Definition of a Nucleotide Binding Site on Cytochrome c by Photoaffinity Labeling*

(Received for publication, April 12, 1996, and in revised form, May 13, 1996)

David B. McIntosh, Jonathan C. Parrish, and Carmichael J. A. Wallace

From the Medical Research Council Biomembrane Research Unit and Department of Chemical Pathology, University of Cape Town Medical School, Observatory 7925, Cape Town, South Africa and the Department of Biochemistry, Dalhousie University, Halifax, Nova Scotia, Canada B3H 4H7

We have used TNP-8N3-AMP (2'3'-O-(2,4,6-trinitrophenyl)-8-azidoadenosine monophosphate) and TNP-8N3-ATP to probe the ATP binding site(s) of cytochrome c. Irradiation of cytochrome c with close to stoichiometric amounts of TNP-8N3-AMP at low ionic strength derivatized approximately half of the protein, with the mono-derivatized species being associated with four peaks (B, 6%; C, 17%; D, 24%; E, 4%) eluted from a cation exchange column. Irradiation in the presence of ATP suggested that the main peaks C and D resulted from more specific nucleotide binding. Thermolysin digestion and TNP-peptide purification and sequencing revealed that peak C was associated with derivatization of mainly Lys-86 and to a lesser extent Lys-72 and peak D with mainly Lys-87 and less so with Lys-72. Minor peaks B and E could not be identified. TNP-8N3-ATP photolabeling produced similar results, showing favored interaction of the adenyl ring with Lys-86 and Lys-87 and to a lesser extent with Lys-72. The results are compatible with previous findings that suggest that the principal locus of ATP binding is at nearby Arg-91 (Corthesy, B. E., and Wallace, C. J. A. (1986) Biochem. J. 236, 359–364). Molecular modeling with energy-minimized docking of ATP between the 60s helix and the 80s stretch with the γ-phosphate constrained to interact with Arg-91, places the 8 position close to Lys-86 and Lys-87 in the anti conformation, the glycosidic bond and to Lys-72 in the syn conformation, and the ribose hydroxyls within H-bonding distance of Glu-69.

Cytochrome c plays a key role in mitochondrial respiration by mediating single electron transfer between the cytochrome bc1 and cytochrome oxidase complexes of the inner mitochondrial membrane (1). It is a small, positively charged, soluble protein with several lysyl residues implicated in its interaction with its redox partners (2). Cytochrome c also associates with the inner mitochondrial membrane, and it is this membrane-bound pool, probably complexed to acidic phospholipids, which may ensure rapid electron transfer between the membrane-embedded redox proteins (3, 4). The cytochrome c/redox partner and cytochrome c/membrane interactions are largely electrostatic and are dependent on anion binding and the ionic strength of the medium (5–7).

It has been known for some time that cytochrome c binds phosphate, ADP, and ATP and that anion binding is dependent on the redox state of the protein (8–10). More recently, gel filtration and equilibrium dialysis have indicated that ferro- and ferricytochrome c bind two and three molecules of ATP, respectively, at low ionic strength (11, 12), and both forms bind one at higher, closer to physiological, ionic strength (13). The binding of ATP is tighter than ADP.

Nucleotide binding modulates cytochrome c function. ATP causes the dissociation of cytochrome c and cytochrome oxidase (14). Derivatization of cytochrome c with 8-azo-ATP inhibits both the ability of the protein to exchange electrons with its redox partners and its association with the inner mitochondrial membrane (15–17). ATP displaces cytochrome c from liposomes constituted of acidic phospholipids (18, 19). It was proposed that the rate of electron transfer through the respiratory chain depends on the proportion of ATP-free cytochrome c, and ATP inhibition of cytochrome c function is a means of sensing and regulating the ATP/ADP status of the cell (13).

Two or three regions on cytochrome c have been implicated in phosphate and/or nucleotide binding. An early NMR study placed a phosphate, ADP, and ATP binding site in the Lys-25/His-26/Lys-27 locus (9). This region together with that around Lys-8 and Lys-87 has been linked to phosphate binding because of the influence of phosphate on the functional and CM-cellulose binding properties of protein derivatized on these lysyl residues (2, 20). Cationic residues around Thr-89 (Lys-86, -87, -88, and Arg-91) are implicated in phosphate-enhanced, chemical modification of this residue (22). This same region is associated with the more specific ATP binding site as derivatization of Arg-91 to 2,2'-dimethylpyrimidylornithine restricts ATP binding to the two nonspecific sites (11), and replacement of neighboring Glu-66 with lysine diminished the affinity of the site for ATP (23). The binding of ATP in this latter region, serving a regulatory function, would provide a satisfying explanation for why Arg-91 is absolutely conserved in all mitochondrial cytochromes c.

