Studies of 8-Azido-ATP Adducts Reveal Two Mechanisms by Which ATP Binding to Cytochrome *c* Could Inhibit Respiration†

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ABSTRACT: We have proposed that the binding of ATP at a site of substantial affinity and specificity could regulate the activity of cytochrome *c* with its physiological partners and thus the overall efficiency of mitochondrial electron transport. We now describe the use of ATP affinity-labeled protein to test the effect of occupancy of that site, which includes the invariant arginine 91, on the activity of cytochrome *c* with purified cytochrome *c* reductase and oxidase and its association with the mitochondrial inner membrane. Electron-transfer activities with the reductase and oxidase were inhibited by site occupancy to 41% and 11−15% of native values, respectively. The marked difference in the degree of inhibition of activity that distinguishes the reactions with the two major physiological partners was sufficient to cause, in whole mitochondria, a demonstrable shift from a situation in which there is a rate-limiting transfer from the reductase to cytochrome *c*, to a state where rates are more evenly matched for transfers between cytochrome *c* and the two redox partners. Site occupancy also substantially reduces the ionic strength necessary for half-maximal dissociation of cytochrome *c* from the membrane. These data imply that the decreased efficiency of electron transfer caused by ATP attachment can be attributed to a decrease in the protein's activity with individual physiological partners, possibly compounded with a decrease in its affinity for the inner mitochondrial membrane, and suggest that feedback regulation by ATP of cellular respiration operates in like manner.

The control of mitochondrial respiration rate and oxidative phosphorylation is fundamental to cellular energy metabolism and has attracted intense scrutiny from a number of groups [for reviews, see Tager et al. (1983), Kacser (1987), Brand & Murphy (1987) and Balaban (1990)]. The development of understanding in this area is based on metabolic control analysis (Kacser & Burns, 1973; Heinrich & Rapoport, 1974), and although the field remains controversial, there is general agreement that (a) control is shared among a number of regulatory sites within the respiratory and phosphorylation systems (Groen et al., 1982; Padovan et al., 1989) and (b) the relative contribution of each of these control points to the overall process is highly dependent on the metabolic status of the mitochondrion, e.g., whether in state 3 (excess of ADP) (Tager et al., 1983; Hafner et al., 1990), or whether isolated or in intact cells (From et al., 1990). Except in state 3 (conditions which are possibly not physiologically relevant), respiration rates correlate well with cellular phosphorylation potential (Wanders & Westerhoff, 1988), but the mechanisms by which this parameter is detected by the various control points have not been fully elucidated.

We have been concerned for some time (Wallace & Rose, 1983; Corthesy & Wallace, 1986, 1988; Craig & Wallace, 1991, 1993) to clarify the role of arginine 91 in cytochrome *c* function. Although absolutely conserved in all mitochondrial cytochromes *c*, the residue is dispensible to the basic function of the protein as electron shuttle between respiratory complexes III and IV (Rose & Wallace, 1983). It was subsequently found to be part of an ATP binding site of substantial affinity and specificity that is functional in all species tested and at physiological ionic strength and [ATP] (Craig & Wallace, 1991, 1993). The position of cytochrome *c*, at the outer face of the inner mitochondrial membrane and in the intermembrane space, locates it in a space continuous, at least in small molecule terms, with the bulk of the cytoplasm. Thus, it could in principle transduce cytoplasmic signals to the respiratory chain. Indeed, it is known that the rate of oxidation of cytochrome *c* by complex IV is sensitive to the concentration of polyanions, in particular ATP (Ferguson-Miller et al., 1976; Smith et al., 1980). The data have normally been interpreted in terms of an interaction between the nucleotide and cytochrome oxidase, and indeed there exists a body of evidence that ATP has a direct regulatory effect on oxidase (Kadenbach, 1986), but it is also recognized that the binding of ATP to cytochrome *c* could be influential, too (Osheroff et al., 1980).

We have canvassed a range of ATP analogues as affinity labels that would lead to charging of the binding site on cytochrome *c* in the absence of free nucleotide, in order to avoid nonspecific effects or interaction with other respiratory chain components, and found the most useful to be 8-azido-ATP (Craig & Wallace, 1993). We now report the effect that this stabilized interaction has on the efficiency of electron transfer in the oxidative phosphorylation system and isolated respiratory components, and upon the affinity of the cytochrome for the intact inner mitochondrial membrane.

The results of these assays support our initial hypothesis that cytochrome *c* represents a site for feedback inhibition of respiration by ATP and permit some consideration of the mechanisms by which the signal is transduced. Most simply,

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ATP binding to cytochrome *c* may render it less reactive with individual components of the respiratory chain. This possibility can be easily checked by assays of isolated reductase (complex III) and oxidase (complex IV) with the cytochrome *c*−ATP adduct.

It is also possible that the interaction modulates the availability of the cytochrome to the respiratory chain. The most widely accepted view, following Chance et al. (1970), is that two pools of cytochrome *c* exist within mitochondria: one free in the intermembrane space which reacts rapidly with the soluble cytochrome *c* peroxidase and slowly with membrane-bound cytochrome *c* oxidase and the second, membrane-associated, reacting rapidly with the oxidase and slowly with the peroxidase (Pettigrew & Moore, 1987). Studies involving the outer membrane-associated NADH−cytochrome *c* reductase (Bernardi & Azzone, 1981) and the intermembrane space sulfite oxidase (Matlib & O'Brien, 1976) agree with this two-pool model. Matlib and O'Brien (1976) propose that physiological conditions may exist whereby the affinity of cytochrome *c* for the inner membrane may be altered, thus altering its ability to transfer electrons to or from the intermembrane space and outer and inner membrane-associated metabolic systems.

