The First Nucleotide Binding Domain of Cystic Fibrosis Transmembrane Conductance Regulator Is a Site of Stable Nucleotide Interaction, whereas the Second Is a Site of Rapid Turnover*

Received for publication, December 9, 2001, and in revised form, February 21, 2002
Published, JBC Papers in Press, February 22, 2002, DOI 10.1074/jbc.M111713200

Luba Aleksandrov, Andrei A. Aleksandrov, Xiu-bao Chang, and John R. Riordan‡
From the Mayo Foundation and Mayo Clinic Scottsdale, S. C. Johnson Medical Research Center, Scottsdale, Arizona 85259

As in other adenine nucleotide binding cassette (ABC) proteins the nucleotide binding domains of the cystic fibrosis transmembrane conductance regulator (CFTR) bind and hydrolyze ATP and in some manner regulate CFTR ion channel gating. Unlike some other ABC proteins, however, there are preliminary indications that the two domains of CFTR are not equivalent in their nucleotide interactions (Szabo, K., Szakacs, G., Hegeds, T., and Sarkadi, B. (1999) J. Biol. Chem. 274, 12209–12212; Aleksandrov, L., Mengos, A., Chang, X., Aleksandrov, A., and Riordan, J. R. (2001) J. Biol. Chem. 276, 12918–12923). We have now characterized the interactions of the 8-azido-photoactive analogues of ATP, ADP, and 5‘-adenyl-β,γ-imidodiphosphate (AMP-PNP) with the two domains of functional membrane-bound CFTR. The results show that the two domains appear to act independently in the binding and hydrolysis of 8-azido-ATP. At NBD1 binding does not require a divalent cation. This binding is followed by minimal Mg2+-dependent hydrolysis and retention of the hydrolysis product, 8-azido-ADP, but not as a vanadate stabilized post-hydrolysis transition state complex. In contrast, at NBD2, Mg2+ATP is hydrolyzed as rapidly as it is bound and the nucleoside diphosphate hydrolysis product dissociates immediately. Confirming this characterization of NBD1 as a site of more stable nucleotide interaction and NBD2 as a site of fast turnover, the non-hydrolyzable N3AMP-PNP bound preferentially to NBD1. This demonstration of NBD2 as the rapid nucleotide turnover site is consistent with the strong effect on channel gating kinetics of inactivation of this domain by mutagenesis.

The ubiquity of adenine nucleotide binding cassette (ABC) proteins in all kingdoms of life and their involvement in many human disease processes (1–3) emphasizes the need to understand their structure and function. The presence of two homologous nucleotide binding domains where ATP binding and hydrolysis can occur is the defining feature of these molecules. It is assumed that the energy of ATP binding is used to do the mechanical work of the molecule, in most cases the vectorial translocation of a solute across a biological membrane (4, 5). There are currently two prominent models for the utilization of the two NBDs in this process. In the first, ATP hydrolysis occurs alternatively and in a mutually exclusive fashion at each NBD, to drive solute export (6). In a second model, hydrolysis at one NBD drives transport whereas at the second “resets” the protein for the subsequent step (7). In both of these models, the NBD at which the initial hydrolysis occurs is chosen randomly; the two domains are distinct only temporarily. Indeed, in the P-glycoprotein multidrug transporter in which studies supporting each of these models have been performed, the amino acid sequences of the two domains are very similar and only minor functional asymmetry has been observed (8). However, in some other members of the large family, including the ABCC subfamily, this similarity is far less and distinctive properties have been observed in the case of SUR1 (9, 10), MRP1 (11–14), and CFTR (15, 16). Utilizing [γ-32P]8-N3ATP, both NBDs of CFTR could be photolabeled (15, 16). However, a non-hydrolyzable analogue, 8-N3-AMP-PNP, competed much more strongly for N3ATP binding and capture at NBD1 than at NBD2 (16). We have now further explored this apparent asymmetry of the domains in photolabeling experiments employing [γ-32P]- as well as [α-32P]8-N3ATP, [α-32P]8-N3-AMP-PNP, and [α-32P]8-N3-AMP-PNP. The latter compound selectively labeled NBD1. NBD1 could be labeled by each of these compounds in the absence of a divalent cation such as Mg2+. Labeling of NBD2 that occurred only when azido-nucleotides were not washed out prior to photoactivation was Mg2+-dependent.

When [γ-32P]8-N3ATP was employed at temperatures and divalent cation concentrations where hydrolysis could occur, only NBD1 was labeled, indicating that the intact nucleoside triphosphate was bound and retained there but not at NBD2. This revealed that the labeling of NBD2 detected with [α-32P]8-N3ATP must have reflected bound [α-32P]8-N3-AMP-PNP formed by hydrolysis. This hydrolysis must have been rapid and complete because no [γ-32P]8-N3-ATP radioactivity could be detected at NBD2. The [α-32P]8-N3-AMP-PNP bound at NBD2 after presentation of the protein with [α-32P]8-N3-ATP could have arisen from hydrolysis at either NBD1 or NBD2. To distinguish between these possibilities, the Walker A lysine mutants K464A and K1250A were used; K464A ablated labeling of NBD1 without influencing that at NBD2, and hence the N3ADP that labeled

---

*This work was supported by Grant DK51619 from the NIDDK, National Institutes of Health and by the Cystic Fibrosis Foundation.