Reactive analogs of ATP have been used to prepare covalent adducts of nucleotide and protein. 8N3-ATP and 2',3'-dialdehyde ATP, under select conditions, derivatize cytochrome c in the vicinity of Arg-91, and lysyl residues are implicated in the reactions (15). 5'-Fluorosulfonylbenzoyladenosine appears to react elsewhere, perhaps at a nonspecific ATP site (15). Not only can such adducts be used in the study of the physiological role of ATP binding, but they have the potential to aid in triangulation of the binding site. Knowledge of the precise orientation of the nucleotide at the protein surface is essential to deciphering the mechanism by which binding can regulate electron transfer efficiency. However, identification of amino acid residues modified by 8N3-ATP has proved difficult (17).

* This work was supported by research grants from the Medical Research Council of South Africa and the Natural Sciences and Engineering Research Council of Canada. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Dept. of Biochemistry, Sir Charles Tupper Medical Bldg., Dalhousie University Halifax, Nova Scotia, Canada B3H 4H7. Tel.: 902-494-1118; Fax: 902-494-1355; E-mail: cwallace@sc.dal.ca.

1 D. B. Craig and C. J. A. Wallace, unpublished results.
ATP Binding Locus on Cytochrome c

and results herein), and therefore we turned to another related analog.

In this study, we use TNP$^2$-8N$_3$-AMP and TNP-8N$_3$-ATP as photoaffinity probes. TNP-nucleotides have been used extensively to probe ATP binding sites of proteins (24, 25, and references therein) because of their chromophoric and fluorescent properties and their tendency to bind tighter to ATP binding sites than ATP itself. The 8-azido-TNP derivatives have been used to locate different ATP binding modes of the Ca$^{2+}$-ATPase of sarcoplasmic reticulum at the catalytic site and to identify a lysyl residue at this site (26–28). They appear to be ideal photoaffinity probes, combining tight binding, absorption in the visible range, and an unusual chemical group that expedites purification of derivatized fragments of the target protein. The contact residues identified by use of these probes, together with the previously identified site components, allow definition of a binding surface. Molecular modeling and energy minimization techniques have been used to optimize the match between this surface and ATP, providing a satisfactory picture of how the two molecules interact.

EXPERIMENTAL PROCEDURES

Materials—Horse heart (type III) cytochrome c, SP Trisacryl Plus-M, and Na$_2$ATP were purchased from Sigma; thermolysin was from Boehringer Mannheim. TNP-8N$_3$-AMP and TNP-8N$_3$-ATP were synthesized as described previously (26, 27). Na$_2$ATP was synthesized from ATP (25) and $^{32}$P incorporated into the γ position by the method of Glynn and ChapPELL (29).

Photolabeling—Cytochrome c (6 or 22 mg, purified by cation exchange chromatography) was dissolved with TNP-8N$_3$-AMP, TNP-8N$_3$-ATP, or $^{32}$P-ATP (0.8 mM cytochrome, 0.7 mM nucleotide) in 5 mM BES/Tris, pH 7.0, and irradiated in an optical silica 1-cm cuvette for 60 min or in a 2-mm quartz cuvette (volume of cuvette = 6 ml) for 10 min, with frequent stirring, and with toluenefilters, as described before (26). In some experiments, indicated in the legend to the appropriate figure, the medium contained ATP and/or KCl. The cuvette was maintained at close to 0°C by frequent cooling in ice and, in the case of the figure, the medium contained ATP and/or KCl. The cuvette was main-

50–100%B (linear) over 10 min, and 100%B for 5 min. The elution was moni-

tored at 210 and 408 nm. Peaks absorbing at 408 nm were collected manually and repurified on the same C$_{18}$ column and the same gradient system except with 1 mM potassium phosphate in the solvents. The advantage of this second system is that the elution of the peptides is predictably close to the retention time of the first run and usually with sufficient spread of peaks to effect reasonable purification. The relevant peaks were collected and reduced in volume by partial lyophilization and were stored in liquid N$_2$.

Peptide Sequencing—Sequence analysis was performed by W. Brandt, Department of Biochemistry, University of Cape Town, Ronde-

bosch, Cape Town, 7700, using a gas phase sequenator constructed in a way that is similar to the commercial machines. The amino acids were converted to the phenylthiohydantoin derivatives and analyzed by HPLC using standard procedures.

Molecular Modeling—Modeling of the ATP/horse heart cytochrome c interaction was performed on a Silicon Graphics Iris Indigo, using the programs Insight II and Discover (Biosym Technologies, San Diego). The x-ray crystal structure for horse heart cytochrome c at 1.9 Å resolution was obtained from G. Brayer at the University of British Columbia (30). Since the presence of the 8-azido group in ATP is expected to cause the nucleotide to adopt the synth conformation about the glycosidic bond, the modeling and minimization was performed in both the syn and anti states. In both cases, modeling proceeded along the same lines. ATP in the appropriate conformation was positioned on the surface of cyto-

chrome c with the γ-phosphate in close proximity to Arg-91 and the adenosine group between positions 72 and 86/87. The crystallographic waters in steric conflict with ATP were removed, and the entire structure was surrounded with an 8 Å layer of water. As a comparison, the horse cytochrome c structure was also subjected to the same minimiza-

tion in the absence of ATP.

