is ruptured, the inner membrane is intact and electron transfer and phosphorylation remain coupled. ATP produced during the course of the assay is quickly utilized by hexokinase-catalyzed phosphorylation of glucose before any appreciable concentration can accumulate (Jacobs & Sanadi, 1960; Wallace & Proudfoot, 1987). The assay measures the overall electron flow through the pathway by monitoring oxygen consumption

over a range of cytochrome *c* concentrations. Plots of O_2 consumption rates versus the added amount of cytochrome *c* are generated, and the initial slopes are determined. Differences in the obtained slopes are attributed to differences in the activities of the cytochromes *c* employed. The complexity of the assay, which involves the activity of cytochrome *c* with both its reductase and oxidase, make values determined for K_m and V_{max} difficult to interpret. Thus, in this assay cytochrome *c* activities measured represent the relative abilities of different cytochromes *c* to restore oxygen consumption to cytochrome *c*-depleted respiratory chains. In assaying the adducts used in this study, what is measured is the effect of site occupancy on the overall metabolic flow along the electron transport chain.

The 8-azido ATP adduct peaks 1 and 2 showed biological activities of 16 and 15% of the native protein (Table 2). Peaks 1 and 2 of the FSBA modification showed activities of 32 and 44%, and activities of the oATP peaks 2 and 3 showed activities of 32 and 68%. The oATP peak 1 adduct, which is modified with two molecules of oATP, showed an activity of 27%, which is a value similar to that expected if the effects of each modification were cumulative.

The modification with 8-azido ATP produces an adduct that more closely resembles the physiological ATP–cytochrome *c* interaction than does that produced by oATP and FSBA. Neither of these labels incorporates a triphosphate moiety onto the protein, and the site of attachment of the latter is unclear, whereas 8-azido ATP modifies the protein in such a manner as to occupy the binding site with a highly negatively charged ATP analog, thus mimicking the steric and electrostatic consequences of ATP binding.

The covalent attachment of 8-azido ATP to the arginine[91]-containing binding site causes a substantially larger decrease in the activity of cytochrome *c* than that induced by reaction with oATP. Although this label is specifically attached at the high affinity ATP-binding site, it causes no greater inhibition than that induced by FSBA, which presumably is not. This observation leads one to conclude that the binding of the nucleoside moiety is not a specific inhibitory factor, and the much greater effect of derivatization with 8-azido ATP implies that the primary effector is the triphosphate unit.

Cytochrome *c* binds its physiological partners primarily through electrostatic interactions. The affinity of binding is diminished by charge-altering modifications of lysine residues at positions 13, 72, 86, 87, 8, 27, 73, 25, and 7 (shown in decreasing order of inhibitory effect) of cytochrome *c* (Kinemage 1). These residues form a ring of positive charge surrounding the heme crevice of cytochrome *c* with which cytochrome *c* oxidase interacts (Ferguson-Miller et al., 1978; Osheroff et al., 1980). The binding site on cytochrome *c* for cytochrome *c* reductase has been found to be indistinguishable or overlapping to a large extent with that of the oxidase (Ahmed et al., 1978; Rieder & Bosshard, 1980; Smith et al., 1980). Arginine[91] is directly adjacent to lysine[86] and lysine[87], therefore the ATP-binding site is either in or adjacent to this ring of positive charge (Kinemage 1). Two types of inhibition can be envisaged. Noncompetitive inhibition may result from the binding of ATP next to this ring causing conformational change in residues that function in the catalysis of electron transfer. This type of inhibition seems unlikely because the binding of ATP was found to cause no changes in conformation detectable by spectroscopic titration methods (data not shown) or redox potential changes (Corthésy & Wallace, 1988). More probable is a competition for the lysine residues of the docking site between the negative charge of the triphosphate element and the negatively charged binding domain of cytochrome *c* oxidase or reductase. In addition to electrostatic interference, the binding of a bulky ATP molecule may sterically hinder the interaction, as it appears to do in the nucleoside-only oATP and FSBA adducts.

The cytochrome *c*–cytochrome *c* oxidase reaction approaches a diffusion rate limit. It is therefore unlikely that this interaction arises from random collisions of all possible orientations. Cytochrome *c* has a strong dipole, due to a high surface charge and the concentration of almost all of its negative charge on a small region on the back surface of the protein. It is thought that this dipole orients the protein in order to maximize the efficiency of collisions (Koppenol et al., 1978; Margoliash & Bosshard, 1983). The binding of a highly negative ATP molecule onto the surface of the protein would be expected to have a significant effect on the dipole and thus also decrease the probability of collision in the proper orientation.

Tripolyphosphate has been shown to bind to cytochrome *c* in the same region as arginine[91], and this binding facilitates aggregation of the protein (Concar et al., 1991). Because the tripolyphosphate tail of ATP is central to its ability to bind to cytochrome *c*, induction of aggregation represents a potential mechanism of inhibition.

