Prolonged Nonhydrolytic Interaction of Nucleotide with CFTR’s NH₂-terminal Nucleotide Binding Domain and its Role in Channel Gating

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Abstract CFTR, the protein defective in cystic fibrosis, functions as a Cl⁻ channel regulated by cAMP-dependent protein kinase (PKA). CFTR is also an ATPase, comprising two nucleotide-binding domains (NBDs) thought to bind and hydrolyze ATP. In hydrolyzable nucleoside triphosphates, PKA-phosphorylated CFTR channels open into bursts, lasting on the order of a second, from closed (interburst) intervals of a second or more. To investigate nucleotide interactions underlying channel gating, we examined photolabeling by [α³²P]8-N₂ATP or [γ³²P]8-N₂ATP of intact CFTR channels expressed in HEK293T cells or *Xenopus* oocytes. We also exploited split CFTR channels to distinguish photolabeling at NBD1 from that at NBD2. To examine simple binding of nucleotide in the absence of hydrolysis and gating reactions, we photolabeled after incubation at 0°C with no washing. Nucleotide interactions under gating conditions were probed by photolabeling after incubation at 30°C, with extensive washing, also at 30°C. Phosphorylation of CFTR by PKA only slightly influenced photolabeling after either protocol. Strikingly, at 30°C nucleotide remained tightly bound at NBD1 for many minutes, in the form of nonhydrolyzed nucleoside triphosphate. As nucleotide-dependent gating of CFTR channels occurred on the time scale of seconds under comparable conditions, this suggests that the nucleotide interactions, including hydrolysis, that time CFTR channel opening and closing occur predominantly at NBD2. Vanadate also appeared to act at NBD2, presumably interrupting its hydrolytic cycle, and markedly delayed termination of channel open bursts. Vanadate somewhat increased the magnitude, but did not alter the rate, of the slow loss of nucleotide tightly bound at NBD1. Kinetic analysis of channel gating in Mg8-N₂ATP or MgATP reveals that the rate-limiting step for CFTR channel opening at saturating [nucleotide] follows nucleotide binding to both NBDs. We propose that ATP remains tightly bound or occluded at CFTR’s NBD1 for long periods, that binding of ATP at NBD2 leads to channel opening whereupon its hydrolysis prompts channel closing, and that phosphorylation acts like an automobile clutch that engages the NBD events to drive gating of the transmembrane ion pore.

Key words: ABC transporters • photolabeling • 8-azido nucleotides • ATP binding and hydrolysis • orthovanadate

Introduction The CFTR Cl⁻ ion channel is encoded by the gene found mutated in cystic fibrosis patients (Riordan et al., 1989). CFTR (ABCC7) belongs to subfamily C in the large family of ABC (ATP-binding cassette) proteins. Other subfamily C members include the multidrug resistance–related protein, MRP1, believed to transport cytotoxic substances out of cells, and the sulfonylurea receptors, such as SUR1, that regulate ATP-sensitive inwardly-rectifying K⁺ channels with which they closely associate. Full-length CFTR, MRPI, and SUR1 proteins each incorporate two nucleotide binding domains (NBDs) anticipated to bind and hydrolyze ATP to drive the conformational changes that underlie their regulatory or transport function. Hydrolysis of ATP has been demonstrated directly for CFTR (Li et al., 1996; Ramjeesingh et al., 1999) and MRP1 (Chang et al., 1998), and inferred for SUR1 (e.g., Matsuo et al., 1999), but in none of these cases is the link between nucleotide binding and hydrolysis and ABC protein function well understood.

In principle, insights into functional mechanisms of these ABC proteins can be gained by examining interactions of their NBDs with the photoactivatable nucleotides [α³²P]8-N₂ATP and [γ³²P]8-N₂ATP in parallel with the functional consequences of those interactions. This approach was exploited in studies (Urbatsch et al., 1995a,b) of the ABC-B subfamily member, multidrug resistance protein, MDR (also called P-glycoprotein, P-gp), that analyzed inhibition of ATP hydrolysis by the

Abbreviation used in this paper: NBD, nucleotide-binding domain.
inorganic phosphate analogue orthovanadate (V_i). In other ATPases, V_i was shown to bind tightly in the catalytic site in place of released phosphate to form an extremely stable complex with the other hydrolysis product, ADP, and thereby prevent further cycles of hydrolysis (e.g., Lindquist et al., 1975). In P-gp, cross-linking of V_i-trapped [^{32}P]8-N_9ADP by UV light revealed that ATPase activity was fully inhibited when only a single NBD in each P-gp was photolabeled, but that labeling was found more-or-less equally in NH_2- and COOH-terminal NBDs (Urbatsch et al., 1995a,b; but cf. Hrycyna et al., 1999). This suggested that the two NBDs randomly hydrolyzed ATP, but displayed obligatory catalytic cooperativity (Senior et al., 1995; Urbatsch et al., 1995b) such that inhibition of ATP hydrolysis at one catalytic site prevented even a single hydrolysis event at the other (Urbatsch et al., 1998). The implied functional similarity between the two NBDs corresponds to their substantial sequence identity, and is corroborated by the finding that mutant P-gp with a second copy of NBD1 in place of NBD2 could still extrude drug from cells (Hrycyna et al., 1999).