Complexes were subjected to minimization using a consistent va-

cence force field with no constraints. A cutoff distance of 12 Å was used with a switching function applied over the last 1.5 Å. Minimization proceeded stepwise as follows. First, residues in close proximity to the ATP binding were relaxed by steepest descents for 1,000 cycles to allow for removal of any steric conflicts produced by manual positioning of the ATP. Second, all hydrogens were allowed to move. Finally, all atoms were allowed to move. The last three steps proceeded using conjugate gradient minimization for 1,000, 1,000, and 10,000 steps, respectively.

RESULTS

TNP-8N$_3$-AMP—Irradiation of cytochrome c in the presence of just substoichiometric TNP-8N$_3$-AMP and at low ionic strength resulted in the derivatization of approximately 50% of the protein. The modified protein eluted in six peaks, labeled A–F, from a Trisacryl cation exchange column (Fig. 1), mirroring the pattern obtained with 8N$_3$-ATP (15) and TNP-8N$_3$-ATP.
The early eluting peak, A (19%), represents mainly double-derivatized cytochrome c (close to 2 mol of TNP-nucleotide/mol), artificially lumped together as a result of the concentration of the elution buffer increasing from 20 to 40 mM. Peaks B (6%), C (17%), D (24%), and E (4%) contained close to 1 mol of TNP-nucleotide/mol and represent at least four different forms of mono-derivatized species. Peak F (2%) contained less than 1 mol of TNP-nucleotide/mol and may be contaminated by a small amount of deamidated cytochrome c, even though most of the latter species had been removed. Nonspecific attachment accounted for 28% of the total cytochrome c. The mono-derivatized peaks make up 53% of the total derivatized cytochrome c.

Peaks A–F were each digested with thermolysin and the peptides separated on a reverse phase C 18 column. The elution profiles of each, as monitored by the absorbance at 408 nm, are shown in Fig. 2. Only the peak A 210 nm absorbance trace is shown as the others were similar. It can be seen that each of the 408 nm traces is different, but some peaks overlap. The broad peak eluting after 80 min in each chromatograph was identified from scans as heme-containing. All of the other main peaks were identified as containing TNP. Each of the main peaks was collected and rechromatographed at a lower phosphate concentration to obtain further purification, and then the main peaks were again collected and sequenced. The sequences, together with yields, are shown in Table I.

Peak A was predictably the most complex and contained TNP-peptide sequences identified in peaks C, D, E, and F (see below).

Peak B exhibited a major peak at 47 min (Fig. 2), which was resolved further into five peaks with the main one at 36 min (Fig. 3). Sequencing failed to reveal any amino acids in this fraction.

Peak C exhibited major peaks at 41, 42, 44.8, and 79 min (Fig. 2). The peaks at 41 and 42 min were pooled, and further purification at a lower phosphate concentration appeared to reverse their elution retention time. The smaller species eluted earlier at 43 min and the larger at 43.5 min (Fig. 3). Sequencing of the former revealed an I KK species, where X is Lys-86 and is presumably that which is derivatized, and contamination with another peptide beginning with phenylalanine (Table I). The larger species eluting at 43.5 min consisted of the same derivatized sequence with a larger proportion of the contaminating peptide. The two derivatized forms may represent alternative ways of the nitrene linking with the amino group of Lys-86, different conformational isomers, or different tautomeric forms. The species eluting at 44.8 min (Fig. 2), purified as a single species (Fig. 3), and was also derivatized on Lys-86 but differed from the earlier eluting species by having at least four more amino acids attached to it (Table I). The relatively minor peak at 47 min (Fig. 2) was considered to be a contaminant from peak D (see below) and was not sequenced. The peak at 79 min (Fig. 2) purified as a large and a small species (Fig. 3), and the larger was associated with derivatization of Lys-72 (Table I).

Peak D exhibited major peaks at 45, 47, and 75 min (Fig. 2). The smaller ones at 40 and 41 min were presumed to be contaminants from peak C. The 45 min species purified as essentially a single species (Fig. 3), and sequencing revealed that it was associated with derivatization of Lys-87 (Table I). The principal peak at 47 min (Fig. 2) purified as two species, only the larger of which, that at 43 min, was sequenced. This showed that it was also associated with derivatization of Lys-87. The sequencing was not continued beyond cycle 8, and so the reason for the different retention times was not established. The species at 43 min was mildly contaminated with a peptide beginning with leucine. The 75 min peak (Fig. 2) purified as a single species (Fig. 3), and sequencing revealed that it was again associated with derivatization of Lys-72.