Cytochrome *c* is positively charged, and its interactions with the inner membrane and associated redox partners are electrostatic in nature (Pettigrew & Moore, 1987). Upon binding a negatively charged ATP molecule, resulting in a decrease in the net positive charge of the protein, it might be expected that the affinity of the protein for the membrane would be decreased, shifting the distribution between pools.

However, the two-pool model has been called into question. Through the use of a fluorescent probe, the ionic strength of the intermembrane space has been estimated to be similar to that of the cytosol, which is 100−150 mM (Cortese et al., 1991). At physiological ionic strength Gupte and Hackenbrock (1988) propose that little if any cytochrome *c* remains bound to the inner mitochondrial membrane but that it is rather entirely free in the intermembrane space. We have therefore made direct determination of the affinity of both cytochrome *c* and adducts for the inner membrane at a wide range of ionic strengths and tested the effect of this parameter on the availability of cytochrome *c* to either the membrane-bound succinate oxidase (complex II)−cytochrome *c* reductase (complex III) assembly or the soluble sulfite oxidase, in order both to clarify this issue and to quantitate the effect of ATP binding upon cytochrome *c* distribution between pools.[1]

EXPERIMENTAL PROCEDURES

Materials

Horse heart cytochrome *c*, 8-azido-ATP, hexokinase, cholic acid, deoxycholic acid, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD), and 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone (decylubiquinone) were obtained from Sigma, and Tris and ATP were from Boehringer Mannheim. Cacodylic acid, KCl, glucose, K_2HPO_4, ammonium acetate, diethyl ether, and cyclohexane were from Fisher Scientific. Column chromatography resins were purchased from Pharmacia LKB. Potassium ferricyanide, $MgCl_2$, and Tween 80 were bought from Baker, and ethanol was obtained from Consolidated Alcohols Ltd. Sucrose, $(NH_4)_2SO_4$, and HCl were purchased from Anachemia, and the protein assay kit was from Bio-Rad. Sodium succinate was obtained through MC&B; MOPS was from ICN; and acetic acid, ascorbic acid, sodium borohydride, and NaCl were from BDH.

Methods

Modification of Cytochrome c with 8-N_3ATP. Ten milligrams of horse ferricytochrome *c*, purified by cation-exchange chromatography to remove any deamidated forms, was dissolved with 0.5 mg of 8-N_3ATP (molar ratio, 1:1) in 1 mL of 5 mM Tris−cacodylate buffer (pH 6.95). The sample was irradiated with 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 onto 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).

Preparation of Mitochondria. A rat liver was homogenized in 8.5% sucrose, and cellular debris was removed by centrifugation at 600*g* for 10 min at 4 °C. Mitochondria were pelleted from the supernatant by centrifugation at 8500*g* for 10 min at 4 °C. The pellet was resuspended in 8.5% sucrose, the 8500*g* centrifugation cycle was repeated twice, and the pellet was resuspended in 8.5% sucrose (Jacobs & Sanadi, 1960).

Rupturing of the Outer Mitochondrial Membrane and Removal of the Endogenous Cytochrome c. The pellet obtained from the last centrifugation of the mitochondria preparation was drained of all of its sucrose and resuspended in 15 mM KCl for 10 min at 4 °C to rupture the outer membrane by hypotonic shock. The suspension was centrifuged at 6000*g* for 10 min at 4 °C. The supernatant contained the soluble components of the mitochondrial intermembrane space, including the enzyme sulfite oxidase, for which cytochrome *c* is electon acceptor (Pettigrew & Moore, 1987), and was stored at 4 °C. The pellet was resuspended in 150 mM KCl for 10 min to allow the endogenous cytochrome *c* to dissociate from the inner membrane (Jacobs & Sanadi, 1960). The centrifugation and resuspension in 150 mM KCl was repeated twice. The cytochrome *c*-depleted mitochondria were resuspended in 10 mM KCl.

Cytochrome c Mitochondria Binding Assay. Equal volumes of cytochrome *c*-depleted mitochondria suspended in 10 mM KCl were aliquoted; to each was added cytochrome *c* and concentrated KCl, and the volumes were adjusted such that all aliquots had a cytochrome *c* concentration of 1 μM, KCl concentrations that ranged from 10 to 160 mM KCl, and identical volumes. The samples were equilibrated for 20 min and centrifuged at 6000*g* for 10 min at 4 °C. Cytochrome *c* concentrations in the supernatants were determined spectrophotometrically by the height of the cytochrome *c* Soret band over baseline.

Purification and Assay of Cytochrome c Oxidase. Bovine cytochrome *c* oxidase was purified by the method described in Darley-Usmar et al. (1987). The final supernatant

[1] Enzymes: Ferrocytochrome *c*:O_2 oxidoreductase (E.C. 1.9.3.1), ubiqinol:ferricytochrome *c* oxidoreductase (E.C. 1.10.2.2), and sulfite: ferricytochrome *c* oxidoreductase (E.C. 1.8.2.1).

consisting of purified oxidase (0.25 nmol/μL) dissolved in 20 mM potassium phosphate (pH 7.4) containing 0.5% (v/v) Tween 80 was aliquoted and stored at −20 °C.

