After phosphorylation by protein kinase A, gating of the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel is regulated by the interaction of ATP with its nucleotide binding domains (NBDs). Models of this gating regulation have proposed that ATP hydrolysis at NBD1 and NBD2 may drive channel opening and closing, respectively (reviewed in Nagel, G. (1999) Biochim. Biophys. Acta 1461, 263–274). However, as yet there has been little biochemical confirmation of the predictions of these models. We have employed photoaffinity labeling with 8-azido-ATP, which supports channel gating as effectively as ATP to evaluate interactions with each NBD in intact membrane-bound CFTR. Mutagenesis of Walker A lysine residues crucial for azido-ATP hydrolysis to generate the azido-ADP that is trapped by vanadate indicated a greater role of NBD1 than NBD2. Separation of the domains by limited trypsin digestion and enrichment by immunoprecipitation confirmed greater and more stable nucleotide trapping at NBD1. This asymmetry of the two domains in interactions with nucleotides was reflected most emphatically in the response to the nonhydrolyzable ATP analogue, 5′-adenylyl-β,γ-imidodiphosphate (AMP-PNP), which in the gating models was proposed to bind with high affinity to NBD2 causing inhibition of ATP hydrolysis there postulated to drive channel closing. Instead we found a strong competitive inhibition of nucleotide hydrolysis and trapping at NBD1 and a simultaneous enhancement at NBD2. This argues strongly that AMP-PNP does not inhibit ATP hydrolysis at NBD2 and thereby questions the relevance of hydrolysis at that domain to channel closing.

The cystic fibrosis transmembrane conductance regulator (CFTR) has two nucleotide binding domains (NBDs) that are believed in some manner to regulate permeation of chloride ions (1). Whereas the two NBDs of another ABC protein, the P-glycoprotein multidrug transporter, are highly homologous and believed to be functionally equivalent (2), the domains in members of the ABCC subfamily to which CFTR belongs show less sequence similarity (3). This asymmetry is reflected functionally in two members of this subfamily, SUR1 (4, 5) and MRP1 (6–8). Both ATP and ADP act on SUR1 to regulate the Kir6.2 potassium channel; ATP is bound at NBD1 and also bound and hydrolyzed at NBD2, and the ADP produced stabilizing ATP binding to NBD1 (9). Similarly in the case of MRP1, which transports conjugated anions, ATP binding was detected exclusively at NBD1 and was enhanced allosterically by the trapping of ADP produced by hydrolysis at NBD2 (6).

Schemes of ATP binding and hydrolysis by CFTR have been proposed solely on the basis of channel-gating responses (10–12). Implicit in these models is the idea that channel opening is driven by ATP binding and hydrolysis at one NBD and closing at the other (13). Intact CFTR (14) and bacterial fusion proteins containing either of the NBDs (15, 16) hydrolyze ATP. It has been reported also that photoaffinity labeling of CFTR by 8-azido-ATP at NBD1 occurred in the absence of orthovanadate, whereas NBD2 labeling required its presence (17). Here we show that 8-azido-ATP, which supports CFTR channel activity as well as ATP, labels both NBDs in a vanadate-stimulatable manner indicating that hydrolysis and trapping occurs at both domains.

Furthermore, the nonhydrolyzable ATP analogue AMP-PNP, which has been relied upon heavily in the formulation of models of the action of the NBDs in gating (18, 19), was found to have entirely opposite effects on the two domains. Strong competitive inhibition of nucleotide trapping at NBD1 without prevention of channel opening (1) makes it unlikely that hydrolysis at NBD1 drives channel opening. Similarly stimulation rather than inhibition of trapping at NBD2 does not support the idea that AMP-PNP prevents channel closing by inhibition of hydrolysis there.

**EXPERIMENTAL PROCEDURES**

**Materials**—BHK-21 cells stably expressing wild-type human CFTR were prepared and maintained as described previously (20) as were cells expressing the K464A and K1250A mutants. The mouse monoclonal antibodies, L12B4 and M3A7, used for immunoprecipitation and Western blotting, were those described by Kartner et al. (21). The antibody MM13-4 recognizes an epitope between residues 24 and 35. 8-Azido-[α-32P]ATP was purchased from ICN Biomedicals. AMP-PNP and other nucleotides and TPCK-treated trypsin were from Sigma as were all protease inhibitors except Pefabloc, which was from Roche Molecular Biochemicals. 8-Azido-AMP-PNP was synthesized by Affinity Labeling Technologies, Inc.