‡ To whom correspondence should be addressed: Mayo Clinic Scottsdale, S. C. Johnson Medical Research Center, Scottsdale, Arizona 85259. Tel.: 480-301-6206; Fax: 480-301-7017; E-mail: riordan@mayo.edu

1 The abbreviations used are: ABC, adenine nucleotide binding cassette; CFTR, cystic fibrosis transmembrane conductance regulator; NBD, nucleotide binding domain; AMP-PNP, 5‘-adenyl-β,γ-imidodiphosphate; BHK, baby hamster kidney; 8-N3-ATP, 8-azidoadenosine 5‘-triphosphate; TPCK, tosylphenylalanyl chloromethyl ketone; DDM, n-dodecyl-β-D-maltoside.

This paper is available online at http://www.jbc.org
NBD2 could not have arisen from hydrolysis at NBD1. This implicates NBD2 as a site of rapid hydrolysis, whereas hydrolysis is very limited at NBD1 where there is labeling to a similar extent with \([\alpha-32P]-\) and \([\gamma-32P]8-N_3\) ATP. Not only is hydrolysis at NBD2 rapid but release of the product, NADP, is also as it is completely removed by washing away of the free compound prior to photo-activation. In contrast, after incubation with \([\alpha-32P]-\) or \([\gamma-32P]8-N_3\) ATP or \([\alpha-32P]8-N_3\) ADP, bound nucleotide is retained at NBD1 to the same extent with or without washout of free azido-nucleotide.

**EXPERIMENTAL PROCEDURES**

**Materials—** \([\alpha-32P]8-N_3\) ATP, \([\gamma-32P]8-N_3\) ATP, \([\alpha-32P]8-N_3\) ADP, and \([\alpha-32P]8-N_3\) AMP-PNP were obtained from Affinity Labeling Technologies, Inc. Non-radioactively labeled 8-N-AMP-PNP was from the same source; other nucleotides were purchased from Sigma as were other reagents employed, including TPKC-treated trypsin. Stable BHK-21 cell lines expressing wild-type and K464A and K1250A variants of CFTR were established and cultured as described previously (16, 17). Membranes for photo-labeling experiments were isolated from these cells by methods described previously (16) and outlined below for purification of CFTR from them.

**Photoaffinity Labeling with 8-Azido-32P-labeled Nucleotides—** Photoaffinity labeling of CFTR in BHK membranes with the different 8-azido-nucleotides was carried out exactly as we have described previously for 8-N-[\alpha-32P] ATP (16). Membrane suspensions (50–200 \(\mu\)g of protein) were incubated for 10 min with the concentration of 8-azido-nucleotide indicated in the figure legends under the conditions also specified there. The membranes were irradiated directly or pelleted and washed with and resuspended in 40 mM Tris-HCl, pH 7.4, 0.1 mM EGTA and then irradiated for 2 min on ice in a Stratalinker UV cross-linker (λ=254 nm) on ice, and membranes were treated on ice with TPKC-treated trypsin for 15 min. After solubilization in radioimmune precipitation buffer, CFTR fragments were immunoprecipitated with monoclonal antibody 596, resolved by SDS-PAGE (6% acrylamide), and electrophoretically for autoradiography.

**RESULTS**

**Photo labeling of CFTR Nucleotide Binding Domains by Hydrolyzed and Non-hydrolyzed 8-Azido-ATP—** Previous experiments had shown that, when incubated with \([\alpha-32P]8-N_3\) ATP under hydrolysis conditions, i.e. at 37°C in the presence of Mg\(^{2+}\) ions, CFTR was photo-labeled more strongly at NBD1 than at NBD2 (15, 16). However, because this labeling was only slightly enhanced by orthovanadate, known to trap the NADP product of hydrolysis at the active site of other ATPases (20), it was unclear whether labeling was with NADP or NADP or both. To resolve this issue and further characterize nucleotide interactions of the two NBDs of CFTR, we have tested conditions that influence hydrolysis and trapping. Fig. 1A shows the results of labeling with \([\alpha-32P]8-N_3\) ATP after incubation at different temperatures and in the presence or absence of Mg\(^{2+}\) ions and orthovanadate, i.e. conditions expected to influence hydrolysis and NADP trapping, respectively. From lanes 1 and 2 with incubation at 0°C where hydrolysis should be minimal, Mg\(^{2+}\) is seen to slightly stimulate but not be essential for labeling of NBD1, whereas labeling of NBD2 is more dependent on the divalent cation. With incubation at 37°C (lanes 3–5) where hydrolysis is enabled, there is much stronger labeling of NBD1 than at 0°C. This labeling is increased by Mg\(^{2+}\) but less so by orthovanadate. Strikingly, labeling of NBD2 is almost completely dependent on Mg\(^{2+}\) ions and is also increased by vanadate. Additional clarification of whether labeling was with NADP or NADP was obtained by using \([\gamma-32P]8-N_3\) ATP (Fig. 1B). In that case after \(\gamma\)-phosphate is removed by hydrolysis, labeling by NADP would not be visible. At 0°C (lanes 1 and 2), there was labeling at NBD1 that was not strongly influenced by Mg\(^{2+}\) ions. Hence, consistent with the result with \([\alpha-32P]NADP\) at 0°C, the first domain was clearly labeled by the intact unhydrolyzed nucleoside triphosphate. There was also weak labeling of NBD2 by the \(\gamma-32P\)-labeled nucleotide at 0°C. It also labeled NBD1 more strongly at 37°C than at 0°C, again indicating strong labeling of that domain with the unhydrolyzed nucleoside triphosphate. However, the incremental amount of labeling of NBD1 with the \(\alpha-32P\)-reagent on shifting from 0°C to 37°C (Fig. 1A, lanes 1 and 2 compared with lanes 3 and 4) is even greater than that with the \(\gamma-32P\)-reagent (Fig. 1B, lanes 1 and 2 compared with lanes 3 and 4). This is not surprising because hydrolysis, which is reflected as well as binding in the former case, is expected to have a large temperature coefficient typical of enzymatic reactions. These observations are consistent with hydrolysis occurring at both domains but more completely at NBD2 than NBD1 during the 10-min pre-incubation prior to photoactivation because labeling of NBD2 is...
detected only using the nucleotide with $^{32}$P in the α-phosphate position and not in the γ-phosphate position.