Peak E exhibited a major peak at 48 min (Fig. 2) which split into three TNP peaks on rechromatography, the larger of which was subjected to sequencing but failed to show amino acids. The second highest peak at 39 min purified as a single TNP species and yielded a sequence beginning with Ile-57 (Table I). The amount of peptide was very low, and the point of attachment of the nucleotide, if there was one, was not clear. This peak eluted just in front of a non-TNP peptide (Fig. 3), and it is possible that the sequence is associated with this peptide, and the TNP peak represents an unattached nucleotide that had...
ATP Binding Locus on Cytochrome c

Peaks B–E are derived from labeled cytochrome c as shown for TNP-8N₃-AMP in Fig. 1. The first retention time (min) refers to the first HPLC in 10 mMKPi, and the second is that obtained on rechromatography in 1 mMKPi. The numbers in parentheses indicate pmol of amino acid residue. Arginyl residues could not be detected and are shown in parentheses when the sequence predicts that they should occur. A horizontal line indicates where the sequencing was terminated. A dash indicates that no significant residue was found, except that attributable to carry over from the previous cycle. In the case of the sequence shown under peak D, 75/73, the fraction actually sequenced was obtained from another irradiation experiment and not that shown in Fig. 3. Where ? appears in parentheses, the quantitation was uncertain because of interference from a neighboring peak.

TABLE I
Sequence analysis of peptides photolabeled with TNP-8N₃-AMP (top) and TNP-8N₃-ATP (bottom) purified by HPLC

| Cycle no. | Peak B | Peak C | Peak D | Peak E |
|-----------|--------|--------|--------|--------|
| 35/30     |        |        |        |        |
| 42/34     |        |        |        |        |
| 42/36     |        |        |        |        |
| 79        |        |        |        |        |
| 33/27.5   |        |        |        |        |
| 40/36     |        |        |        |        |
| 41/37     |        |        |        |        |
| 1         | I(25)  | I(78)  | T(19)  | L(123) |
| 2         | —      | L(94)  | E(102) | K(43)  |
| 3         | —      | K(31)  | E(233) | M(38)  |
| 4         | K(28)  | Y(220) | K(47)  | P(45)  |
| 5         | —      | T(8)   | Y(4)   | K(80)  |
| 6         | —      | —      | K(49)  | —      |
| 7         | —      | —      | —      | Y(28)  |
| 8         | —      | —      | —      | I(6)   |
| 9         | —      | —      | —      | P(34)  |
| 10        | —      | —      | —      | G(13)  |
| 11        | —      | —      | —      | T(3)   |
| 12        | —      | —      | —      | K(15)  |
| 13        | —      | —      | —      | M(2)   |

come adrift from its peptide.

Peak F exhibited major TNP species at 47, 62, and 63 min (not shown). The latter species are not present in the other peaks and probably represent a point of attachment of the nucleotide, but the amounts were too small to sequence.

Peak A contained species associated with derivatization of each of the lysines seen in peaks C and D. Derivatization with TNP-8N₃-AMP, and all the subsequent separations, digestions, HPLCs, and sequencing, were repeated once. Essentially the same results were obtained. In some cases more judicious peak collecting from the HPLCs eliminated some of the contaminating peptide fragments, and some of the longer peptides appeared to be slightly longer than shown in Table I. Again, peak D had very little of the 79 min species of peak C, and the latter digest had an insignificant amount of the 75 min species. This indicates that peak C contained a Lys-72 adduct different from that in peak D.

Averaging the quantitation of TNP peaks in the HPLC profiles of the two runs suggested that both peaks C and D contained approximately 80% Lys-86 or Lys-87 and 20% Lys-72. It can be seen from the Trisacryl elution profile in Fig. 1 that the Lys-87 derivatization (peak D) is favored slightly over the Lys-86 (peak C) one.

TNP-8N₃-ATP—Irradiation of cytochrome c with TNP-8N₃-ATP resulted in approximately 60% derivatization, which could be resolved into four main peaks on the Trisacryl column. These were all mono-derivatized species (the di-derivatized species would not be expected to bind to the column). They will be shown to be related to peaks B, C, D, and E of the monophosphate experiments (Fig. 1) and occurred in similar proportions, peaks C and D being about equal and larger than B and E (not shown).

HPLC of the digested adduct of peak B did not yield any TNP peaks.

Peak C yielded several TNP peaks at the 30–45 min elution time of which the two largest were selected for further purification. The first produced a single TNP species with the sequence I₉X₉K with Lys-86 as the probable derivatized residue (Table I). The peak at 42 min resolved into two species, the first of which is associated with derivatization of the same residue and the second with Lys-87. The late eluting peak at 79 min did not require further purification and yielded a sequence implicating Lys-72 as the attachment site.

Peak D produced two main peaks, the first of which resolved into two TNP peaks, and the larger species failed to show a sequence. The second purified as a single species and implicated Lys-87 (Table I).