**Membrane Isolation**—CFTR-expressing BHK cells were homogenized in an ice-cold hypotonic buffer containing 10 mM HEPES, pH 7.2,
Radioactivity associated with the CFTR band was determined by elec-
a
trometric analysis by SDS-PAGE and 150–200 lane m for standard (x-ray film) and electronic (Packard Instant Imager)

Precipitates were subjected to SDS-PAGE and transferred to nitrocel-

limited proteolysis and immunoprecipitation—

Photoaffinity labeling with 8-azido-[α-32P]ATP—The photoactivatable radionucleotide at the concentration indicated in each experiment was incubated with the resuspended membranes (15–30 μg of protein for direct analysis by SDS-PAGE and 150–200 μg of protein for immunoprecipitation) for 10 min at 37 °C in the presence or absence of sodium orthovanadate (0.5 mM). After transfer to ice the membrane suspension was irradiated for 2 min in a Stratalinker UV cross-linker (λ = 254 nm) either before or after pelleting to wash out unbound 8-azido-[α-32P]ATP and resuspension in 40 mM Tris-HCl, pH 7.4, 0.1 mM EDTA. The membranes then were solubilized directly in gel loading buffer for SDS-PAGE or digested with trypsin prior to immunoprecipitation.

Limited proteolysis and immunoprecipitation—Photoaffinity-labeled membranes were incubated with TPCK-treated trypsin for 15 min on ice at an enzyme to membrane protein mass ratio of 1:180. Digestion was terminated with excess soybean inhibitor, and membranes were solubilized in RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% deoxycholate, 1% Triton, 0.1% SDS, 150 mM NaCl) and immunoprecipitated with either of the monoclonal antibodies L12B4 or M3A7 (21). The immunoprecipitates were subjected to SDS-PAGE and transferred to nitrocel-

ulose for standard (x-ray film) and electronic (Packard Instant Imager) autoradiography.

results

Fig. 1. Photoactivation-dependent radiolabeling of CFTR with 8-azido-[α-32P]ATP. A, membranes (30 μg of protein) from BHK-21 cells expressing CFTR were incubated with 5 μM 8-azido-
[α-32P]ATP in the absence (−) or presence (+) of vanadate as described under “Experimental Procedures.” After irradiation, samples were sub-

Submitted to SDS-PAGE and autoradiography. Lane m had membranes from mock-transfected BHK cells not expressing CFTR. B, results of an experiment identical to the one in A but with no UV irradiation. C, inhibition of photolabeling by increasing concentrations of ATP (○) and 8-azido-ATP (●) present during incubation prior to photoactivation. 32P radiolab-

ativity associated with the CFTR band was determined by electron autoradiography (Packard Instant Imager).

1 mM EDTA, and a protease inhibitor mixture consisting of leupeptin (1 μg/ml), aprotinin (μg/ml), E64 (3.5 μg/ml), and benzamidine (120 μg/

ml). Nuclei and unbroken cells were removed by centrifugation for 15

min at 600 × g. Membranes were pelleted by centrifugation for 30 min at 100,000 × g and then resuspended in 40 mM Tris-HCl, pH 7.4, 5 mM

MgCl2, 0.1 mM EGTA. The membranes then were solubilized in RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% deoxycholate, 1% Triton, 0.1% SDS, 150 mM NaCl) and immunoprecipitated with either of the monoclonal antibodies L12B4 or M3A7 (21). The immunoprecipitates were subjected to SDS-PAGE and transferred to nitrocel-

ulose for standard (x-ray film) and electronic (Packard Instant Imager) autoradiography.