In contrast, in the ABC-C family members, MRP1, SUR1, and CFTR, the two NBDs diverge not only structurally (e.g., only 27% identity between NBD1 and NBD2 sequences in CFTR), but also functionally. Thus, analysis of photolabeling measurements with [^{32}P]8-N_9ATP with and without V_i suggested that ATP hydrolysis in MRP1 (Chang et al., 1998) occurred predominantly at NBD2 (Gao et al., 2000; Hou et al., 2000; Nagata et al., 2000). Also, in SUR1, although both NBDs were photolabeled after incubation with [^{32}P]8-N_9ATP, only NBD1 (and not NBD2) was photolabeled by [^{32}P]8-N_9ATP (Ueda et al., 1997; Matsuo et al., 1999), suggesting that hydrolysis likely occurs at NBD2 but not NBD1. Further mechanistic interpretations have been hampered, however, because transport assays for MRP1 are largely limited to steady-state population measurements (e.g., Gao et al., 2000; Hou et al., 2000), and functional assays for SUR1 are complicated by the likely octameric nature of the entire SUR1–K^+ channel complex (Shyng and Nichols, 1997).

CFTR, on the other hand, comprises a Cl^- ion channel whose activity is regulated by events at its NBDs (e.g., Gadsby and Nairn, 1999; Sheppard and Welsh, 1999), so that electrophysiological measurements afford high-resolution assays of function, even at the single-channel level. In photolabeling studies, nucleotide at NBD1 in CFTR was found to survive washes with ice-cold buffer before photocrosslinking (Szabó et al., 1999; Aleksandrov et al., 2001), and labeling with [^{32}P]8-N_9ATP suggested that the surviving nucleotide could include nonhydrolyzed ATP (Aleksandrov et al., 2002). Because NBD2 in CFTR could be labeled by [^{32}P]8-N_9ATP, but not by [^{32}P]8-N_9ATP (Aleksandrov et al., 2002), it appeared that, as suggested for the other ABC-C proteins, NBD2 of CFTR hydrolyzes ATP. However, the relationships, if any, between these nucleotide interactions and gating of CFTR channels remain unexplained.

In this work, we attempted to correlate measurements of nucleotide labeling of wild-type and mutant CFTR under nonhydrolytic (0°C) and hydrolytic (30°C) conditions, with or without Mg^{2+} or V_i, with measurements of CFTR channel activity. Because CFTR channel gating requires phosphorylation by PKA (e.g., Gadsby and Nairn, 1999; Sheppard and Welsh, 1999), we also examined the influence of phosphorylation on CFTR photolabeling. We found that the nucleotide remained at NBD1 in the form of nonhydrolyzed nucleoside triphosphate for several minutes at a temperature at which CFTR channels open and close every second or two. This suggests that the nucleotide association and dissociation events, together with any ATP hydrolysis, that underlie CFTR channel gating occur predominantly at NBD2. We found that V_i delayed the termination of open bursts, presumably by disrupting the hydrolytic cycle at NBD2, and somewhat increased the magnitude, without altering the rate, of the slow nucleotide loss from NBD1. Phosphorylation of CFTR by PKA had little effect on either binding or retention of nucleotide at NBD1. Moreover, by comparing channel gating kinetics during activation by Mg^{2+}ATP or MgATP, we conclude that the slow step that at saturating [nucleotide] limits the rate of CFTR channel opening to a burst follows nucleotide binding to both NBDs.

**Materials and Methods**

**Molecular Biology**

pRES-CFTR and pRES-Flag-CFTR were constructed by subcloning CFTR or Flag-CFTR from pGEMHE-CFTR or pGEMHE-Flag-CFTR (Chan et al., 2000) into the EcoRV site of the pRES vector (Clontech Laboratories, Inc.). We have previously described pGEMHE-CFTR, pGEMHE-Flag3–835 (Chan et al., 2000), and pGEMHE-K464A (Vergani et al., 2003). pGEMHE-Flag837–1480 (with Flag sequence inserted in the extracellular loop between transmembrane helices 7 and 8; cf. Howard et al., 1995) was a gift from Dr. R. Frizzell (University of Pittsburgh School of Medicine, Pittsburgh, PA).

**CFTR Expression and Preparation of Membranes**

HEK293T cells were maintained in DMEM medium (GIBCO) supplemented with 10% Fetal bovine serum (GIBCO BRL). Cells plated in 100-mm dishes were transfected with 2 μg of pRES-Flag-CFTR using Effectene transfection reagent (QIAGEN), and were harvested 2 d later for membrane preparation. To obtain membranes, cells scraped from two dishes were resuspended in 1 ml of ice-cold lysis buffer containing 10 mM HEPES (pH 7.5), 5 mM EDTA, 50 mM NaCl, 1 mg/ml BSA, and protease inhibitor cocktail (Calbiochem; final concentrations, 1 mM AEBSF HCl, 300 mM aprotinin, 2 μM E-64, 2 μM leupeptin hemisulfate), and disrupted by sonication. *Xenopus laevis* stage V-VI oocytes were
isolated as described (Chan et al., 2000), injected with 25 ng of each cRNA (in 50 nl), and incubated at 18°C for 3 d. For membrane preparation, ~200 oocytes were resuspended in 1 ml of lysis buffer and homogenized at 4°C. Membrane suspensions from HEK293T cells or oocytes were centrifuged twice at 3,000 g for 10 min at 4°C to remove debris, and then 2 ml of lysis buffer was added to the supernatant and the membranes pelleted at 175,000 g for 1 h at 4°C. The pelleted membranes were washed with 5 ml of modified lysis buffer (with 10% glycerol and no BSA), centrifuged again, and then resuspended in 0.2 ml modified lysis buffer and stored at -80°C. Protein concentration was measured with bicinchoninic acid (Pierce Chemical Co.).