Reaction of purified oxidase with cytochrome *c* and adducts was followed using a Clark-type oxygen electrode as described in Darley-Usmar et al. (1987). Five microliters of purified oxidase (1.25 nmol) was added to 5 mL of 50 mM MOPS containing 0.3% Tween 80 (pH 7.4). Seventy microliters of 0.5 M ascorbate (pH 5) and 30 μL of 50 mM TMPD were added, and a steady baseline oxygen consumption rate was reached. Recrystallization of the TMPD in acidified 80% ethanol is necessary to ensure a low baseline oxygen consumption rate. Multiple injections of 1−5 μL of 10−20 μM cytochrome *c* were made, and the oxygen consumption rate was determined after each injection. The relatively low cytochrome *c*:cytochrome *c* oxidase ratios ensure that it is the high-affinity phase of the reaction that is observed (Darley-Usmar et al., 1987). O_2 consumption (electron-transport rate) is plotted against total cytochrome *c* concentration for each addition. Initial rates obtained from these curves are expressed as a percentage of that with native cytochrome *c*.

Purification and Assay of Cytochrome c Reductase. Bovine cytochrome *c* reductase was purified from the same fresh beef heart used for the oxidase preparation, by a distinct protocol described by Darley-Usmar et al. (1987). The final reductase pellet was suspended in 50 mM Tris-HCL (pH 8.0) containing 0.67 M sucrose and stored at −20 °C.

This preparation was assayed by the spectroscopic method described in the same work (Darley-Usmar et al., 1987). Ten micromoles of decylubiquinone was dissolved in 1 mL of ethanol and adjusted to pH 2. A few crystals of $NaBH_4$ were added, followed by 1 mL of dH_2O. The solution was vortexed with 3 mL of 2:1 diethyl ether/cyclohexane, and the upper phase was collected and vortexed with 1 mL of 2 M NaCl. The upper phase was dried with N_2 and redissolved in 1 mL of ethanol (pH 2).

Five microliters of purified cytochrome *c* reductase was added to 0.75 mL of 50 mM Tris−cacodylate (pH 7) containing 1−15 μM cytochrome *c*. Five microliters of ubiquinol solution was added, and the absorbance at 550 nm was followed using a Beckman DU-65 spectrophotometer. For this assay plots of ΔA_{550} versus total added cytochrome were used to obtain initial rates for comparison with that of native protein.

Equilibrium Redox State of Cytochrome c in Active Mitochondria. Assay buffer containing 300 mM sucrose, 75 mM glucose, 1 mM $MgCl_2$, 12 mM K_2HPO_4, 11 mM succinate, 2 mM ADP, and 3.5 mg/100 mL hexokinase (pH 7) was prepared. An aliquot of cytochrome *c*-depleted mitochondria suspended in 8.5% sucrose was added to buffer to make a final volume of 1 mL. Native or 8-azido-ATP-modified cytochrome *c* was added to a final concentration of 1 μM. Spectra were taken from 350 to 450 nm with a Beckman DU-65 spectrophotometer at 3-min intervals until O_2 was depleted. As wavelength controls, spectra were also drawn of fully reduced (dithionite-treated and desalted) or fully oxidized (ferricyanide-treated and desalted) ion-exchange-purified cytochrome *c* or adduct added to depleted mitochondria in the standard assay buffer in either the presence of cyanide or the absence of succinate.

Succinate Oxidase Assay. The succinate oxidase activity of mitochondria, with intact outer membranes and with their endogenous cytochrome *c* retained, was determined by following the rate of oxygen consumption with an O_2 electrode (Jacobs & Sanadi, 1960). Mitochondria were suspended in 300 mM sucrose containing 50 mM glucose, 2 mM ADP, 2 mM phosphate, 10 mM sodium succinate, and 3.5 mg of hexokinase/100 mL (pH 7). Variable amounts of 300 mM sucrose were replaced by equal volumes of 150 mM KCl to determine the effects of increased ionic strength on activity.

Sulfite Oxidase Assay. The effect of increased ionic strength on the sulfite oxidase activity of intact mitochondria via endogenous cytochrome *c* was monitored in the same way as for the effect on the succinate oxidase assay. The buffer employed was identical, save for the replacement of the 10 mM succinate with Na_2SO_3. As a control of the effect on increasing [KCl] on the intrinsic activity of sulfite oxidase, the reduction of cytochrome *c* was followed spectrophotometrically at 550 nm using the intermembrane space extract obtained from centrifugation of osmotically shocked purified mitochondria as described above. The assay buffer contained 300 mM sucrose, 75 mM glucose, 1 mM $MgCl_2$, 12 mM K_2HPO_4, and 10 mM sodium sulfite, adjusted to pH 7. To this was added cytochrome *c* to a final concentration of 4 μM and KCl to give a concentration of 30, 80, 130, or 230 mM. An aliquot of the crude sulfite oxidase (5 μL/mL of assay mixture) was added and rapidly mixed, and the absorbance increase was followed at 550 nm for 10 min. Initial rates of ΔA_{550} were compared.