Influence of Walker A Lysine Mutagenesis and Domain-spe-
cific Antibody Binding—In P-glycoprotein (2) and MRPI (6) mutagenesis of the Walker A lysine residues in either NBD1 or

NBD2 prevents ATP hydrolysis and hence trapping of 8-azido-
[α-32P]ADP. Because neither CFTR channel gating (22) nor hydrolysis of ATP (23) is prevented completely by substitution of these residues, it was of interest to determine their effect on photolabeling. Fig. 2A shows that labeling of the NBD2 mutant

Fig. 2. Walker A lysine mutagenesis and antibody binding in-
hibit photolabeling of NBD1. A, membranes from BHK cells expressing wild-type and the K1250A (15 μg of protein) and K464A variants (60 μg of protein) were incubated with 20 μM 8-azido-[α-32P]ATP in the presence of 0.5 mM orthovanadate and processed as under “Experimen-
tal Procedures.” A Western blot of the same gel probed with M3A7 is shown below this autoradiogram. Note that 4 times more K464A mem-
brane protein was used than wild type and K1250. The cells expressing K464A had also been grown at 26 °C to promote maturation of the mutant CFTR. B, 15 μg of BHK membrane protein containing wild-type CFTR was incubated at 0 °C for 1 h with 50 μg/ml purified immuno-

globulin from each of the mouse monoclonal antibodies. Prior to photo-

labeling membranes were pelleted and rinsed with binding buffer be-

fore incubation with 8-azido-ATP. C, a sketch indicating locations of epitopes recognized by antibodies L12B4, M3A7, and MM13-4 and approximate major trypsin digestion sites (T) is shown.
(K1250A) is reduced only a small amount compared with wild type indicating that normally there may be little hydrolysis and trapping at NBD2. In contrast the comparable substitution in NBD1 (K464A) greatly diminished labeling compared with wild type indicating that the majority of labeling and trapping may occur at NBD1. However, the effects of this mutation have to be considered in light of the fact that it also interferes with biosynthetic maturation of the protein as is reflected in the much lower ratio of the larger mature band to the smaller immature band in the Western blot shown below the autoradiogram. Nevertheless the amount of labeling is reduced substantially relative to the amount of mature protein present.

As an additional indirect means of assessing the role of the two NBDs in the photolabeling of the whole protein the influence of the binding of different monoclonal antibodies was measured. L12B4, which binds near the N terminus of NBD1 (21), reduced labeling to a very low level again suggesting that much of the labeling occurred at NBD1. The sites responsible for the 40-kDa band detected by L12B4 are not clearly established. Digestion near the N terminus of NBD2 is responsible for the 30–35 kDa bands detected by M3A7. The assignment of the bands was confirmed by additional antibodies recognizing epitopes within NBD1 and NBD2 (data not shown).

When immunoprecipitations were carried out after digestion to enrich the NBD-containing fragments, the results shown in Fig. 3B were obtained. These blots confirmed that the L12B4 antibody has immunoprecipitated the NBD1-assigned fragments, and the M3A7 antibody has immunoprecipitated the NBD2 fragments (Fig. 3B, lanes 2 and 3). However, unexpectedly this experiment also showed that the large fragments containing the two NBDs remained strongly associated after detergent solubilization the same two antibodies employed above were then used to immunoprecipitate polypeptide fragments containing each domain. It was first necessary to identify these fragments by immunoblotting after their separation by SDS-PAGE. These results are shown in Fig. 3A. The L12B4 antibody detected undigested CFTR at the top of the gel, a tight set of bands in the 70–80 kDa range, and a band at 40 kDa. The same procedure using the M3A7 antibody detected in addition to undigested CFTR a strong band at 95–105 kDa that is actually a doublet and a set of bands in the 30–35 kDa range. The large fragments detected by each antibody reflect trypsin cleavage(s) in the R-domain (Fig. 2C). The sites responsible for the 40-kDa band detected by L12B4 are not clearly established. Digestion near the N terminus of NBD2 is responsible for the 30–35 kDa bands detected by M3A7. The assignment of the bands was confirmed by additional antibodies recognizing epitopes within NBD1 and NBD2 (data not shown).

Localization of Labeling Sites—To assess hydrolysis and trapping at each of the NBDs more directly, membranes were treated with trypsin to cleave between the domains. After
trypsin digestion. That is, probing the NBD2 immunoprecipitate with antibody to NBD1 revealed the presence of the large NBD1 bands (Fig. 3B, lane 1). Similarly probing the NBD1 immunoprecipitate with antibody to NBD2 detected the lower member of the large NBD2 doublet (Fig. 3A, lane 4). These immunoprecipitations were done in RIPA buffer with its multiple detergents, but even when the trypsin digests were incubated in 2% SDS prior to dilution to RIPA conditions these same co-immunoprecipitations occurred. These data do not absolutely prove that the strong interaction between these fragments are exclusively between the NBDs, because the large NBD2-containing fragment also contains all of the second membrane-associated domain and part of the R-domain. Other studies with the domains expressed separately have found interactions between NBD1 and NBD2 (24), but the observations reported here are the first suggesting that the strong binding occurs in the intact functional protein. This is not crucial to the main point of this paper, which is to demonstrate the asymmetry of nucleotide interactions at the two NBDs and their relevance to channel regulation, but it will be important in more detailed future studies of the mechanism. The positions of the reduced immunoglobulin bands present in the immunoprecipitates are shown in Fig. 3C and account for the bands of these mobilities in Fig. 3B.