**Immunoprecipitation and Protein Detection**

Immunoprecipitation with anti-Flag M2-agarose beads (affinity gel; Sigma-Aldrich) was essentially as described (Picciotto et al., 1992). Membranes were dissolved in 300 µl solubilization buffer (25 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5 mM EDTA, 2.5% nonidet P-40, 0.5% SDS). After centrifugation at 15,000 g for 10 min, 40 µl of antibody beads was added to the supernatant, and the mixture rocked overnight at 4°C. The samples were spun at 15,000 g for 10 min and washed with 500 µl of a high-salt buffer (10 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.5% nonidet P-40, 0.05% SDS), then isotonic buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% nonidet P-40, 0.05% SDS), and finally NaCl-free buffer (10 mM Tris-HCl, pH 7.5, 0.05% SDS). The immunoprecipitated proteins were released from the beads by resuspension in 50 µl of 2.5% Laemmli buffer (2.5% SDS) and centrifugation for 10 min at 15,000 g. The proteins in the supernatant were separated (15–200 µg per lane for immunoblots, 300–400 µg for 32P detection) on 6% or 8% SDS-PAGE gels, which were dried and exposed for 32P detection, or transferred onto nitrocellulose membranes (300–400 µg of protein) were resuspended in 100 µl reaction buffer with or without 1 mM V_i (and with or without Mg2+), as specified, and incubated for 15 min at 30°C. After this incubation, before cross-linking, the membranes were washed once with 900 µl ice-cold wash buffer (composition specified above, under in vitro phosphorylation), pelleted at 15,000 g for 20 min at 4°C, and washed a second time with 1,000 µl ice-cold wash buffer; then, before repeating the centrifugation, the membranes were subjected to a 0–15-min (usually 5 min) postincubation period at 30°C (compare Ueda et al., 1999) still in the nucleotide-free wash buffer. After centrifugation, the membrane pellet was resuspended in 47.5 µl cold wash buffer and the samples were irradiated with UV for 10 min on ice. Finally, 2.5 µl 20% SDS was added to solubilize the membranes and, after 10 min at room temperature, a further 100 µl 1% SDS was added and CFTR then immunoprecipitated. Photolabeling with [γ32P]ATP followed the same protocol, but controls without UV exposure were included to assess covalent incorporation of γ32P into CFTR via phosphorylation by endogenous membrane-associated kinases (compare Hou et al., 2000).

**Electrophysiology**

HEK293T cells were transfected with pIRES-CFTR directly on poly-L-lysine-coated glass coverslips. Oocytes were injected with 0.1–10 ng of cRNA transcribed from pGEMHE-CFTR (WT or K464A). Starting 12 h after transfection or cRNA injection, HEK293T cells or oocytes were transferred to a Petri dish containing standard (designed to minimize cation channel currents) bath solution (in mM): 138 NMG, 2 MgCl2, 5 HEPES, 0.5 EGTA, 134 sulfamic acid (pH 7.1 with sulfamic acid), 2–7-MΩ fire-polished pipettes contained pipette solution with (in mM): 138 NMG, 2 MgCl2, 5 HEPES, 136 HCl (pH 7.4 with HCl). After seal formation (>100 GΩ), an inside-out patch was excised and moved to a chamber where its cytosolic face was continuously superfused (0.2–0.6 ml/min) with bath solution supplemented, as indicated, with 300 nM PKA and/or nucleotides, at room temperature (21–26°C). Na8N3ATP (ICN Biomedicals, Inc.) was kept as a 100 mM stock solution (pH 7.0 with NMG) in bath solution plus equimolar Mg-sulfamate. Solutions were switched by
Data Analysis

Data analysis was as described (Chan et al., 2000; Csányi et al., 2000; Vergani et al., 2003). CFTR channels open from long closures into open bursts (average duration, \( r_b \)) interrupted by brief flickery closures (Haws et al., 1992), and dwell-time analyses of single-channel records reveal, within the bandwidth of our measurements, one population of open states (average duration = mean interval between flickery closures) but two distinct populations of closed states. The durations of the brief, intraburst flickery closures and of individual openings within bursts are not influenced by [MgATP] (e.g., Vergani et al., 2003), but the duration (\( r_b \)) of the long-lived intraburst closures declines as [MgATP] is increased (Gunderson and Kopito, 1994; Winter et al., 1994). Here we compare the influence of [MgATP] with that of [Mg8-N3ATP] on CFTR channel gating kinetics in the presence or absence of \( V_i \), using three kinds of measurements. We estimated absolute gating rates in patches with a known small number (\( \leq 8 \)) of active channels, we determined rates relative to a standard condition when the number of active channels was uncertain, and we measured rates of macroscopic current relaxation in patches with large numbers of active channels.