Peak E produced several TNP species on digestion, and purification of the largest showed it to be a single TNP species but without a sequence.

Derivatization with TNP-8N₃-ATP produced TNP fragments that eluted in broader peaks than is the case with TNP-8N₃-AMP, and in consequence they were more difficult to purify. A comparison of the HPLC profiles of these TNP-peptides with those of the monophosphate derivatization in Fig. 2 showed a satisfying complementarity but also one difference. With both nucleotides, peak C is largely associated with derivatization of Lys-86 and peak D with Lys-87. In the case of the monophosphate, there are two species of Lys-72 adducts, one eluting under peak C and another under peak D. They seem to
be present in equal amounts. With the tripophosphate, the only Lys-72 adduct detected is associated with peak C.

8N3-ATP—The experiments were also performed with 8N3-[γ-32P]ATP (not shown). Irradiation with the same proportions of nucleotide and cytochrome c as above resulted in approximately 60% derivatization and an elution profile from the Trisacryl column similar to that shown for TNP-N3-AMP (Fig. 1). It has been shown previously that derivatization produces two main adducts (15). Digestion with thermolysin and HPLC separation revealed that the earlier eluting peak produced a single significant radioactive peak at the void volume of the column. Repeated HPLC purifications in 100 mM phosphate, pH 3.0, resulted in a semipure 32P peak with a sequence of IKKK as one of the possibilities. The later eluting peak off the Trisacryl column produced six significant early eluting radioactive peaks following digestion and HPLC. Repeated HPLC purifications of the peaks in high phosphate led to either loss of the radioactivity or indefinite sequences.

Ionic Strength and ATP Inhibition—The effects of 150 mM KCl and 7 mM ATP (10-fold excess over TNP-8N3-AMP) on TNP-8N3-AMP derivatization and the elution profile from the Trisacryl column were examined (results not shown). Both KCl and ATP diminished the amount of derivatization, and this was attributable to lowered levels of peaks C and D; the other peaks were not affected. In the presence of the KCl peaks C and D still remained the major peaks. In the presence of ATP, peak D was the major peak, albeit much smaller, with peaks B, C, E, and F being about equal.

Since peaks C and D, in the case of derivatization with TNP-8N3-AMP, are each associated with the derivatization of two lysines (Lys-86 + Lys-72 and Lys-87 + Lys-72, respectively, see above) it was of interest to determine whether ATP selectively protected against the derivatization of any one of the lysines in each peak. Accordingly, peaks C and D derived from labeling in the presence of ATP (pooled peaks from labeling performed in the presence 3.5 and 7 mM ATP were used) were digested with thermolysin as above and subjected to HPLC analysis. The results provided no evidence for the selective inhibition of one of the lysines and are not shown. This suggests that the labeling of Lys-86, Lys-87, and Lys-72 and the ATP inhibition thereof are via a single site.

Molecular Modeling—Examination of the surface topology of cytochrome c indicates that Lys-86 and Lys-87 are located on a ridge produced by an extended helical structure usually referred to as the 80s stretch. On either side of the ridge (and the two lysines) there are valleys, in the one case leading to the amino-terminal end of the 60s helix and a 70s loop and in the other to the amino-terminal helix. Both valleys are potential nucleotide binding sites. However, for reasons explained later (see "Discussion"), only the former valley, demarcated by the 60s helix and 70s loop and incorporating Arg-91, which has been shown to be critical for ATP binding, was formally modeled.

Another consideration is that bulky 8 substitutions in the adenine ring cause the nucleotide to adopt a syn conformation about the glycosidic bond with the 8 substituent pointing away from the furanose ring, rather than the anti of ATP, where the 8H is located above the furanose ring (31–34). Consequently both syn and anti structures were modeled onto cytochrome c.

Energy-minimized structures of horse cytochrome c-ATP complex, with the nucleotide positioned in the appropriate valley and the γ-phosphate constrained to within electrostatic contact distance of the guanidino group of Arg-91, are shown in Fig. 4. In the first frame (Fig. 4, upper left), ATP is docked in an anti conformation, energy minimization performed, and finally Lys-86 flexed to allow close approach to the 8 position of the adenine ring. The amino group can reach to within 3.12 Å of the C8 atom. The sides of the crevice into which the adenine ring fits are provided by Ala-83, the aliphatic side chain of Lys-86, and Asn-70, giving significant hydrophobic character to the cleft. The second frame (upper right) is similar except Lys-87, the principal nitrene target, approaches the 8 position. The amino group can reach to 6.4 (strained) to 7 Å of the C8 atom, and allowing for the presence of the nitrene and the ability of a structure to "breathe," this distance may be optimal. The third frame (lower left) shows ATP docked in an energy-minimized complex in a syn conformation and then Lys-72 rotated to give the closest approach to the C8 position (3.53 Å). Again, reaction of the amino group with a nitrene radical is feasible. Minimization of the complex in both the anti and syn nucleotide conformations led to similar alterations in the final structures.