RESULTS AND DISCUSSION

Adduct Preparation and Characterization. The reaction of 8-N_3ATP with cytochrome *c* proceeded as previously described (Craig & Wallace, 1993), resulting in preparations of two isomers (peaks I and II). Formation of both is abolished by prior modification of arginine 91 (D. B. Craig and C. J. A. Wallace, unpublished results), so it was presumed that they represent reaction with alternative side chains within the binding site, since the nitrenes generated by UV irradiation of azides are catholic in their reactivity. Slight differences in charge distribution on the resulting adducts, and hence position of the nucleotide, are signaled by the ability to resolve them by cation-exchange chromatography, despite their having the same net charge (Craig & Wallace, 1993) and the small variation in biological properties noted below.

We have previously noted the instability of the bonding of these labels to cytochrome *c* under cleavage conditions used prior to peptide mapping and that what residual label was detectable was located on the 81−104 CNBr fragment. The same problem was noted by Lin et al. (1995), who were unable to determine the site of attachment, and is not unique to cytochrome *c*: Wright and Walsh (1993) used [^{32}P]-8-azidoATP to label two ligases but could not recover labeled peptide. However, it has proved possible to identify the lysine residue in the ATP binding site of the sarcoplasmic reticulum Ca^{2+}-ATPase using the related 2′,3′-*o*-(2,4,6-trinitrophenyl)-8-azido-AMP (McIntosh et al., 1992). With cytochrome *c*, this agent reacts to give a pattern of modification identical (though displaced due to the lower net charge change) to that observed on ion-exchange chromatography for the 8-N_3ATP adducts (D. B. McIntosh and C. J. A. Wallace, unpublished data). Reaction is inhibited by free

Table 1: Biological Activities of the 8-Azido-ATP-Modified Cytochrome *c* Adducts[a]

	intact chain[b]	complex III[c]	complex IV[d]
native	100	100	100
8-N_3ATP pk1	16 ± 6	41 ± 6	15 ± 7
8-N_3ATP pk2	15 ± 6	41 ± 6	11 ± 3

[a] The activities of the adducts are computed from initial slopes to Michaelis−Menten plots (equivalent to K_{cat}/K_m) and are tabulated relative to native cytochrome *c* for succinate oxidase, complex III, and complex IV assays. See Experimental Procedures for details of the assays. [b] From Craig and Wallace (1993). [c] Average of four assays with two separate reductase preparations. [d] Average of 12 assays with two separate oxidase preparations.

ATP. The TNP-AMP adducts can be cleaved by thermolysin, and labeled peptides may be isolated on reversed-phase HPLC. Sequences obtained from the major peptide peaks are as follows: for isomer I, IXKK (Lys[86] labeled): for isomer II, IKXKT (Lys[87] labeled). The reason for the greater stability of the TNP-containing adducts is not known, but the advantage is clear. The instability in mapping and sequencing procedures of the 8-N_3ATP adducts raises questions concerning stability in the functional studies described in this paper. We have examined this issue by storing adducts in frozen solution at −20 °C for up to 2 years and then checking chromatographic properties on Memsep (Millipore) ion-exchange membrane cartridges. In one sample of isomer I, 30% of the adduct appeared unchanged, while 40% of the material eluted at the native cytochrome *c* position, implying loss of all phosphate and possibly nucleoside derivative; another 30% was at intermediate positions, implying phosphate loss. In a sample of isomer II, >80% was unchanged triphosphate adduct. Despite this evidence of substantial stability to storage, adducts were prepared fresh before each round of experiments.

Biological Properties of ATP−Cytochromes c. Three types of biological assay have been used to compare the electron-transfer efficiency of cytochrome *c*−ATP adducts and the native protein. The comparisons are summarized in Table 1: data for the depleted-mitochondria assay (succinate oxidase) have been reported previously (Craig & Wallace, 1993). The percentages given are for initial slopes to the K_{et} vs [cyt *c*] curves and are thus equivalent to relative K_{cat}/K_m values, or catalytic strength. This is the standard method of comparing data obtained from the depleted mitochondria assays. We have chosen to adopt the same approach with the oxidase and reductase assays for comparability. In addition, calculating reliable individual V_{max} and K_m values requires obtaining data up to very high substrate (cytochrome *c*) concentrations. This is both physiologically irrelevant, given the normal ratios of cytochrome *c* and physiological partners in the mitochondria, and difficult in the absence of large quantities of purified adducts. Notwithstanding this difficulty, Lin et al. (1995) have made Eadie−Hofstee plots and measured these parameters for oxidase under comparable conditions and also observe very substantial decreases in electron transport. Examples of Michaelis−Menten plots for the oxidase and reductase reactions are shown in Figure 1.

Both oxidase and reductase activities are affected by the attachment of ATP at the Arg[91]-containing binding site, since both complexes bind to the same surface of cytochrome *c* and this region includes or is directly adjacent to Arg[91]