Table 2. *Biological activities of the adducts*[a]

Adduct	Biological activity (as % of native)
Native cytochrome *c*	100
8-Azido ATP cytochrome *c* peak 1	16 ± 6
8-Azido ATP cytochrome *c* peak 2	15 ± 6
oATP cytochrome *c* peak 1	27 ± 8
oATP cytochrome *c* peak 2	32 ± 6
oATP cytochrome *c* peak 3	68 ± 22
FSBA cytochrome *c* peak 1	32 ± 9
FSBA cytochrome *c* peak 2	44 ± 11

[a] The biological activities of the adducts are shown as a percentage of native cytochrome *c*.

Cytochrome *c* was found to elute in the void volume from a cytochrome *c* affinity column in both low and physiological ionic strength, and this elution volume was not affected by the presence of 10 mM PPPi or ATP. The inability of these ligands to provide sufficient cytochrome *c*–cytochrome *c* interactions to cause a detectable change in retention time indicates that aggregation is unlikely to be the means by which ATP inhibits cytochrome *c* activity.

Cytochrome *c* has been shown to be capable of binding ATP at an evolutionarily conserved site, which involves the absolutely conserved residue arginine[91] (Kinemage 1). Under physiological conditions, the K_d of this interaction is in the middle of the cellular ATP concentration range, and the occupancy of the site is dependent upon the [ATP]/[ADP] ratio. The covalent attachment of ATP analogues at this site causes no long-range effects and has permitted assays of the effect of this binding in the absence of free ATP, which might interact with other proteins of the respiratory chain and cause significant inhibition of overall electron transport. We believe that the behavior of the 8-azido ATP–cytochrome *c* adduct is a good model for that of the noncovalent ATP–protein complex. Because cytochrome *c* operates in the mitochondrial intermembrane space, and this space is continuous with the cytoplasmic ATP pool, we propose that the binding of ATP to cytochrome *c* is a means by which mitochondrial respiration is regulated by the cytoplasmic ATP concentration. Arginine[91] is essential to this ability to bind ATP and thus to regulate mitochondrial ATP production, so explaining its absolute evolutionary conservatism.

We may speculate that under normal metabolic circumstances, when the [ATP]/[ADP] ratio is high, cytochrome *c* would bind ATP and operate at a decreased rate. However, if a heavy metabolic load developed, [ATP]/[ADP] ratios in the cytoplasm would begin to drop, leading to dissociation of the cytochrome *c*–ATP complex, and an immediate increase in throughput of electrons in the respiratory chain to prevent any large drops in phosphorylation potential. Such a system would be of particular value in a tissue such as muscle that is subject to sudden demands, and it is of interest to note that the greatest sensitivity of our putative regulatory system is in the range corresponding to muscle ATP concentrations.

Materials and methods

Materials

Horse heart cytochrome *c* (type III), *Pseudomonas aeruginosa* cytochrome c_{551}, CHD, 8-azido ATP, oATP, FSBA, pentasodium tripolyphosphate, and hexokinase were purchased from Sigma and Tris from Boehringer-Mannheim. Column chromatography resins were obtained from Pharmacia LKB. Acetic acid, boric acid, NaCl, glycine, *N*-ethylmorpholine, and sodium borohydride were bought from BDH; sodium dithionite and sodium acetate from Merck; and succinic acid from Fluka. Sucrose, HCl, and urea were purchased from Anachemia. Ferricyanide, ferrocyanide, $MgCl_2$, and NaOH were obtained from Baker, and glucose, KCl, $NaHCO_3$, cacodylic acid, and KH_2PO_4 came from Fisher Scientific.

Modification of cytochrome c with CHD

Horse cytochrome *c*, devoid of any of its deamidated forms, was modified with CHD to yield N^7N^8-(1,2-dihyroxycyclohex-1,2-ene)diyl-L-arginine[91] cytochrome *c* (DHCH-arginine[91] cytochrome *c*) as described by Corthésy and Wallace (1988). One millimolar cytochrome *c* 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 dialyzed 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), to allow any denatured protein to refold when buffer exchanged into 0.1 M borate (pH 8). The sample was subjected to ion-exchange chromatography on Trisacryl SP using 120 mL of 0.1 M borate (pH 8) containing 0–0.4 M KCl.

Affinity chromatography

Cytochrome *c* binds strongly to ATP immobilized on agarose. One can thus compare the affinities of different cytochromes *c* for ATP by their abilities to be retained on an agarose–hexane ATP affinity column. Although this method does not allow quantification of the binding strengths it does permit the determination of the relative strengths while requiring only a small amount of sample. The retention times of native cytochrome *c*, DHCH-arginine[91] cytochrome *c*, and *P. aeruginosa* cytochrome c_{551} were determined using 35 mM phosphate (pH 7.00) for elution with continuous monitoring at 280 nm. Retention times were determined as the difference between elution time and void time.