In both cases the γ-phosphate remained in close contact with Arg-91 (in fact, the latter's guanidinium group "pops out" of the
protein interior to be closer to the phosphoryl group), and lysines 86, 87, and 88 moved toward the negatively charged phosphate groups (before manual rotations of the former two). The adenine ring fell between Ala-83 and Asn-70, and the furanose ring was in close contact with Glu-69. The hydroxyl groups on the ribose formed hydrogen bonds with the carboxyl group of Glu-69, in particular O2' in the anti conformation and O3' in the syn conformation. It can be seen however that the fit of the adenine ring is better in the anti conformation compared with the syn; the former is set deeper in the crevice and in
contact with more of the protein. The exocyclic amino group is surface exposed in both cases but could contact Asn-70 and/or Lys-86 in the anti conformation and not in the syn. The hydrophobic aliphatic chain of Lys-86 is not in contact with the adenine in the syn conformation.

A space-filling model of the anti nucleotide with a close approach of Lys-86 (identical complex to that in Fig. 4, upper left) is shown in the side view in Fig. 4, lower right. It can be seen how the nucleotide fits snugly into the crevice with Lys-86 wrapped around the adenine.

An energy minimization routine similar to that described above was also performed on the syn nucleotide with the 8 position in close proximity to Lys-86 and Lys-87. This requires that the whole molecule is turned upside down (compared with its position in Fig. 4, lower left), and the ribose hydroxyls now point upward and set in the direction of Lys-88. This results in a much poorer fit of the nucleotide to the protein, and, in particular, the adenine ring cannot be placed in a suitable crevice, the phosphates are not interacting with the lysines, and the γ-phosphate is at best 6–8 Å from Arg-91.

TNP-8N₃-AMP and TNP-8N₃-ATP were not formally docked and energy minimized onto cytochrome c, but the TNP moiety can be accommodated in the models of Fig. 4 if the furanose hydroxyls are lifted and angled slightly so that the spiro-linked cyclohexyldienylidene ring is flattened along the side of the 60s helix interacting with Lys-73 and on top of submerged Tyr-67. Modeling suggests that this change could be accommodated without much shift in the position of the adenine or phosphates. The cyclohexyldienylidene ring has a delocalized formal negative charge, and this could interact favorably with Lys-73.

**DISCUSSION**

Previously, cytochrome c has been found to possess three ATP binding sites at low ionic strength, one of which can be considered to be biologically relevant since it is present at physiological ionic strength (11, 13). The latter site is close to Arg-91 since derivatization of the arginine (12) inhibits ATP binding here. In this study, we demonstrate that irradiation of close to equimolar concentrations of cytochrome c and TNP-8N₃-AMP or TNP-8N₃-ATP results in two main adducts that are the result of derivatization of Lys-86, Lys-87, and to a lesser extent Lys-72, the formation of which is inhibited selectively by competition with ATP. These residues are in proximity to Arg-91, and the results both confirm the existence of a binding site at this locus and suggest a probable orientation of the ATP molecule.

The use of the TNP-nucleotides greatly assisted in elucidation of the derivatized amino acids. The peptide adducts are quite stable, bind much more tightly to reverse phase columns than the non-TNP nucleotides, absorb in the visible range, and during Sequencing phenylisothiocyanate derivatization of the adduct amino acid does not yield phenylthiohydantoin species with elution properties like that of any standard amino acid. This latter characteristic allows positive identification of the derivatized amino acid. The monophosphate TNP-nucleotide yields derivatized peptides that elute more sharply on HPLC than the triphosphate species, a help in obtaining purer fractions. In this study and in another in which TNP-nucleotides were used to probe the binding site of sarcoplasmic reticulum Ca²⁺-ATPase (26, 27) the monophosphate otherwise provided very similar results to the triphosphate species.

On the other hand, the TNP moiety could wrongly orientate the nucleotide or inhibit binding to the natural ATP site. However, like the TNP-nucleotides, irradiation with 8N₃-ATP produces two main adducts, one of which was linked to the sequence IKKK. It has been shown previously that derivatization with 8N₃-ATP is associated with cyanogen bromide fragment 81–104, and lysyl residues have been implicated in the reaction because modification of all lysines is inhibitory (13). Formation of both adducts is prevented by prior modification of Arg-91, a residue adjacent to Lys-86 and Lys-87 (15). ATP binding specifically inhibits formation of the two TNP adducts (present results). It seems likely that the two adducts formed with the non-TNP nucleotide are associated with covalent attachment to Lys-86 and Lys-87 and that the presence of the TNP moiety has not substantially altered the position of the adenyl ring. The TNP moiety may not interact tightly with the protein; the affinity for the TNP-nucleotides does not appear to be higher than that of ATP.