The most striking revelation from the use of $[^{32}\text{P}]N\text{A}_{3}\text{ATP}$ at 37 °C is the complete lack of labeling of NBD2 (Fig. 1B, lanes 3–5). This can only mean that the compound is hydrolyzed as soon as it is bound to that domain; labeling detected with the $[^{32}\text{P}]$-labeled compound (Fig. 1A, lanes 4 and 5) is by the hydrolysis product $[^{32}\text{P}]N\text{A}_{8}\text{ATP}$. These results suggest that NBD2 is a site of more rapid hydrolysis than NBD1.

Distinct Divalent Cation Requirements at CFTR Nucleotide Binding Domains—One notable aspect of the above experiments was the observation that N$_{A}$ATP binding to NBD1 was not dependent on Mg$^{2+}$ ions. A similar finding was made earlier with another ABC protein, SUR1 (21). It is of particular interest for CFTR because the chloride channel can be activated by ATP in the absence of Mg$^{2+}$, providing one piece of evidence that hydrolysis is not essential for activation (22–24).

The ion is required for photolabeling of NBD2 and an increment in labeling of NBD1 at 37 °C (Fig. 1) and for ATP hydrolysis by the whole CFTR protein (25). This is confirmed in Fig. 2A, which shows that a maximal rate of hydrolysis occurred with approximately equimolar concentrations of ATP and Mg$^{2+}$ (~2 mM in this assay). It is also seen that Mn$^{2+}$ is more potent than Mg$^{2+}$ on a molar basis. Because there appears to be more rapid and complete hydrolysis at NBD2 than at NBD1, it might be anticipated that this greater effect of Mn$^{2+}$ on hydrolysis by the whole protein might reflect its action on NBD2. Fig. 2C indicates that this is the case, i.e. Mn$^{2+}$ promotes much stronger labeling of NBD2 than Mg$^{2+}$ with $[^{32}\text{P}]N\text{A}_{3}\text{ATP}$ as substrate. Because labeling of NBD2 using this substrate is entirely a result of binding $[^{32}\text{P}]N\text{A}_{3}\text{ADP}$, this indicates that greater hydrolysis has occurred at NBD2 with Mn$^{2+}$. Thus, there is a parallel between the divalent cation requirements for hydrolysis by the whole protein and that which occurs at NBD2. No divalent cation-independent nucleotide binding was detected at NBD2. That which occurs at NBD1 may account for the non-hydrolytic channel activation that occurs (22–24).

8-Azido-ADP Is Retained by NBD1 but Rapidly Dissociates from NBD2—In the photolabeling experiments in Fig. 1, the unbound photolabile nucleotides were not removed after incubation with CFTR-containing membranes prior to UV irradiation. We had previously shown that labeling of NBD2 with $[^{32}\text{P}]N\text{A}_{3}\text{ATP}$ was greatly reduced if the reagent was washed out before photolabeling (16). Although we had thought this might reflect just a low affinity of the domain for the NTP, the finding that only the NDP was detected at NBD2 (Fig. 1) suggested that the hydrolysis product might not be retained at NBD2 as it is at NBD1. To examine this possibility, photolabeling was performed using $[^{32}\text{P}]N\text{A}_{8}\text{ADP}$ as well as $[^{32}\text{P}]N\text{A}_{8}\text{ATP}$ with and without washout of the compounds before irradiation. The results were remarkably similar with both compounds (Fig. 3), i.e. NBD1 was strongly labeled even after washout, but NBD2 was not. Hence, 8-azido-ADP is bound stably at NBD1 whether presented directly or as the product of hydrolysis of 8-azido-ATP, and this is not a result of trapping by orthovanadate as a mimic of a post-hydrolysis transition state complex. In contrast, the photolabile NDP labels NBD2 only when present in solution at the time of photoactivation; if it is removed after the 10-min period of incubation before irradiation, little or no labeling of NBD2 is detected. When combined with the results of Fig. 1, this provides strong evidence that Mg$^{2+}$ATP is bound and efficiently hydrolyzed by NBD2. The hydrolysis product, 8-azido-ADP, then dissociates more rapidly from NBD2 than NBD1 because none is detected at the former but is at the latter.