Cytochrome *c* affinity columns were prepared by attachment of cytochrome *c* to CNBr-activated Sepharose 4B. Five milligrams of cytochrome *c* per milliliter swollen gel was incubated in an end-over-end mixer for 1 h in 100 mM $NaHCO_3$ containing 500 mM NaCl (pH 8.3) followed by 1 h in 200 mM glycine (pH 8). The resin was then washed with 100 mM $NaCOOCH_3$ containing 500 mM NaCl (pH 4.0). Cytochrome *c* was loaded onto the column and eluted with 10 mM Tris-cacodylate or 10 mM Tris-cacodylate containing 150 mM KCl and in the presence of ATP or PPPi. Runs were performed with both immobilized and free cytochrome *c* in their reduced and oxidized states and with reduced immobilized and oxidized free cytochrome *c*.

Modification of cytochrome c's lysine residues

[$N^{\epsilon}N^{\epsilon}$-dimethylamino]$_{19}$-lysine, [N^{ϵ}-isopropylamino]$_{19}$-lysine, and [N^{ϵ}-acetimidyl]$_{19}$-lysine cytochromes *c* were prepared as described by Wallace and Corthésy (1987).

Modification of cytochrome c with 8-N_3 ATP

Ten milligrams of horse ferricytochrome *c*, devoid of any of its deamidated forms, was dissolved with 0.5 mg of 8-N_3 ATP in 1 mL of 5 mM Tris-cacodylate buffer (0.8 mM cytochrome *c* and 0.7 mM 8-azido ATP, pH 6.95). The sample was irradiated with a long-wave UV light from a Chromato-vue model CC20 lamp at a range of 3 cm for 60 min. The reaction mixture was then loaded on a Trisacryl SP ion-exchange column, and any reduced protein present was reoxidized with ferricyanide. The sample was eluted from the column while utilizing continuous UV detection at 280 nm with 500 mL of a 25–60-mM phosphate buffer (pH 7). [$N^{\epsilon}N^{\epsilon}$-dimethylamino]$_{19}$-lysine, [N^{ϵ}-isopropylamino]$_{19}$-lysine, and [N^{ϵ}-acetimidyl]$_{19}$-lysine cytochromes *c* were also modified with 8-azido ATP.

Modification of cytochrome c with oATP

Ten milligrams of horse ferricytochrome *c*, devoid of any of its deamidated forms, was incubated for 2 h at room temperature in 1 mL of 10 mM *N*-ethylmorpholine-HCl buffer (pH 8) containing 0.5 mg oATP (0.8 mM cytochrome *c* and 0.8 mM oATP). The sample was loaded onto a Trisacryl SP ion-exchange column, treated with ferricyanide, and eluted with 500 mL of 50–100 mM phosphate buffer (pH 7) while utilizing continuous UV detection at 280 nm.

Modification of cytochrome c with FSBA

Ten milligrams of horse ferricytochrome *c*, devoid of any of its deamidated forms, was dissolved in 1 mL of 10 mM *N*-ethylmorpholine-HCl buffer (pH 8) containing 0.5 mg FSBA (0.8 mM cytochrome *c* and 0.9 mM FSBA). The sample was incubated at room temperature for 3 h and loaded onto a Trisacryl SP ion-exchange column. The sample was treated with ferricyanide and eluted with 500 mL of 50–100 mM phosphate buffer (pH 7). The eluant was continuously monitored at 280 nm. Each of the above affinity-labeling reactions was also applied in the presence of 10 mM ATP and 150 mM NaCl, and to DHCH-arginine[91] cytochrome *c*.

Redox potential

The redox potentials of the adducts were determined by the method of mixtures as discussed by Wallace et al. (1986).

Spectroscopy

UV-visible spectra in the 220–750 nm range were drawn with either a Beckman DU-40 or DU-65 spectrophotometer. Difference spectra were made of the adducts, and absorbance coefficients were determined by peak ratio to that of cytochrome *c*'s Soret band, which has an absorbance coefficient at 410 nm of 106,100 M^{-1} cm^{-1}. The decrease in the intensity of the 695 nm band with increasing pH from 7 to 11 was followed as discussed by Wallace (1987).

CNBr cleavage

Cytochrome *c* was incubated for 24 h in 70% formic acid containing 20 mg/mL CNBr (190 mM). The sample was fractionated by gel filtration chromatography on Sephadex G-50 with elution with 7% formic acid. Fragments 1–65, 66–80, and 81–104 were further purified by ion-exchange high-performance liquid chromatography on a Waters phenyl SP5PW cation exchange column using 4–400 mM phosphate (pH 7) for elution. Spectra of the purified fragments were drawn with a Beckman DU-65 spectrophotometer.

Biological assays

The adducts were tested for their abilities to restore oxygen consumption in cytochrome *c*-depleted mitochondria using the succinate oxidase assay of Jacobs and Sanadi (1960) as discussed by Wallace and Proudfoot (1987).

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