For kinetic analysis, digitized segments of records in which individual channel opening and closing events could be discerned were baseline subtracted (to remove slow drifts and small, \(<0.5 \) pA, changes in the magnitude of the seal current accompanying solution exchange), and idealized using half-amplitude threshold crossing. The resulting events lists were used to generate dwell-time distributions at all conductance levels (0, 1, 2, 3... channels open) that were then simultaneously fitted with a maximum likelihood algorithm (Csányi, 2000) to determine rate constants (\( r_{OC} \), average number of transitions from state A to state B occurring per unit of dwell time in state A, measured in \( s^{-1} \)). Likelihood of the three-state “C-O-CF” scheme,

\[
\frac{r_{CO}}{r_{OC}} \frac{r_{OF}}{r_{FO}} CF
\]

was optimized with respect to the rate constants indicated: “C” represents the long interburst closed state, “O” the open state, and “CF” the short flickery closed state. The rates of channel opening to a burst (often simply called channel opening rate) and closing from a burst (often called channel closing rate) are given directly by the simple rate constants \( r_{CO} \) and \( r_{OC} \) (where \( 1/r_{OB} = r_{CO} \), \( 1/r_{BC} = r_{OC}/(1 + (r_{OF}/r_{FO})) \)). An artificial dead time of 5–6.5 ms (larger than filter dead time of \( \approx 3.6 \) ms) was imposed to implement a correction for events missed due to the limited bandwidth (Csányi, 2000).

During exposure to saturating 5 mM MgATP in the presence of PKA, channel open probability, \( P_o \), was usually sufficiently high (\( >0.5 \)) that in patches with few channels the observed maximum number of simultaneously open channels could be shown by statistical tests (Csányi et al., 2000) to reasonably approximate the true number of active channels. In patches with 8–18 simultaneously open channels under those conditions, we could then also estimate absolute opening and closing rates in 5 mM MgATP after withdrawal of PKA: \( r_{OC} = 0.27 \pm 0.03 \) s\(^{-1} \), \( n = 18 \), \( r_{OC} = 3.28 \pm 0.21 \) s\(^{-1} \), \( n = 18 \) (Vergani et al., 2003). As 2 mM MgATP and 2 mM Mg8-N3ATP are both saturating concentrations (see Fig. 1, below), in each of 17 patches with few channels we directly compared opening and closing rates of the same channels (hence with the same phosphorylation status) under these two conditions, bracketing the measurements where possible: the ratios of these rates then yielded, via the absolute rates determined at saturating [MgATP], absolute gating rates at saturating [Mg8-N3ATP]. To collect enough gating events for analysis at lower [nucleotide], when \( P_o \) is lower, we used patches with more channels. Due to practical limits imposed by computer-processing times (Csányi, 2000), the maximum likelihood fitting programs do not allow the number, \( N \), of active channels to exceed 8. So we analyzed records only from patches with maximum conductance levels of \( \leq 8 \) open channels under all test and reference conditions used, and we assumed \( N = 8 \) for the maximum likelihood fit. In those cases, we extracted not absolute values for \( r_{CO} \) and hence \( r_{OB} \), but values relative to those in the same channels in the same patch during bracketing exposures to a reference condition (e.g., 2 mM [Mg8-N3ATP]; see Fig. 1, below). These normalized values (which should be relatively insensitive to \( N \)) were then scaled by the absolute rates at saturating nucleotide (described above) to yield the data in Fig. 1, B and C (below).

The marked prolongation of burst duration caused by \( V_i \) made it difficult to collect enough events for the kinetic analyses just described. To estimate \( r_{OB} \) under those conditions, we analyzed macroscopic current decay reflecting closure of large numbers of channels after sudden nucleotide removal. Records were digitally refiltered at 10 Hz, and then fitted with single or double exponential decay functions by nonlinear least squares (Sigmaplot; Jandel Scientific). Unless otherwise noted, data are given as mean \( \pm \) SEM (\( n \)), where \( n \) represents the number of observations.

RESULTS

Mg8-N3ATP Supports CFTR Cl\(^-\) Channel Gating but with Altered Single-channel Kinetics

Use of 8-azido-substituted nucleotides as surrogates rests on the assumption that the substitution does not grossly affect nucleotide interactions with the subject protein. To this end, Mg8-N3ATP was found to support substrate transport by MRP1 with comparable maximal rate and apparent affinity (10–50 \( \mu \)M) as for ATP (Gao et al., 2000; Hou et al., 2000), and 1 mM Mg8-N3ATP activated macroscopic Cl\(^-\) current in excised patches containing PKA-phosphorylated CFTR channels (Travis et al., 1993). The results in Fig. 1 A confirm that Mg8-N3ATP supports activity of phosphorylated wild-type (WT) CFTR channels expressed in Xenopus oocytes, but they also show that the rates of channel opening to a burst (\( r_{CO} \), see MATERIALS AND METHODS, and of closing from a burst (\( r_{OC} \)), are both lower during exposure to 2 mM Mg8-N3ATP than they are in 2 mM MgATP. Kinetic analysis (Csányi, 2000) showed that, at the saturating concentration of 2 mM, the 8-N3 group reduced the maximal rates of opening to a burst \( \sim 2 \) fold (\( r_{CO2Mm8-N3ATP}/r_{CO2MmATP} = 0.42 \pm 0.04 \), \( n = 17 \)), and of closing from a burst \( \sim 5 \) fold (in 2 mM MgATP, \( r_{OC} = 3.83 \pm 0.25 \) s\(^{-1} \); in 2 mM Mg8-N3ATP, \( r_{OC} = 0.74 \pm 0.09 \).
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N3ATP averaged 60