Examination of the surface topography of cytochrome c in the vicinity of the three targeted lysines showed that there are two possible valleys on either side of the 80s stretch into which a nucleotide could be accommodated. The choice of smaller cleft incorporating Arg-91 was primarily guided by previous findings that have shown this residue to be critical for ATP binding (11–13). Also, derivatization of Arg-91 with 1,2-cyclohexanedi-one blocks the covalent attachment of 8N₃-ATP (15). Locating the site where we did explains how all three lysines are labeled from a single locale, in agreement with the ATP competition experiments. Attachment in the neighboring valley would be expected to inhibit cytochrome c function completely as it is at the site of interaction of the redox partners (2, 35). Although this may be construed as compatible with the approximately 85% lowering of electron transfer to cytochrome oxidase shown by the two 8N₃-ATP adducts (16), it would not be compatible with the smaller 60% inhibition of the transfer from the reductase (16) and the minimal effect that derivatization with 8N₃-ADP has on binding of cytochrome oxidase and electron transfer (17), assuming the same site is involved.

An energy-minimized docking procedure shows that a satisfactory placing of nucleotide can be achieved in the cleft incorporating Arg-91 and Glu-69. The fit and contact points for the adenine ring are highly favorable if the base is in an anti conformation about the glycosidic bond. In this position and conformation, reaction of the light-generated nitrene with Lys-86 and Lys-87, but not Lys-72, is feasible. In the alternate syn conformation the adenine is more exposed to the medium, and the nitrene is capable of interacting with Lys-72, but not Lys-86 and Lys-87. A problem with this arrangement is that the syn conformation is the one expected to be favored for the 8N₃-nucleotides. Docking of the syn conformation with the nitrene within reactive distance of the principal targets, Lys-86 and Lys-87, is energetically highly unfavorable according to our energy-minimized modeling studies.

It is possible that favorable binding interactions either induce a switch of syn to anti conformation on the cytochrome surface or the small proportion of anti conformers binds preferentially. There is a precedent for this. Crystal structures of 8Br-ADP-ribose and 8Br-NAD⁺ with dehydrogenases indicate the anti rotamer, which is the conformation of bound natural coenzyme NAD⁺ (36, 37). Another possibility is that in solution, photochemical conversion of the azido group to the nitrene radical permits rotation to the anti conformer, and this is the form that binds preferentially. It would require that the nitrene radical be fairly long lived. The lifetimes of purine nitrenes have not been determined, as far as we are aware. The high efficiency of photolabeling which is obtained with the 8N₃-nucleotides and cytochrome c, namely approximately 60% irradiation period, at a site(s) that is not expected to be of high affinity or particularly enclosed, suggests that the reaction with water and medium components is unfavorable. The limit to even higher efficiency of labeling is likely to be caused by the generation of inert photolysis products during irradiation.
which compete for the binding site, as occurs with labeling of Lys-492 of the sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase (26, 27).

It is not immediately clear how ATP binding at this site could modify the interaction of cytochrome c with its redox partners. The effect is not likely to be steric as the bulk of the molecule is outside of the surface interactive zone. An alternative is an effect on electrostatics. Lys-86, Lys-87, and Lys-72 are implicated, along with Lys-8 and Lys-13, in hydrogen bonding or salt linking with its redox partners, especially at low ionic strength (2, 20, 35). The importance of the lysine connections is borne out by the 35–85% decrease in the rate of cytochrome c oxidase by a soluble subunit II fragment of Paracoccus denitrificans cytochrome c oxidase when single mutations in the latter protein are made to three invariant carboxylic acids, a glutamate, or glutamine (38), which likely interact with the lysines on cytochrome c. It follows that, at least at low ionic strength, the incorrect orientation of a single interactive lysine on cytochrome c could adversely affect electron transfer. Interaction of Lys-86, Lys-87, and Lys-72 with the nucleotide could draw these lysines away from the docking protein.

On the other hand, the importance of the γ-phosphate in mediating 8N3-nucleotide adduct effects on function (17) suggests that other mechanisms, especially at physiological ionic strength, must operate as well. Cytochrome c has a strong dipole due to the unequal distribution of surface charges, and this is considered to be important in orienting the redox pairs and increasing the frequency of productive collisions (21, 39). As suggested previously (15, 16) the binding of ATP, and possibly not the diphosphate and monophosphate species, could sufficiently modify the dipole moment as to affect adversely the steering and hence collisional frequency. Electrostatic binding of cytochrome c to the inner mitochondrial membrane could also be regulated by diminution of the dipole moment through ATP binding (16).

In conclusion, identification of Lys-86 and Lys-87, and to a lesser extent Lys-72, as the major nitrone targets, and the satisfactory modeling of ATP at a single site in proximity to these residues, provide substantial additional evidence of a relatively specific ATP binding site on cytochrome c. As elaborated on previously (11–13, 15, 16), ATP binding to cytochrome c may be an important mechanism for regulating ATP synthesis in the mitochondrion.