Quantification of Nucleotide Interaction at Each NBD—Experiments employing both α- and γ-$[^{32}\text{P}]N\text{A}_{3}\text{ATP}$ and temperatures promoting (37 °C) or precluding (0 °C) hydrolysis have enabled an evaluation of nucleotide binding and hydrolysis at each of the NBDs. Once it is appreciated what occurs at each domain under different conditions, it is possible to estimate the proportions of N$_{A}$ATP and N$_{3}$ADP associated with each. As already shown in Fig. 1, when the two domains are resolved after limited trypsin digestion, there is no labeling of NBD2 with $[^{32}\text{P}]N\text{A}_{3}\text{ATP}$ (Fig. 4A, lanes 3 and 4). Therefore, all the labeling of NBD2 not washed out prior to photoactivation (lane 1) is by the hydrolysis product $[^{32}\text{P}]N\text{A}_{8}\text{ATP}$, which has dissociated, as indicated by lane 2 when reagents were washed out before irradiation. This comparison is also extremely informative for NBD1 whereby simply comparing the relative amounts of radioactivity associated with it in lanes 2 (4603 cpm in $[^{32}\text{P}]N\text{A}_{3}\text{ATP}$ and -N$_{3}$ADP combined) and 4 (2049 cpm in $[^{32}\text{P}]N\text{A}_{3}\text{ATP}$ only), it can be seen that approximately equal amounts of the NTP and NDP are bound to NBD1 after 10 min of hydrolysis at 37 °C.

A similar conclusion can be reached without using limited trypsin digestion to separate fragments containing NBD1 and NBD2 if labeling is done at both 0 and 37 °C. For example, in Fig. 4B, labeling of NBD1 with radioactive N$_{A}$ATP and N$_{3}$ADP from $[^{32}\text{P}]N\text{A}_{3}\text{ATP}$ at 37 °C with washout before irradiation is indicated in lane 4 and the amount of NBD1 labeling by just N$_{A}$ATP from $[^{32}\text{P}]N\text{A}_{3}\text{ATP}$ of the same specific radioactivity is indicated in lane 6. Comparison of these two lanes yields the same conclusion as that obtained from the experiment in Fig. 3A, i.e. that approximately equal amounts of N$_{A}$ATP and N$_{3}$ADP are associated with NBD1. This interpretation is validated by the fact that binding of $[^{32}\text{P}]N\text{A}_{3}\text{ATP}$ is similar at 0 and 37 °C (lanes 5 and 6 compared with lanes 7 and 8). These experiments support the designation of NBD1 as a slow ATP turnover site and NBD2 as a rapid turnover site.
experiments have shown distinct differences between the N3ATP labeling of the NBD1 band. This occurs, however, with washing of membranes after incubation with [α-32P]8-N3ATP (A) or [γ-32P]8-N3ADP (B) before photoactivation. Incubation of wild-type CFTR-containing membranes, photoactivation, and analysis was as in Fig. 1; except where indicated, membranes were pelleted and washed with 40 mM Tris-HCl, pH 7.4, 0.1 mM EGTA prior to UV irradiation. The presence or absence of 0.5 mM sodium orthovanadate is indicated above each lane.

Photolabeling of Each NBD Is Independent of That at the Other—We have interpreted the results of these experiments assuming that photolabeling of one NBD reflected events only at that domain. However, structural determination of NBDs of some ABC proteins has suggested that residues from both domains may participate in the binding and hydrolysis of each ATP (26–28). To assess the extent to which this may occur with CFTR, we determined the influence of mutation of the Walker A lysine residues in the P-loop of each domain, which contacts the β-phosphate of the bound nucleotide (Fig. 5). As expected, the mutations K464A and K1250A prevented photolabeling with either 8-azido-ATP or 8-azido-ADP of NBD1 and NBD2, respectively. However, there was no indication that the mutation in one domain had any influence on the labeling of the other, i.e. in K464A, NBD2 was labeled as in wild-type and in K1250A, NBD1 was not different from wild type. Because labeling with [α-32P]N3ATP under these conditions (Fig. 5A) reflects both binding of the nucleoside triphosphate and its diphosphate hydrolysis product at NBD1 and solely the latter at NBD2, it can be concluded that neither binding nor hydrolysis at one domain is entirely dependent on those of the other domain. Binding of the NDP at each domain detected with [α-32P]N3ATP as substrate clearly is not dependent on an intact Walker A motif in the other domain. Although these observations do not exclude cooperativity between the two domains at some level, they do indicate that the NDP, which dissociates so readily from NBD2 after NTP hydrolysis there, does not contribute significantly to the labeling of NBD1. The occlusion of the NDP at NBD1 and lack thereof at NBD2 obviously precludes any movement of the nucleotide in the opposite direction.