\[ \text{FIG. 5. AMP-PNP inhibits photolabeling of NBD1 with 8-azido-[a-32P]ATP.} \]

A, membranes were incubated with 25 \( \mu \text{M} \) 8-azido-[a-32P]ATP in the presence of the indicated concentrations of AMP-PNP. After irradiation and trypsin digestion membranes were solubilized in RIPA buffer, immunoprecipitated with L12B4, and electrophoresed. B, similar experiments were performed using increasing concentrations of ATP, ADP, or dATP. The amount of 32P radioactivity associated with NBD1 and NBD2 was determined by electronic autoradiography and plotted as the histograms show.

\[ \text{FIG. 6. 8-Azido-AMP-PNP inhibits photolabeling of NBD1 and stimulates labeling of NBD2.} \]

The experiment was performed as in Fig. 5A but 8-azido-AMP-PNP replaced AMP-PNP. A, autoradiogram; B, plots of the relative amounts of radioactivity associated with NBD1 and NBD2 as determined by electronic autoradiography. The NBD2 curve is that of strictly competitive inhibition using a \( K_i \) of 2 \( \mu \text{M} \). The experimental points fit this curve well.

8-Azido-AMP-PNP inhibits photolabeling of NBD1 and stimulates labeling of NBD2. The experiment was performed as in Fig. 5A but 8-azido-AMP-PNP replaced AMP-PNP. Fig. 4A reveals that both domains are labeled, but there is more labeling of NBD1 than NBD2. In this experiment the free 8-azido-ATP incubated with membranes was not removed prior to photoactivation. When the nucleotides were washed out before irradiation labeling of NBD1 was not altered, but labeling at NBD2 was reduced greatly (Fig. 4B). This was evident particularly in the large decrease in intensity of the small NBD2 bands but was seen also in the large NBD2 bands. This result suggests there may be lesser affinity for the nucleotide at NBD2 than at NBD1. This is substantiated by the concentration dependence shown in Fig. 4C, which also indicates saturation of labeling at NBD1 at a lower 8-azido-[a-32P]ATP than at NBD2.
AMP-PNP Inhibits Labeling of NBD1 and Increases Labeling of NBD2—The nonhydrolyzable ATP analogue, AMP-PNP, has been used extensively in attempts to determine the role of nucleotide binding and hydrolysis in CFTR channel gating (1, 25). Its ability when present together with ATP to lock the channel open has been attributed to its suggested binding to NBD2 and prevention of hydrolysis there proposed to be necessary for channel closing (13). Incubation of increasing concentrations of AMP-PNP together with 8-azido-[α-32P]ATP prior to photoactivation strongly inhibited labeling at NBD1 but not at NBD2 (Fig. 5A). In fact there was increased labeling of the NBD2 bands at the lower AMP-PNP concentrations. In contrast to the differential effects of AMP-PNP, other nucleotides including ATP, ADP, and dATP competed for labeling at either domain approximately equally (Fig. 5B). dATP was more potent than ATP and ADP, consistent with other observations in which the deoxynucleotide had a lower \( K_m \) for hydrolysis by CFTR and a strong influence on channel gating (data not shown). However, the opposite effects of AMP-PNP on NBD1 and NBD2 were most striking, because it is the only compound we have found yet to influence the two domains differentially. This observation is important, because it is the first direct test of AMP-PNP interactions predicted on the basis of the its locking-open of the channel in the presence of ATP (18). The enhancement of nucleotide trapping at NBD2 would not seem to be in agreement with the predicted inhibition of hydrolysis there. The pronounced reduction in trapping at NBD1 could reflect an inhibition of hydrolysis at that domain.