We incubated membranes from HEK293T cells ex-

topus CFTR with an NH2-terminal Flag epitope, which in

drolysis. To facilitate immunoprecipitation, we used

We examined simple binding of 8-N3ATP to CFTR at

32P]8-N3ATP (in the presence of 10 mM Mg2

MgATP) = 0.27 ± 0.03 s⁻¹ and rOC(5 mM MgATP) = 3.3 ± 0.2 s⁻¹ (n = 18; Vergani et al., 2003). For Mg8-N3ATP we multiplied those rates by the factors (0.42 for rCO, and 0.19 for rOC) determined by direct comparison (as in A, top trace), assuming that 2 and 5 mM MgATP are both saturating concentrations. Curves in B show Michaelis-Menten fits, with parameters (mean ± SEM; 2 ≤ n ≤ 7) Kd = 55 ± 5 μM and 11 ± 3 μM and Vmax = 1.02 ± 0.02 and 1.01 ± 0.04, for MgATP and Mg8-N3ATP, respectively. The high apparent affinity for Mg8-N3ATP is directly evident in Fig. 1 A, bottom trace: thus, rCO at 15 μM Mg8-

N3ATP (Fig. 2 A). Assuming a Kd of 5 μM for Mg8-

N3ATP, Kd for Mg2

N3ATP, in A), kinetic parameters were normalized to

The opening rates were half-maximal at 55 ± 5 μM MgATP (Fig. 1 B, black circles) or 11 ± 3 μM Mg8-

N3ATP (Fig. 1 B, red triangles).

Binding of [32P]8-N3ATP to CFTR at 0°C

We examined simple binding of 8-N3ATP to CFTR at

0°C, a temperature anticipated to largely prevent hy-
drolysis. To facilitate immunoprecipitation, we used

CFTR with an NH2-terminal Flag epitope, which in Xe-

nopus oocytes differed from WT CFTR only in its ∼3-
fold lower maximal opening rate (Chan et al., 2000).

We incubated membranes from HEK293T cells ex-

pressing Flag-CFTR on ice with varying concentrations of [α32P]8-N3ATP (in the presence of 10 mM Mg2⁺) for 5 min, and then subjected them to UV irradiation on ice, with no intervening wash. Autoradiography of the photolabeled CFTR after immunoprecipitation and SDS-PAGE showed that, in agreement with others (Travis et al., 1993; Aleksandrov et al., 2002), the labeling saturated at low μM [α32P]8-N3ATP (<50 μM; e.g., Fig. 8 B, below).

Relative binding affinities at 0°C were determined by including various concentrations of unlabeled competing nucleotide during the 5-min incubation with 5 μM Mg[α32P]8-N3ATP before photocrosslinking. For example, the labeling by 5 μM Mg[α32P]8-N3ATP was re-

duced to about half by 10–50 μM unlabeled (cold) MgATP (Fig. 2 A). Assuming a Kd of 5 μM for Mg8-

N3ATP binding (see Fig. 8 B, below) and simple competi-
tion between Mg8-N3ATP and other Mg-nucleotide complexes, least-squares fits yielded Kd values of 1 ± 1 μM for MgADP, 6 ± 2 mM for MgAMPPNP, and 16 ± 4 μM for MgATP (Fig. 2 C). In separate experiments, we found that omission of Mg2⁺ (and addition of 1 mM EDTA) reduced photolabeling by 5 μM [α32P]8-N3ATP only to 77 ± 9% (n = 4) of the level found with Mg2⁺. By including this constraint (i.e., for Mg2⁺-free [α32P]8-

N3ATP, Kd = 8 μM) in the fit, we estimate a Kd for Mg2⁺-

free (Tris) ATP of 69 ± 20 μM (Fig. 2 C).

UV irradiation after incubation with 5 μM Mg

[α32P]8-N3ATP on ice resulted in a comparable level of photolabeling of CFTR (Fig. 2 B) to that seen with 5 μM Mg[α32P]8-N3ATP (Fig. 2 A).

Binding of Low Concentrations of 8-N3 Nucleotide at 0°C Occurs Predominantly at NBD1

To examine the relative labeling of the two NBDs, we coexpressed in Xenopus oocytes two Flag-tagged halves of CFTR: the NH2-terminal half, Flag-3–835, containing NBD1, and the COOH-terminal half, Flag-837–1480, containing NBD2. Gating of full-length CFTR with an extracellular Flag epitope, as in Flag-837–1480, is re-

portedly identical to that of WT CFTR (Schultz et al., 1997). We previously characterized in detail gating of Flag-3–835 + 837–1480 split CFTR (with a single Flag
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tained for the different nucleotides. Given the finite duration of UV irradiation, these apparent binding affinities might be less accurate than the ratios of the values obtained for free 8-N3ATP. Ki values from the fits were 0.2 ± 0.1 μM for MgATP (filled circles, n = 4), 16 ± 4 μM for MgADP (filled circles, n = 4), and 69 ± 20 μM for TrisATP (empty circles, n = 3). Given the finite duration of UV irradiation, these apparent binding affinities might be less accurate than the ratios of the values obtained for the different nucleotides.