As another means of evaluating the influence of nucleotide interaction at one domain on that of the other, the apparently selective interaction of AMP-PNP with NBD1 was employed. The concentration dependence of photolabeling with [α-32P]N3AMP-PNP is shown in Fig. 6A and reveals a much higher affinity for NBD1 than for NBD2. Little interaction with NBD2 is detected at concentrations below 100 μM. Concentrations in this range were found to almost completely inhibit labeling of NBD1 with [α-32P]N3ATP (16). This effect is illustrated again in Fig. 6B, where 150 μM N3AMP-PNP ablates the N3ATP labeling of the NBD1 band. This occurs, however, without any diminution of labeling of the NBD2 band. Hence, as with a mutation (K464A) that prevents labeling of NBD1 by N3ATP, occupation of the domain by AMP-PNP leaves the labeling of NBD2 with [α-32P]N3ATP entirely intact.

Cooperative Interaction of N3ATP with NBD2—The above experiments have shown distinct differences between the NBDs of CFTR in their interactions with nucleotides including divalent cation dependence, extent of hydrolysis, and dissociation. To focus strictly on the binding events, we utilized [α-32P]N3ADP and [γ-32P]N3ATP at 0 °C over a wide range of concentrations. Fig. 7A reveals that the NDP exhibits apparently conventional saturable binding to both domains with no marked difference in affinity for the two domains under these conditions where the nucleotide is kept present in solution until the time of photoactivation. Both sets of data are fit well by a single hyperbolic function. If the azido-nucleotide is removed from solution prior to irradiation, however, there is dissociation from NBD2 as would be expected from an equilibrium binding reaction. In contrast, NBD1 exhibits non-equilibrium behavior by occluding the NDP; hence, essentially the same curve is obtained when the nucleotide is washed out before photoactivation (data not shown). Nevertheless, the inherent binding affinities of the two domains for N3ADP do not appear to be significantly different. The binding curves for
Influence on labeling with \( [\alpha^{32}\text{P}]8\text{-N3ATP} \) of NBDs by \( [\alpha^{32}\text{P}]8\text{-N3NTP} \) photolabeling of NBDs by \( [\alpha^{32}\text{P}]8\text{-N3ADP} \). Membranes from BHK cells expressing wild-type and K464A and K1250A variants of CFTR were incubated as in Figs. 1 and 3 but only at 37 °C without washing before photoactivation. The mobility of the large 95-kDa, NBD2 band is increased in the K464A variant, which does not mature and completely acquire the complex oligosaccharide chains, which contribute to the apparent size of the wild-type band.

Fig. 5. Influence of Walker A lysine residue substitutions on photolabeling of NBDs by \( [\alpha^{32}\text{P}]8\text{-N3ATP} \) (A) and \( [\alpha^{32}\text{P}]8\text{-N3NTP} \) photolabeling of NBDs (B). Membranes from BHK cells expressing wild-type and K464A and K1250A variants of CFTR were incubated as in Figs. 1 and 3 but only at 37 °C without washing before photoactivation. The mobility of the large 95-kDa, NBD2 band is increased in the K464A variant, which does not mature and completely acquire the complex oligosaccharide chains, which contribute to the apparent size of the wild-type band.

[\gamma^{32}\text{P}]N3ATP at 0 °C, however, appear quite different (Fig. 7B). Simple saturable binding occurs at NBD1, but the NBD2 curve is shifted to the right at low concentrations and is sigmoidal. The latter could be indicative of cooperativity in the interaction at NBD2 but provides no information as to which other domains or molecules might contribute to the cooperativity. In addition to these different behaviors of the domains, comparison of the two curves does suggest a higher affinity of NBD1 than NBD2 for the NTP. These observations combined with those in the previous figures characterize NBD1 as a higher affinity NTP binding site where there is a relatively low rate of hydrolytic turnover and occlusion of the NDP product, whereas NBD2 is a lower affinity site where a more complex binding interaction occurs followed by rapid hydrolysis and product dissociation.

DISCUSSION

Most experimental data contributing to interpretations of the activities of the two NBDs of CFTR have come from assays of its chloride channel activity in response to different nucleotides and conditions expected to influence their binding and hydrolysis, as well as mutagenesis of important residues within the domains (29–31). Earlier interpretations of such studies had led to suggestions that ATP hydrolysis of NBD1 might contribute most to channel opening, with hydrolysis at NBD2 then enabling channel closing (29). However, considerable more recent data are not adequately explained by this model (16, 23, 31). Although there have been some assays of binding and hydrolysis by the whole CFTR protein (15, 25) and individual recombinant domains (32–41), as yet it has not been clearly established to what extent the two domains play similar or different roles. In the present study we have employed photo-affinity labeling with different azido-nucleotide derivatives under different conditions as one means of assessing binding and hydrolysis at each domain in the intact functional membrane-bound protein. Even though the technique does not provide rapid kinetic measurement of equilibrium binding reactions, which will be required for more complete elucidation of mechanisms, it has enabled comparison of events at the two domains and revealed distinct differences in nucleotide binding, hydrolysis, and product dissociation, which had not been previously appreciated. This new information in itself does not explain how these different interactions at the two NBDs are mediated to channel gating. However, it does provide some constraints on models of how this may occur.