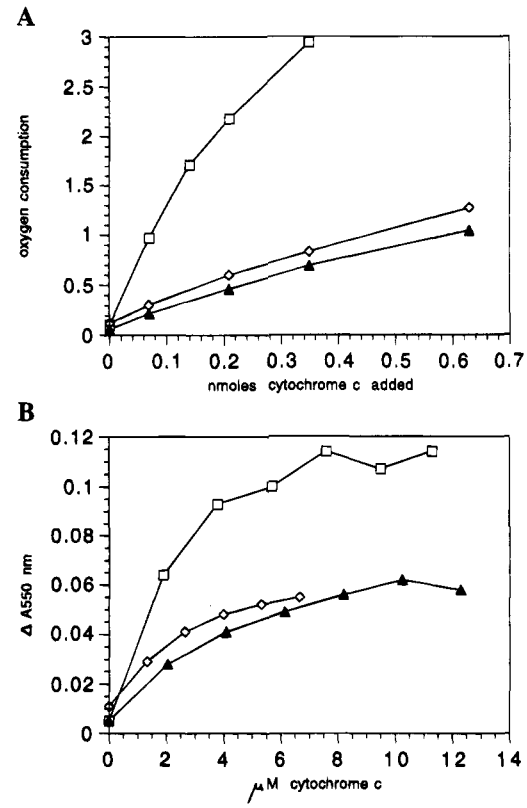

FIGURE 1: Assays of native and ATP-ligated cytochromes with isolated components of the mitochondrial respiratory chain. (A) Polarographic assay of ferrocytochrome oxidation by isolated complex IV (oxidase): (□) native horse cytochrome *c*; (◇) isomer I of cytochrome *c*−ATP adduct; (▲) isomer II. Oxygen consumption is expressed at % total dissolved O_2/min. (B) Spectrophotometric assay of ferricytochrome reduction by complex III (reductase). Symbols as in (A). Details of the assays are given in the text.

(Rieder & Bosshard, 1980). However, the marked difference between them in the amount of inhibition under comparable conditions produced by site occupancy implies that the elements of interaction on the surface of the oxidase and reductase differ from one another, in either their composition or their sensitivity to external influences. Binding to either partner is mediated largely by electrostatic influences (Margoliash & Bosshard, 1983), and it seems likely that it is the perturbation of individual charge−charge interactions by ATP, or an influence on the dipole axis that "steers" the cytochrome *c* to its target and orients it there, that leads to the rate reduction.

Two of the residues that are implicated in the orientation and docking of cytochrome *c* are lysines 86 and 87, which we have tentatively assigned as the attachment points of ATP in the two adduct isomers studied. This observation immediately raises the issue of whether the effects on biological properties of adduct formation are in fact due to the surface association of ATP or are simply a consequence of chemical modification of these crucial lysine residues that affects their electrostatic interactions. It should first be noted that it is far from certain that attachment of the affinity label will modify charge. The product of nitrene reaction with amino groups is suggested to be a substituted hydrazine (Knowles, 1972) which might well be substantially protonated at pH 7.4 [p*K*(hydrazine) > 8)]. An important piece of evidence is provided by Lin et al. (1995). They also prepared the cytochrome *c* adduct with 8-N_3ADP. This exhibits kinetic and affinity properties substantially different from the ATP-

containing adducts, implying that the primary influence on these parameters is the electrostatic properties of the nucleotide, not the attachment point. This conclusion is supported by the observed decrease in reductase activity. It has been noted that at low ionic strengths the effect of lysine modifications on the reductase−cytochrome *c* transfer is small (Speck & Margaliash, 1984), so that under our assay conditions even attachment that results in a charge change on a lysine residue should not produce a near 60% decrease in activity.

Nevertheless, it seems likely that the effect of free ATP on binding and electron transfer between cytochrome *c* and oxidase (which could be mediated *via* either or both proteins) is less than that induced by adduct formation (Ferguson-Miller et al., 1976; Lin et al., 1994; Craig, 1993). There are difficulties involved in making such comparisons: apart from direct effects on oxidase activity, it is likely that oxidase will sequester a portion of the ATP in the system (Huther & Kadenbach, 1987) and possible that oxidase preparations have residual ATPase activity. It is for these reasons that the cytochrome *c*−ATP adducts were developed. However, it is important to recognize that the ATP in them is tethered and thus cannot be, for example, displaced from the binding site upon docking with oxidase. Thus, the adducts, while reflecting the effects of ATP binding on the interaction, may exaggerate them.

Effect of ATP Binding on Equilibrium Redox State of Cytochrome c in the Mitochondrion. The rate-determining step in succinate oxidase assays has been proposed to be the reduction of cytochrome *c* by the cytochrome bc_1 complex (Wallace, 1984; Wallace & Proudfoot, 1987). Indeed, since complex IV outnumbers complex III by 2:1 (Wikstrom & Saraste, 1984; Hackenbrock, 1981) and its maximum specific activity in isolated systems is 3 times that of the reductase (Wallace, 1984), total oxidase capacity is likely to be substantially greater than that of reductase. One problem with these comparisons is an uncertainty about how well isolated reductase (or indeed oxidase) reflects the behavior of the complex in the mitochondrial membrane. Therefore, spectroscopic determinations of the redox state of native cytochrome *c* and adducts in reaction with intact mitochondrial inner membrane were made as a check of these past and present observations.

If the inhibitory effect of ATP binding site occupancy is also more marked for oxidase in situ than it is for reductase, a higher intrinsic oxidase activity for the system should result in the effective activities of the two complexes with adduct becoming more similar to one another, or even a switch in rate determination from the reductase to the oxidase transfer. Assuming reaction with the reductase is the limiting step, the majority of the cytochrome *c* in steady-state coupled respiration will be in the oxidized form. A shift toward equality of redox partner activity due to ATP binding would consequently change the equilibrium redox state of the cytochrome *c* toward a higher proportion of the reduced form. The redox states of 1 μM native and 1 μM 8-azido-ATP cytochromes *c* in a suspension of active mitochondria were determined by the position of the absorption maximum of the Soret band. The absorbance maxima in respiring and control systems are presented in Table 2.