To explore these reciprocal effects of AMP-PNP further we had the compound 8-azido-AMP-PNP synthesized. Because 8-azido-ATP was far more effective than ATP in competing for photolabeling by 8-azido-[α-32P]ATP (Fig. 1C) we reasoned that 8-azido-AMP-PNP also might have a more potent influence than AMP-PNP on the interactions of 8-azido-[α-32P]ATP. Fig. 6 shows that this was indeed the case. Qualitatively, the effect was the same as that of AMP-PNP, i.e. there was inhibition of labeling at NBD1 and enhancement of that at NBD2. Quantitatively however these effects occurred at nearly 10-fold lower concentrations and were more clear-cut. The data points relating labeling at NBD1 and 8-azido-AMP-PNP concentration (Fig. 6B) fit very well with a strictly competitive inhibition curve with a \( K_i \) in the range of 1–2 \( \mu \)M. The curve for labeling at NBD2 is almost the mirror image of that at NBD1 at concentrations up to 100 \( \mu \)M. The basis of this effect cannot be interpreted so readily, but it may reflect an indirect consequence of the high affinity interaction at NBD1. At concentrations between 100 \( \mu \)M and 1 mM this effect is overcome probably by direct competition for binding to the lower affinity site on NBD2.

**DISCUSSION**

The advantage gained by utilization of two rather than one NBD by ABC proteins is not obvious. In the case of CFTR, which is unique among these molecules as an ion channel, a possible correspondence of this duality with the two discrete steps in channel gating, i.e. opening and closing, is an attractive idea. Indeed this concept has been central in prevalent models of CFTR channel gating (25). Consistent with this possible separation of function between the domains, nucleotide interactions at the NBDs of other ABC family proteins are markedly asymmetric (6–9). As we show here this also seems to be the case for CFTR, i.e. ATP binding and ADP trapping is tighter at NBD1 than NBD2, and AMP-PNP has opposite effects on the two domains. This latter observation is significant, because studies of the effect of AMP-PNP on CFTR channel gating in the presence of ATP have been interpreted on the basis of the assumption that it binds with high affinity to NBD2 (1). By so doing the nonhydrolyzable nucleotide was proposed to maintain the channel in an open state until it slowly dissociated (18). Explicit in this proposal was the idea that the hydrolysis of ATP at this site would normally be necessary for channel closing. Our present observations show that AMP-PNP competes for nucleotide trapping at NBD1 and at the same time enhances the interaction at NBD2 probably as an allosteric response to the higher affinity binding at NBD1. The fact that the two domains seem firmly associated in the intact protein supports the feasibility of such allosteric interactions. In this regard it is significant that major conformational changes have been observed in P-glycoprotein on binding AMP-PNP (26).

Although most of the attention has been paid to the locking-open effect of AMP-PNP (1, 13, 25) Weinreich et al. (27) more recently have shown that it also slows channel opening. On this basis these authors suggested that there may be two binding sites for AMP-PNP on CFTR. Our data also indicate that the compound has two different types of impact on the protein. It should be noted also that the CFTR channel can be gated in the absence of ATP by high concentrations of AMP-PNP alone (28). However, this requires much larger concentrations (≥5 mM) than those that elicit the two effects described in this paper.

In our present findings indicate that the two NBDs of CFTR are quite asymmetrical in terms of their ability to bind and occlude nucleotide, the interaction at NBD1 being more stable than that at NBD2. The influence of AMP-PNP is quite different than that predicted from its effect on channel gating and hence has implications for models based on these. There is a high affinity competitive interaction at NBD1 and a concomitant stimulatory influence on NBD2 arguing directly against the notion that it inhibits channel closing by preventing hydrolysis at NBD2 (1, 25). These and other data showing that mutagenesis of crucial residues in either NBD does not influence channel opening and closing predictably (11) makes it difficult to attribute each transition separately to nucleotide interactions at one or other of the domains. Instead it may be more likely that opening and closing occur as a consequence of nucleotide binding and dissociation or hydrolysis at a functional unit involving both domains. Such an interpretation is consistent with the fact that the domains are physically associated in the intact protein and with proposals that the NBDs of other ABC proteins may participate jointly in ATP binding and hydrolysis utilizing amino acid residues from each (2, 29).

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Differential Interactions of Nucleotides at the Two Nucleotide Binding Domains of the Cystic Fibrosis Transmembrane Conductance Regulator
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