Major differences in the reactivities of the NBDs were revealed in Fig. 1, which is representative of a large number of experiments in which photolabeling by 8-azido-ATP with \( ^{32}\text{P} \) at either the \( \alpha \)- or \( \gamma \)-phosphate positions was compared. First, there was an obvious and clear-cut difference in labeling of NBD2 at 37 °C; the \( \alpha^{32}\text{P}\)-compound labeled strongly and the \( \gamma^{32}\text{P}\)-compound essentially not at all. Therefore, although the NTP clearly interacts at NBD2, the intact nucleoside triphosphate is not retained there; rather it is hydrolyzed and the NDP is detected (\( \alpha^{32}\text{P}\)-labeled). In distinct contrast, NBD1 is strongly labeled by both the \( \alpha^{32}\text{P}\) and \( \gamma^{32}\text{P}\) reagents, indicating that at least some of the intact NTP is bound at this domain, i.e., it is not all hydrolyzed as it is at NBD2 during the same 10-min preincubation period prior to irradiation under identical conditions. This provides the first significant conclusion, which is confirmed and extended in the subsequent experiments; 8-azido-ATP binds to both NBDs of CFTR but is more rapidly hydrolyzed at NBD2 than at NBD1. The second point made in Fig. 1 is that the photolabeling of CFTR NBDs does not primarily reflect vanadate trapping of the NDP formed by hydrolysis, i.e., both domains are labeled in the absence of added vanadate; this confirms earlier findings (15, 16) and hence is not dwelt upon here. However, it is significant to note that this is different from the situation for P-glycoprotein and MRPI, where vanadate is necessary for NDP trapping (6, 12).

The third major point made by the results in Fig. 1 is that labeling of NBD1 is not dependent on the divalent cation, Mg\(^{2+}\), whereas that of NBD2 is. In fact, there is an augmentation of \( \alpha^{32}\text{P}\)N3ATP labeling of NBD1 at 37 °C where hydrolysis occurs because Mg\(^{2+}\) is required for hydrolysis. The portion of the labeling resulting from the NTP binding (measured by \( \gamma^{32}\text{P}\)N3ATP labeling) is unaffected by Mg\(^{2+}\). The Mg\(^{2+}\) independence of NTP binding to NBD1 is also observed with another ABCB protein, SUR1 (21). One might speculate that the ATP-supported gating in the absence of divalent cations in the so-called “non-hydrolytic mode” might relate to the Mg\(^{2+}\)-independent binding at NBD1 (22). The strong dependence of NBD2 labeling on Mg\(^{2+}\) is of course consistent with its absolute requirement for the hydrolysis that is prominent there.

Because a higher rate of hydrolysis was revealed at NBD2 than NBD1, we asked whether the pattern of divalent cation dependence of hydrolysis by the whole CFTR protein might parallel that at NBD2. This was found to be the case, as Mn\(^{2+}\) was more effective than Mg\(^{2+}\) in both instances (Fig. 2). This result is consistent with the idea that the greater proportion of ATP hydrolysis by CFTR occurs at NBD2.

To confirm the indications from Fig. 1 that some of the labeling with \( \alpha^{32}\text{P}\)N3ATP as substrate was with...
H9251-32P]N3ADP formed by hydrolysis, photolabeling was also performed with H9251-32P]N3ADP as substrate (Fig. 3). Both domains were labeled if the radioactive nucleotide was not washed out before UV irradiation. However, with washing to remove free nucleotide before photoactivation, only NBD1 was labeled, indicating there had been complete dissociation from NBD2 before photolysis. These results combined with those from Fig. 1 demonstrate a major difference not only in the rate of hydrolysis at the two domains but also in the rate of dissociation of the NDP; it is readily released from NBD2 while retained at NBD1. This interpretation is supported quantitatively in the experiments in Fig. 4.

To determine whether the nucleotide interactions at one NBD are dependent on those at the other, the crucial Walker A lysine residues were mutagenized (Fig. 5). This resulted in prevention of labeling of the domain containing the substituted lysine but not of the other domain. Therefore, binding and hydrolysis at one NBD1 is apparently not essential for that to occur at the other. Hence, if there is coupling between the domains, it is not at this level. The retention of activity by one domain when the other is compromised parallels the channel gating that persists when either one of the domains is similarly mutagenized (30), which, along with other observations, has led to the suggestion that either of the NBDs may gate the channel (23). Such individual action of the two domains is different from the situation with one other ABCC subfamily relative of CFTR, the MRP1-conjugated anion transporter in which nucleotide interactions at one NBD strongly influence those at the other (11–13). It is possible that this type of allosteric coupling between NBDs in CFTR could require phosphorylation by cAMP-dependent protein kinase, which is essential for channel activation under normal circumstances (42).