Under these experimental conditions, at zero time, native cytochrome *c* was found to have an absorbance maximum at 410 nm (Figure 2), which shifted to 414 nm between 9 and 12 min of incubation, at the same time that oxygen was depleted in a separate run monitored by an oxygen electrode using a sealed cell. Thus equilibration of the ferri-/ferrocytochrome *c* ratio occurs during the mixing time of <10 s and, as anticipated, native cytochrome *c* showed a redox state favoring the oxidized form (by a ratio of 8:3) in the succinate oxidase assay system employed. This clearly confirms the expectation that total oxidase activity outweighs that of the reductase in this assay system.

Table 2: Absorbance Maxima of the Soret Band of Cytochrome *c* and Adducts[a]

cytochrome species	wavelength (nm)
in phosphate buffer	
ferricytochrome *c*	408.5[b]
ferrocytochrome *c*	414[b]
iso I/II adducts Fe^{3+}	408.5
iso I/II adducts Fe^{2+}	414
ferrocytochrome *c* + CN^-	414[c]
in assay buffer with depleted mitochondria	
cytochrome *c* (initially Fe^{2+} or Fe^{3+}) (buffer contains CN^-)	414
cytochrome *c* (initially Fe^{2+} or Fe^{3+}) (buffer succinate omitted)	408.5
in respiring mitochondria (Figure 2)	
cytochrome *c*−ATP adduct iso I	412
cytochrome *c*−ATP adduct iso II	412
cytochrome *c*	410

[a] Spectra were drawn on a Beckman DU65 spectrophotometer from 390 to 430 nm. Ferri- and ferrocytochrome control samples were oxidized with $K_3Fe(CN)_6$ or reduced with $Na_2S_2O_4$ and desalted. In mitochondria-containing controls Fe^{2+}-cyt *c* was kept reduced by CN^- poisoning of oxidase, or Fe^{3+}-cyt *c* was kept oxidized by omission of succinate. [b] Margoliash and Frohwirt (1958) report values of 410 and 416 nm. The discrepancy is probably due to instrumentation differences. [c] Ferrocytochrome *c* absorbance is not affected by the presence of cyanide in the buffer.

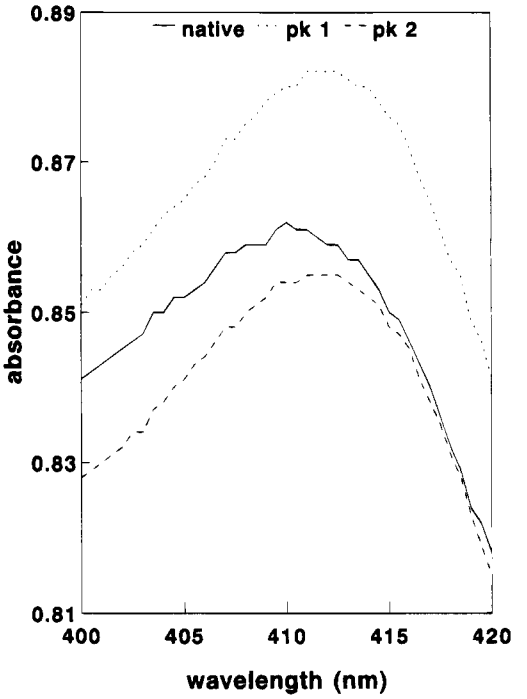

FIGURE 2: Shift in the equilibrium redox state in respiring mitochondria of cytochrome *c* upon ATP ligation shown by the change in the wavelength of maximum absorbance by the Soret band.

In the same system, both isomers of the 8-azido-ATP−cytochrome c adduct showed absorbance maxima at 412 nm (Figure 2) followed by an eventual shift toward a more reduced state upon depletion of available oxygen. (In this case, oxygen depletion required >24 min to occur due to the decreased overall respiration rate supported by the adducts.) Once again the zero time maximum remained unchanged until O_2 was depleted. An absorption maximum at 412 nm equates to a 4:7 ratio of ferri- and ferrocytochrome c, implying that the effective activities with reductase and oxidase remaining after inhibition by occupancy of the binding site are substantially changed by ATP binding in the intact mitochondrial membrane, too, and the differential inhibition noted for the isolated complexes represents the physiological reality.

Simple calculations based on these differential changes in activities and the shift in equilibrium ferri- and ferrocytochrome c concentrations might lead one to expect less overall inhibition of electron flow than is actually observed. Although this difference might still be accounted for by the uncertainties surrounding the physical state and the responsiveness of isolated vs membrane-bound components, we have considered the possibility that direct steric or electrostatic effects on the interaction of cytochrome c with either partner is not the sole contributor to inhibition of electron flow by ATP binding, and have looked at how binding might modulate cytochrome c pool size.