Figure 2. [α32P]8-N3ATP and [γ32P]8-N3ATP binding to Flag-CFTR in HEK293T cell membranes and inhibition by nucleotides. (A) Autoradiogram showing competition by cold MgATP for binding of 5 μM [α32P]8-N3ATP to CFTR at 0°C, followed by UV irradiation, washing, immunoprecipitation, and SDS-PAGE. (B) CFTR incubated with 5 μM [γ32P]8-N3ATP at 0°C was photolabeled only after UV irradiation, indicating lack of phosphorylation by endogenous kinases under these conditions. (C) Quantification of competition for binding of 5 μM [α32P]8-N3ATP by unlabeled MgATP (as in A), MgADP, or MgAMPPNP (all in Mg2+-containing buffer), or by unlabeled TrisATP (in Mg2+-free buffer). Photolabeling was normalized to the signal obtained in the absence of competing nucleotide (note broken abscissa), and the data (error bars show ±SD) fitted assuming simple competition and a Kd values from the fits were 1 ± 1 μM for MgADP (filled triangles, n = 2), 6 ± 2 μM for MgAMPPNP (empty squares, n = 4), 16 ± 4 μM for MgATP (filled circles, n = 4), and 69 ± 20 μM for TrisATP (empty circles, n = 3). Given the finite duration of UV irradiation, these apparent binding affinities might be less accurate than the ratios of the values obtained for the different nucleotides.

Also Figs. 5 A and 6 C, below) was weakly labeled in the autoradiogram. In contrast, no comparably prominent signals that would indicate strong labeling of NBD2 by [α32P]8-N3ATP were seen in the autoradiogram (Fig. 3 A), i.e., at positions corresponding to the bands recognized by the anti–COOH-terminal CFTR antibody (Fig. 3 B). We cannot rule out that relatively weak labeling of NBD2-containing fragments may be partly obscured by the faint nonspecific labeling evident in uninjected oocytes (Fig. 3 A, left lane). Nevertheless, the results suggest that in this assay of simple binding at 0°C low micromolar concentrations of nucleotide label mostly NBD1, and hence that the relative nucleotide affinities assayed in Fig. 2 also largely reflect nucleotide interactions at NBD1.

With Mg8-N3ATP, as with MgATP, Vt Markedly Prolongs the Open Burst State of CFTR Channels

Before using Vt in photolabeling studies, we examined its influence on CFTR channel gating in MgATP and in Mg8-N3ATP. After activating CFTR channels in large oocyte patches with PKA, MgATP, and Vt, sudden nucleotide withdrawal caused rapid closure of about half the channels, with a time constant, τc = 0.2 s (n = 6), and similar to the mean burst duration at saturating [MgATP] in PKA (τburst ~700 ms; Csanády et al., 2000; Vergani et al., 2003): this fast component presumably represents closing of channels that had not in...
teracted with $V_i$. The other half of the open channels (fractional amplitude, $a_j/(a_i + a_j) = 0.48 \pm 0.06$; $n = 6$; Fig. 4E, black bar) closed slowly ($\tau_i = 31.5 \pm 3.8$ s; $n = 6$; Fig. 4D, black bar) reflecting marked stabilization of their open burst state by $V_i$ (Fig. 4, A and C; Baukrowitz et al., 1994; Gunderson and Kopito, 1994). On average, the fraction of its open time a single channel spends in short (or long) bursts equals the fraction of the population of open channels in short (or long) bursts at any instant. At the instant of MgATP removal, the ratio of these population fractions is given by the ratio of the fast/slow fractional amplitudes, $a_f/a_s = 0.52/0.48 = 1$, which thus equals the ratio $n_f/m_s$, where $n$ and $m$ are the mean numbers of short ($\tau_i \approx 1$ s) and long ($\tau_i = 30$ s) bursts, respectively, entered by each channel in any given (sufficiently long) time interval. So, the ratio $n/m$, $\sim 30$ here, indicates that 1 in $\sim 31$ openings resulted in a $V_i$-stabilized open burst.

When Mg8-N3ATP was used to open the channels (Fig. 4B), the slow time constant reflecting the $V_i$-stabilized open state was unaltered ($\tau_i = 30.4 \pm 3.6$ s; $n = 6$; Fig. 4D, gray bar), though the steady-state fraction of such locked-open channels was somewhat reduced ($a_j/(a_i + a_j) = 0.26 \pm 0.04$; $n = 6$; Fig. 4E, gray bar). The fast time constant after Mg8-N3ATP, $\tau_f = 3.0 \pm 0.3$ s ($n = 6$), like the mean burst duration (Fig. 1, A and C), was larger than that with MgATP, supporting our interpretation that the fast component reflects normal channel closing, but appears to alter neither the stability of the $V_i$-locked open-burst state nor the frequency of entry into that state.

$V_i$ similarly affected CFTR expressed in HEK293T cells (Fig. 4, F and G). On nucleotide withdrawal after channel activation with PKA + MgATP, $>90\%$ of the current relaxed rapidly ($\tau_i = 1.9 \pm 0.5$ s; $n = 4$); but, after activation with PKA + MgATP + $V_i$, $39 \pm 4\%$ ($n = 4$) of the current relaxed slowly ($\tau_i = 22.0 \pm 2.6$ s; $n = 4$), reflecting channel closing from $V_i$-stabilized open bursts, whereas the rest decayed rapidly ($\tau_i = 1.9 \pm 0.6$ s; $n = 4$) like channels not exposed to $V_i$. This large slow component was seen only after exposure to $V_i$, regardless of whether that exposure occurred after (Fig. 4F) or before (Fig. 4G) the control activation without $V_i$.