Acknowledgments—We are indebted to Jeanette Gibson and Angela Brigley for technical assistance.

REFERENCES

1. Pettigrew, G. W., and Moore, G. R. (1987) Cytochrome c: Biological Aspects, Springer-Verlag, New York.
2. Ferguson-Miller, S., Brautigan, D. L., and Margoliash, E. (1978) J. Biol. Chem. 253, 149–159
3. Vik, S. B., Georgevich, G., and Capaldi, R. A. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 1456–1460
4. Speck, S. H., Neu, C. A., Swanson, M. S., and Margoliash, E. (1983) FEBS Lett. 164, 379–382
5. Wando, N. W., Eichel, B., and Gould, A. (1960) J. Biol. Chem. 235, 1521–1525
6. Yu, C., Yu, L., and King, T. E. (1975) J. Biol. Chem. 250, 1383–1392
7. Nicholls, D. (1974) Biochim. Biophys. Acta 346, 261–310
8. Margoliash, E., Barlow, G. H., and Byers, V. (1970) Nature 228, 723–726
9. Stellwagen, E., and Shulman, R. G. (1973) J. Mol. Biol. 75, 683–695
10. Margoliash, R., and Schejter, A. (1973) Eur. J. Biochem. 32, 500–505
11. Corthesy, B. E., and Wallace, C. J. A. (1988) Biochem. j. 236, 359–364
12. Corthesy, B. E., and Wallace, C. J. A. (1988) Biochem. j. 252, 349–355
13. Craig, D. B., and Wallace, C. J. A. (1991) Biochem. j. 279, 781–786
14. Ferguson-Miller, S., Brautigan, D. L., and Margoliash, E. (1976) J. Biol. Chem. 251, 1104–1115
15. Craig, D. B., and Wallace, C. J. A. (1993) Protein Sci. 2, 966–976
16. Craig, D. B., and Wallace, C. J. A. (1995) Biochemistry 34, 2686–2693
17. Lin, J., Wu, S., Lau, W., and Chan, S. I. (1995) Biochemistry 34, 2678–2685
18. Rytomaa, M., Mustonen, P., and Kinnunen, P. K. J. (1992) J. Biol. Chem. 267, 22243–22248
19. Rytomaa, M., and Kinnunen, P. K. J. (1994) J. Biol. Chem. 269, 1770–1774
20. Brautigan, D. L., Ferguson-Miller, S., and Margoliash, E. (1978) J. Biol. Chem. 253, 130–139
21. Margoliash, E., and Bosshard, H. R. (1983) Trends Biochem. Sci. 8, 316–320
22. Taborsky, G., and McCollum, K. (1979) J. Biol. Chem. 254, 7069–7075
23. Wallace, C. J. A., and Corthesy, B. E. (1986) Protein Eng. 1, 23–27
24. Nakamoto, R. K., and Inesi, G. (1984) J. Biol. Chem. 259, 2961–2970
25. Hiratsuka, T. (1985) J. Biol. Chem. 260, 4784–4790
26. Seebregts, C. J., and McIntosh, D. B. (1989) J. Biol. Chem. 264, 2043–2052
27. McIntosh, D. B., Woolley, D. G., and Berman, M. C. (1992) J. Biol. Chem. 267, 5301–5309
28. McIntosh, D. B., and Woolley, D. B. (1994) J. Biol. Chem. 269, 21587–21595
29. Yu, C., Yu, L., and King, T. E. (1975) J. Biol. Chem. 250, 147–149
30. Bushnell, G. W., Louis, G. V., and Brayer, G. D. (1990) J. Mol. Biol. 214, 585–595
31. Tavale, S. S., and Sobell, H. M. (1970) Mol. Biol. 48, 109–123
32. Ikehara, M., Uesugi, S., and Yoshida, K. (1972) Biochemistry 11, 830–842
33. Takenaka, H., Ikehara, M., and Tomonura, Y. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 157, 4229–4233
34. Stolarz, R., Hagberg, C., and Shugar, D. (1984) Eur. J. Biochem. 138, 187–192
35. Pelletier, H., and Kraut, J. (1992) Science 258, 1748–1755
36. Abdallah, M. A., Bielmann, J. F., Nordstrom, B., and Branden, C. I. (1975) Eur. J. Biochem. 50, 475–481
37. Gronenborn, A. M., and Clore, G. M. (1982) J. Mol. Biol. 157, 155–160
38. Lappalainen, P., Watham, N. J., Greenwood, C., and Saraste, M. (1995) Biochemistry 34, 5824–5830
39. Koppend, W. H., Vroiland, C. A. J., and Braams, R. (1978) Biochim. Biophys. Acta 503, 499–508