Ionic Strength Effects on Cytochrome c Pool Size and Reactivity and the Influence of Bound ATP. The distribution of native and 8-azido-ATP-modified cytochrome c between the free and bound forms at varying [KCl] was measured in order to determine the effect of site occupancy on the protein's affinity for the inner membrane. Cytochrome c bound to the inner membrane pellets with the mitochondria, whereas that free will be detected in the supernatant. As a control, cytochrome c-depleted mitochondria, to which no cytochrome c had been added, were centrifuged in the presence of 160 mM KCl, and no residual cytochrome c (<50 nM) was detected in the supernatant, indicating that removal of the endogenous cytochrome c was complete. A similar method was utilized by Gupte and Hackenbrock (1988), who report that virtually all of the cytochrome c is dissociated within the putative physiological range of 100−150 mM. However, this protocol included 10−25 mM phosphate in the suspension. Cytochrome c binds phosphate (Corthesy & Wallace, 1986) quite strongly at the site under investigation, and this binding is likely to partially mimic the effect of ATP binding described below. Hence it is likely that the distribution reported by these authors reflects a decreased affinity. When assayed in the *absence* of anions which bind strongly to cytochrome c, we find that although in the range of 100−150 mM KCl the majority of the protein is indeed free, a significant proportion remains bound to the inner membrane (Figure 3). This is consistent with the two-pool model proposed by Chance et al. (1970).

Occupancy of the Arg^{91}-containing binding site causes the affinity of cytochrome c for the inner membrane to decrease. The native protein requires 80 mM KCl to cause 50% dissociation, whereas the 8-azido-ATP-modified cytochromes c require only 20−40 mM (Figure 3). At the putative ionic strength of the intermembrane space of 100−150 mM, virtually all of the modified protein is dissociated, whereas a significant proportion of the native protein remains bound.

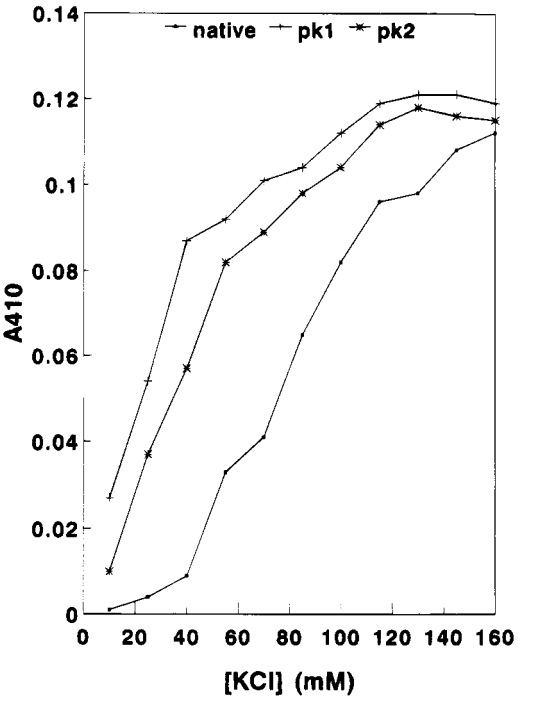

FIGURE 3: Cytochrome c binding to mitochondria. The Soret band absorbances over baseline of native and 8-azido-ATP-modified cytochrome c peaks 1 and 2 (pk1 and pk2) are given for the supernatants of centrifuged mitochondrial suspensions in solutions of variable [KCl].

Table 3: Effect of Changing [KCl] on Sulfite Oxidase Activity of Mitochondrial Intermembrane Space Contents[a]

KCl concn[b] (mM)	enzyme act.[c]
30	0.0165
80	0.0150
130	0.0075
230	0.0045

[a] This fraction was prepared by centrifugation of osmotically shocked mitochondria as described in the text. [b] Assays were performed in buffer containing 12 mM KH_2PO_4 and 10 mM Na_2SO_3 in addition to the indicated [KCl]. [c] Activity was determined as initial slopes to plots of ΔA_{550} vs time. In the absence of SO_3^{2-}, cytochrome c is not reduced in this system, and there is no change in A_{550}.

Succinate oxidase assays, which measure respiratory flow from complexes II to IV, were performed on mitochondria with intact outer membranes and retaining their endogenous cytochrome c in the presence of varying [KCl] in order to determine the effects of cytochrome c dissociation on its ability to support respiration. Removal of cytochrome c into the intermembrane space would also be expected to increase the rate of sulfite oxidation, which involves the transfer of electrons from the intermembrane space complex sulfite oxidase (sulfite:ferricytochrome c oxidoreductase) to cytochrome c. Thus sulfite oxidase assays were also performed in the presence of varying [KCl]. So as to avoid any osmotic effects of varying the ionic strength on the mitochondria, KCl was exchanged for sucrose at a 1:2 molar ratio. To check for possible effects of K^+ or Cl^- on the enzyme, its activity in the absence of mitochondria was examined over a range of [KCl]. No significant change occurred when [KCl] was increased from 30 to 80 mM. Further increases caused a substantial decline in activity (Table 3).