As one attempt to also estimate the relative affinity of the two NBDs for the substrate and product of the ATPase reaction, we determined the concentration dependence of photolabeling with [α-32P]N3ADP and [γ-32P]N3ATP at 0 °C (Fig. 7). Although these are not equilibrium binding measurements, the amount of labeling does reflect the balance between association and dissociation during the 10-min incubation before photolabeling. In distinct contrast to the differential labeling of the

**Fig. 7.** Concentration dependence of photolabeling of wild-type CFTR with [α-32P]N3ADP (A) and [γ-32P]N3ATP (B) at 0 °C. Experiments were performed as in Fig. 1 and analyzed by electronic autoradiography to generate the plots shown. In A similar K_D values of ~14 μM could be estimated for N3ADP binding to both domains. In B, a value of ~34 μM was estimated for N3ATP binding to NBD1. No single value could be determined for NBD2, but, when the data were fitted to the Hill equation, a Hill coefficient of 1.9 was obtained. This experiment has been repeated three times with very similar results.
domains by N\textsubscript{2}AMP-PNP, N\textsubscript{2}ADP labeling of both was very similar (Fig. 7A). However, with the hydrolyzable NTP, there was greater labeling of NBD1 than NBD2 at low concentrations (Fig. 7B). The overall shape of the NBD2 curve was suggestive of a cooperative interaction at that domain, which was not evident at NBD1. This provides one additional piece of evidence that the two NBDs of CFTR are distinct in their interactions with nucleotides. Despite the apparent independence at this level, it is entirely possible that there is functional coupling of the impact of the nucleotide interactions at the two domains, as has recently been demonstrated in the action of SUR2A on K\textsubscript{ATP} channel gating (43). Although our present findings do not explicitly clarify the mechanism whereby the action of ATP at the NBDs of CFTR influences channel gating, they do reveal some of the properties of the domains that must be incorporated into models of this mechanism. To summarize these properties, NBD1 appears to be primarily a binding site where nucleoside triphosphate can interact in the absence of a divalent cation and from which the nucleoside diphosphate, produced by the hydrolysis that does occur when a divalent ion is added, dissociates slowly. NBD2 is a site of high turnover reduced by the hydrolysis that does occur when a divalent ion is present. NBD2 is a site of high turnover where there is both more rapid hydrolysis and dissociation of the nucleoside diphosphate product. Additional studies of both nucleotide binding and hydrolysis employing kinetic assays and channel activity are required to incorporate these characteristics into models of nucleotide-regulated CFTR channel gating.

Acknowledgments—We thank Sharon Fleck and Marv Ruona for the preparation of the manuscript and the figures, respectively.