**Figure 4.** $V_i$ locks open WT CFTR channels activated by PKA and either MgATP or Mg8-N3ATP. (A–C) Macroscopic current, in patches containing hundreds of channels excised from oocytes expressing WT CFTR, relaxed with double exponential time course (smooth fit lines, largely obscured by data) after removal of nucleotides. Nucleotides (0.5 mM in A and B; 5 mM in C) and $V_i$ were applied in the presence of 300 nM PKA. Fit parameters for trace in A are, $a_i = 25$ pA, $a_j = 24$ pA, $\tau_i = 0.5$ s, $\tau_j = 20$ s; for trace in B, $a_i = 14$ pA, $a_j = 7$ pA, $\tau_i = 3$ s, $\tau_j = 35$ s; for first decay in C, $a_i = 50$ pA, $\tau_i = 0.5$ s; second decay in C, $a_i = 27$ pA, $a_j = 18$ pA, $\tau_i = 0.6$ s, $\tau_j = 21$ s. Solution exchange took $<1$ s, estimated from current decay after brief activation of endogenous Ca$^{2+}$-activated Cl$^-$ channels. (D and E) Summary of mean (±SEM, $n = 6$) time constants ($\tau$) and fractional amplitudes ($E$) of slow component of current decay after activation by PKA and 0.5 mM MgATP without (white bars), or with (black bars) 5 mM $V_i$, or by PKA and 0.5 mM Mg8-N3ATP with 5 mM $V_i$ (gray bars). (F and G) Macroscopic current records from inside-out patches excised from HEK293T cells expressing WT CFTR; MgATP (5 mM) and $V_i$ (5 mM) applications were in the presence of 300 nM PKA; the bars indicate omission of 30 s (F) and 100 s (G) of record. (In C, F, and G, offset bars beneath traces signal different solution compositions.)

Tightly Bound [$\alpha^{32P}$]8-N3 Nucleotide after Incubation at 30°C Photolabels Predominantly NBD1

To assay protracted nucleotide interactions with the NBDs, it was essential to wash away freely exchangeable nucleotide before photocrosslinking. After incubation with [$\alpha^{32P}$]8-N3ATP at 30°C, Flag-CFTR-containing HEK293T-cell membranes were diluted 10-fold into ice-cold nucleotide-free buffer, spun down at 4°C, then re-
suspended in more of the same buffer and subjected to a 5-min postwash incubation at 30°C (compare Ueda et al., 1999) before UV irradiation on ice to cross-link tightly bound nucleotide, followed by immunoprecipitation, SDS-PAGE, and autoradiography. We found, like Szabó et al. (1999) (but cf. Aleksandrov et al., 2002), that there was little tight binding without Mg2+; only 11 ± 3% (n = 2) in the absence of Vi and 20 ± 7% in its presence, of that with both Vi and Mg2+. The presence of Mg2+ alone increased labeling to 70 ± 16% of that seen when Vi was also added (cf. Szabó et al., 1999; Aleksandrov et al., 2001, 2002). Varying the concentration of [α32P]8-N3ATP, in the presence of Mg2+ and 1 mM Vi, showed that the 5 μM [α32P]8-N3ATP used in most experiments was not saturating, and that half-maximal tight binding (or occlusion) of nucleotide at 30°C occurred at ≈10 μM [α32P]8-N3ATP (unpublished data), the concentration at which channel opening rate at room temperature was also half maximal (Fig. 1 B). Additional controls, with 5 μM [α32P]8-N3ATP, Mg2+, and Vi, demonstrated that the standard 15-min incubation and 10-min irradiation times yielded robust signals that appeared relatively insensitive to even 2-fold reductions in those times (unpublished data).

When split CFTR channels (Flag-3–835 + Flag-837–1480) in oocyte membranes were incubated at 30°C with 5 μM [α32P]8-N3ATP and Mg2+, with or without Vi, followed by the two washes and 5-min 30°C postincubation just described, the tightly-bound 8-N3 nucleotide predominantly photolabeled NBD1 (compare Szabó et al., 1999; Aleksandrov et al., 2001, 2002). The autoradiogram (Fig. 5 A, left) showed strong labeling, enhanced by Vi, of a single band near ~90 kD, not seen in un.injected oocyte membranes (right lane). Comparison with the immunoblots (Fig. 5 A, right) shows that the photolabeled band corresponds to the principal band recognized by the anti-R-domain antibody (epitope near NBD1), and that none of the bands recognized by the anti-COOH-terminal antibody (Fig. 5 A, right; epitope near NBD2) was strongly photolabeled (including, importantly, the broad band that corresponds to fully glycosylated protein; Chan et al., 2000), indicating that the occluded [α32P]8-N3 nucleotide was virtually exclusively cross-linked to NBD1.

Dwell Time of 8-N3 Nucleotide Occluded at NBD1 with or without Vi

To learn just how long the nucleotide remains associated with NBD1 at a temperature that supports channel gating, we used Flag-CFTR expressed in HEK293T cells and varied the postincubation period at 30°C from 0 to 15 min before photocrosslinking (Fig. 5 B). Three results are clear. First, the standard 5-min postincubation released less than half the label found with no postincubation (just two cold washes), regardless of whether the initial incubation included Vi (Fig. 5 B). Second, the initial presence of Vi increased tightly bound nucleotide on average by <50% for all postincubation times up to 5 min (Fig 5 B). Third, nucleotide loss during postincubation at 30°C could be fitted with the same exponential time course (τ ≈15 min) whether or not Vi had been present during the initial incubation (fitted curves, Fig. 5 B).