We found a greater than 75% decrease in succinate oxidation with a parallel more than tripling in the rate of

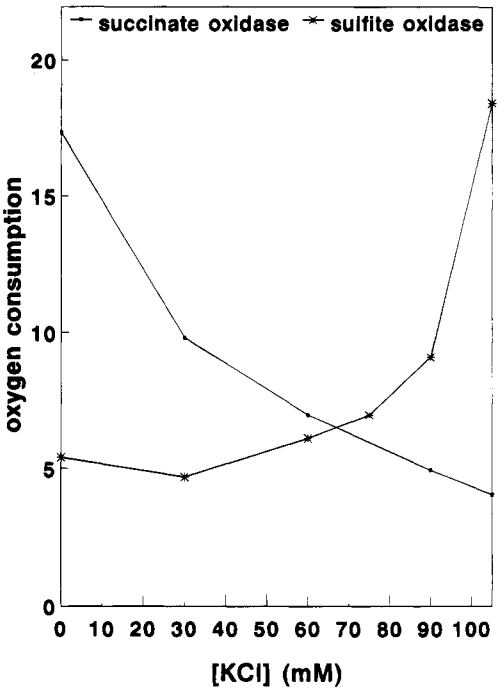

FIGURE 4: Effect of cytochrome *c* dissociation from the inner mitochondrial membrane on its activity in sulfite and succinate oxidase assays. The effects of added KCl on the rate of O_2 consumption (expressed as % of total dissolved O_2/min) in sulfite and succinate oxidase assays are shown.

sulfite oxidation as [KCl] increases (Figure 4) that is clearly not due to direct effects on the enzyme of the increasing [KCl] (Table 3). This is consistent with the proposal that intermembrane space and inner membrane-bound cytochrome *c* pools are distinct with respect to their activities with these two complexes and that dissociation of the protein causes a decrease in its ability to support respiration. Sulfite oxidase activity, measured polarographically, depends on electron transfer from cytochrome *c* to membrane-bound oxidase. The increased throughput under dissociating conditions implies that this latter step is also far from limiting for sulfite oxidation.

Dissociation of cytochrome *c* from the inner membrane will make the protein more accessible to the intermembrane and outer membrane associated redox partners. However, these complexes bind to the same surface on cytochrome *c* as do complexes III and IV, and one would therefore expect that the binding of ATP might also cause some degree of inhibition of their activity. The net effect on overall activity with these secondary partners has not yet been determined.

The similarity between the effects of increases in [KCl] in the cytochrome *c* mitochondria binding assay (Figure 3), which utilizes mitochondria with disrupted outer membranes, and the sulfite and succinate oxidase assays (Figure 4), which utilize mitochondria with intact outer membranes, is consistent with the proposal that the ionic strength of the intermembrane space is continuous with that of the cytosol (Cortese et al., 1991) and suggests that the dissociation of the cytochrome and the diminution of respiration are related phenomena.

The Regulatory Role of Cytochrome c in Respiration. The experiments here reported show that inhibition of cytochrome *c* activity in whole mitochondria can be attributed to two consequences of ATP binding. The equilibrium between cytochrome *c* bound to the inner membrane and free in the intermembrane space is shifted toward dissociation. Such a shift can decrease the frequency of collisions with the protein's physiological partners and correspondingly its activity. The effects of this phenomenon are compounded with a decreased ability to transfer electrons to and from those physiological partners, possibly due to alternative docking conformations for the protein with or without ATP (Lin et al., 1995) and probably related to a shift in dipole axis caused by the triphosphate moiety (Craig & Wallace, 1993), so that in an ATP-charged system cytochrome *c* could prove a very effective regulator of overall respiratory rate.

The proposition that the modification of docking conformation is more likely to be due to electrostatic (dipole axis shift) than to steric effects of the added bulk in the contact interface is supported by the marked difference between the steady-state kinetic parameters for the ADP-containing and the ATP-containing adducts.

The data of Lin et al. (1995) and our own observations suggest that the effects of free ATP may be less marked, on binding and electron transfer, than those of the tethered nucleotide in the adduct. We have discussed above the reasons for thinking that this is not a consequence primarily of lysine modification. The crucial difference probably lies in the permanency of tethering versus the transience of the non-covalent interaction, where the ATP may be competed away by the approaching physiological partner. This temporary shielding of the same surface of the cytochrome as that masked in the adduct can be expected to achieve, albeit to a lesser degree, the same functional effects as those we observe. Despite this effective exaggeration, the difficulties inherent in direct studies of the free ATP−cytochrome *c* interaction in the presence of physiological partners mean that these adducts should continue to be useful models of the intriguing non-covalent interaction.

How do these observations on the regulatory potential of the interaction fit the current consensus on respiratory control? Feedback inhibition by ATP at cytochrome oxidase is easy to contemplate, since the oxidase has been identified as a regulatory site having a high control coefficient. Cytochrome *c* has not received wide consideration as a control point, but the summation of control strengths under even the best-studied conditions (state 3) leaves room for other control elements (Groen et al., 1982). It may prove that under conditions of high metabolic stress, when both cytoplasmic phosphorylation potential and pH fall (ATP affinity is quite sensitive to pH change; Craig & Wallace, 1991), that rate restrictions normally exercised through cytochrome *c* are alleviated by the dissociation of ATP.

The widely held view that the majority of mitochondrial cytochrome *c* is loosely associated with the inner membrane, thus ensuring high-efficiency two-dimensional diffusion in transfers between reductase and oxidase mediated by cytochrome *c* (Salemme, 1977; Pettigrew & Moore, 1987), has been challenged (Gupte & Hackenbrock, 1988). Our data seem to support the latter position, in that at least the majority of the cytochrome will be dissociated at cytoplasmic ionic strength. The difficulty of assessing effective ionic strength in the intermembrane space is substantial. Nevertheless, it is reasonable to suppose that the dissociation of ATP in response to changing concentration and/or pH could tip the balance between negligible and significant association of cytochrome *c* with the inner membrane, thus providing the

amplification requirement of an efficient signal transduction system.

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