REFERENCES
1. Saurin, W., Hofnung, M., and Dassa, E. (1999) J. Mol. Biol. 48, 22–41
2. Dean, M., Rzhetsky, A., and Allikmets, R. (2001) Genome Res. 11, 1156–1166
3. Holland, I. B., and Blight, M. A. (1999) J. Mol. Biol. 283, 381–399
4. Higgins, C. F. (1992) Annu. Rev. Cell Biol. 8, 67–113
5. Rosenberg, M. F., Velarde, G., Ford, R. C., Martin, C., Berridge, G., Kerr, I. D., Hanrahan, J. W., and Riordan, J. R. (1993) J. Biol. Chem. 268, 11304–11311
6. Nagata, K., Nishitani, M., Matsuo, M., Kioka, N., Amachi, T., and Ueda, K. (2000) J. Biol. Chem. 275, 17526–17530
7. Hou, Y.-X., Cui, L., Riordan, J. R., and Chang, X.-B. (2000) J. Biol. Chem. 275, 26463–26467
8. Zhang, S., Szakacs, G., Hegeds, T., and Sarkadi, B. (1999) J. Biol. Chem. 274, 12209–12212
9. Sokol, N., Szakacs, G., Hegeds, T., and Sarkadi, B. (1999) J. Biol. Chem. 274, 12209–12212
10. Aleksandrov, L., Menges, A., Chang, X., Aleksandrov, A., and Riordan, J. R. (2001) J. Biol. Chem. 276, 12918–12923
11. Chang, X.-B., Tabcharani, J. A., Hou, Y.-X., Jensen, T. J., Kartner, N., Alon, N., Hanrahan, J. W., and Riordan, J. R. (1991) J. Biol. Chem. 266, 5110–5119
12. Szabo, K., Augustinas, O., Jensen, T. J., Naimis, A. L., and Riordan, J. R. (1992) Nat. Genet. 1, 321–327
13. Chang, X.-B., Hou, Y.-X., and Riordan, J. R. (1997) J. Biol. Chem. 272, 30962–30966
14. Eckstein, F. (1985) Annu. Rev. Biochem. 54, 367–402
15. Ueda, K., Inagaki, N., and Seino, S. (1997) J. Biol. Chem. 272, 22983–22986
16. Aleksandrov, A. A., Chang, X., Aleksandrov, L., and Riordan, J. R. (2000) J. Physiol. 528, 259–265
17. Ikuma, M., and Welsh, M. J. (2000) Proc. Natl. Acad. Sci. U.S.A. 97, 8675–8680
18. Schultz, B. D., Bridges, R. J., and Frizzell, R. A. (1996) J. Membr. Biol. 151, 63–75
19. Li, C., Ramjeesingh, M., Wang, W., Garami, E., Lee, D., Rommens, J. M., Galley, K., and Bear, C. E. (1996) J. Biol. Chem. 271, 28560–28565
20. Hopfner, K. P., Karcher, A., Shin, D. S., Craig, L., Arthur, L. M., Carney, J. P., and Tainer, J. A. (2000) Cell 101, 789–800
21. Diederichs, K., Dies, J., Groller, G., Muller, C., Breed, J., Schnell, C., Vorhein, C., Boos, W., and Welte, W. (2000) EMBO J. 19, 5591–5601
22. Jones, P. M., and George, A. M. (1999) J. Biol. Chem. 274, 37479–37482
23. Ikuma, M., and Welsh, M. J. (2000) J. Biol. Chem. 275, 28757–28763
24. Seibert, F. S., Chang, X. B., Aleksandrov, A. A., Clarke, D. M., Hanrahan, J. W., and Riordan, J. R. (1993) J. Biol. Chem. 268, 30962–30966
25. Li, C., Ramjeesingh, M., Wang, W., Garami, E., Lee, D., Rommens, J. M., Galley, K., and Bear, C. E. (1996) J. Biol. Chem. 271, 28560–28565
26. Hopfner, K. P., Karcher, A., Shin, D. S., Craig, L., Arthur, L. M., Carney, J. P., and Tainer, J. A. (2000) Cell 101, 789–800
27. Diederichs, K., Dies, J., Groller, G., Muller, C., Breed, J., Schnell, C., Vorhein, C., Boos, W., and Welte, W. (2000) EMBO J. 19, 5591–5601
28. Jones, P. M., and George, A. M. (1999) J. Biol. Chem. 274, 37479–37482
29. Sheppard, D. N., and Welsh, M. J. (1999) J. Biol. Chem. 274, 37479–37482
30. Hou, Y.-X., Cui, L., Riordan, J. R., and Chang, X.-B. (2000) J. Biol. Chem. 275, 20279–20285
31. Hou, Y.-X., Cui, L., Riordan, J. R., and Chang, X.-B. (2000) J. Biol. Chem. 275, 13098–13108
32. Hou, Y.-X., Cui, L., Riordan, J. R., and Chang, X.-B. (2000) J. Biol. Chem. 275, 20279–20285
33. Randak, C., Neth, P., Auerswald, E. A., Eckerskorn, C., Assfalg-Machleidt, I., and Machleidt, W. (1997) FEBS Lett. 410, 140–146
34. Hartman, J., Huang, Z., Rado, T. A., Peng, S., Jilling, T., Muccio, D. D., and Sorscher, E. J. (1992) J. Biol. Chem. 267, 6455–6458
35. Ko, Y. H., Thomas, P. J., Delanney, M. R., and Pedersen, P. L. (1993) J. Biol. Chem. 268, 24303–24308
36. Qu, B. H., and Thomas, P. J. (1996) J. Biol. Chem. 271, 7261–7264
37. Yike, I., Ye, J., Zhang, Y., Manavalan, P., Gerken, T. A., and Dearborn, D. G. (1996) Protein Sci. 5, 89–97
38. Neville, D. C., Rozanov, C. A., Tulk, B. M., Townsend, R. R., and Verkman, A. S. (1998) Biochemistry 37, 2401–2409
39. Lu, N. T., and Pedersen, P. L. (2000) Arch. Biochem. Biophys. 375, 7–20
40. Howell, L. D., Borchartt, R., and Cohn, J. A. (2000) Biochem. Biophys. Res. Commun. 271, 518–525
41. Duffieux, F., Annereau, J. P., Bouchar, J., Miclet, E., Pandl, O., Schneider, M., Stoven, V., and Lallemand, J. Y. (2000) Eur. J. Biochem. 267, 5306–5312
42. Seibert, F. S., Chang, X. B., Aleksandrov, A. A., Clarke, D. M., Hanrahan, J. R., and Riordan, J. R. (1999) Biochem. Biophys. Acta 1461, 275–283
43. Zingman, L. V., Hodgson, D. M., Riesengraber, M., Karger, A. B., Kathmann, E. C., Alekseev, A. E., and Terziev, A. (2002) J. Biol. Chem. 277, 14206–14210
The First Nucleotide Binding Domain of Cystic Fibrosis Transmembrane Conductance Regulator Is a Site of Stable Nucleotide Interaction, whereas the Second Is a Site of Rapid Turnover
Luba Aleksandrov, Andrei A. Aleksandrov, Xiu-bao Chang and John R. Riordan

J. Biol. Chem. 2002, 277:15419-15425.
doi: 10.1074/jbc.M111713200 originally published online February 22, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M111713200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 43 references, 20 of which can be accessed free at http://www.jbc.org/content/277/18/15419.full.html#ref-list-1