Is the Nucleotide Tightly Bound at NBD1 8-N3ADP, or 8-N3ATP?

To learn whether the nucleotide occluded at NBD1 had been hydrolyzed to N3ADP during the postincubation at 30°C, we incubated Flag-CFTR from HEK293T cells with 5 μM [γ32P]8-N3ATP instead of [α32P]8-N3ATP, at 30°C in the presence of Vi, subjected the membranes to the standard two ice-cold
washes with intervening warm 5-min postincubation, and then incubated samples with or without UV irradiation (Fig. 6 A). A low level of covalent incorporation of \([\gamma^{32}\text{P}]\) into CFTR was seen without UV irradiation. We attribute this to phosphorylation of CFTR by endogenous kinases, as it was observed with \([\gamma^{32}\text{P}]\)ATP as well as \([\gamma^{32}\text{P}]\)8-N\(_3\)ATP and was enhanced by \(V_i\), an inhibitor of protein phosphatases (unpublished data), but was absent at 0°C (Fig. 2 B, above). A similar phosphorylation was found with MRPI (Hou et al., 2000). Nonetheless, UV irradiation markedly increased the label incorporated into CFTR (Fig. 6 A, left lane). That UV-dependent component of labeling with \([\gamma^{32}\text{P}]\)8-N\(_3\)ATP represents photocrosslinking of nonhydrolyzed N\(_3\)ATP, which must have survived intact throughout the initial 15-min incubation at 30°C, and the subsequent cold washes and 5-min postincubation at 30°C. So we may conclude that some of the tightly bound nucleotide remained in the form of ATP, but what fraction?

To answer this, we examined radiolabeling of membranes from oocytes expressing split CFTR, Flag-3835 plus Flag-837–1480, after incubation with 5 μM \([\gamma^{32}\text{P}]\)8-N\(_3\)ATP or 5 μM \([\alpha^{32}\text{P}]\)8-N\(_3\)ATP, this time without \(V_i\), followed by a 5-min postincubation at 30°C (Fig. 6 B). Comparison with the immunoblots (Fig. 6 C) confirmed that both \([\alpha^{32}\text{P}]\)8-N\(_3\)ATP and \([\gamma^{32}\text{P}]\)8-N\(_3\)ATP labeled essentially only the NH\(_2\)-terminal half of CFTR, and that the labeling with \([\gamma^{32}\text{P}]\)8-N\(_3\)ATP was stronger than with without UV irradiation (Fig. 6 B, right); the latter presumably reflected R-domain phosphorylation by native kinases. The UV-dependent component of \([\gamma^{32}\text{P}]\)8-N\(_3\)ATP labeling, estimated by subtracting the signal without that from UV exposure, had an intensity comparable (~60% in this case) to that of the labeling by \([\alpha^{32}\text{P}]\)8-N\(_3\)ATP (entirely dependent on UV irradiation; Fig. 7 B). As the specific activities of the \([\alpha^{32}\text{P}]\)- and \([\gamma^{32}\text{P}]\)-labeled nucleotides were identical (within 1%), this finding establishes that most of the nucleotide remaining at NBD1 after a 5-min postincubation at 30°C in nucleotide-free buffer is nonhydrolyzed N\(_3\)ATP. We conclude that, even at 30°C, NBD1 in CFTR hydrolyzes ATP little, if at all.

Influence of Prephosphorylation of CFTR on Nucleotide Binding at 0°C and on Occlusion at 30°C

We first phosphorylated Flag-CFTR in HEK293T cell membranes by incubating them for 20 min at 30°C with cold (nonradiolabeled) MgATP, with or without PKA catalytic subunit; a control sample included \([\gamma^{32}\text{P}]\)ATP with PKA to monitor phosphorylation. All samples were washed with ice-cold nucleotide-free buffer and pelleted twice, to remove PKA and/or ATP, before they were incubated with \([\alpha^{32}\text{P}]\)8-N\(_3\)ATP at 0°C to assess binding or at 30°C to assess occlusion. In vitro phosphorylation appeared (Fig. 7 A) to slightly diminish nucleotide binding at 0°C (on average by ~15%), consistent with the negligible effect reported by Travis et al. (1993). Prephosphorylation also consistently reduced occlusion of \([\alpha^{32}\text{P}]\)8-N\(_3\)ATP at 30°C, to 80 ± 7% (n = 9) of control with \(V_i\), and to 75 ± 15% (n = 6) without it (Fig. 7 B), i.e., regardless of the small augmentation caused by the \(V_i\) itself. The strong signal in the right lane of Fig. 7 B confirms that CFTR remained phosphorylated throughout all the incubations. This reduction in \([\alpha^{32}\text{P}]\)8-N\(_3\)ATP occlusion seems unlikely to be entirely attributable to residual partial occupancy of NBD1 by unlabeled ATP that was bound during the prephosphorylation and remained bound during the subsequent washes. Inclusion of PKA during a 10-min incubation with \([\alpha^{32}\text{P}]\)8-
N3ATP at 25°C also reduced occlusion, by about one third (to 67%) in the presence of Vi, (Fig. 7 C; compare lane 1 to lane 3), and by almost one quarter (to 78%) in its absence (Fig. 7 C; compare lane 2 to lane 4), consistent with the above results. We conclude that the strong facilitatory effect of PKA on CFTR channel gating is not due to enhanced binding or occlusion of nucleotide at NBD1.

**The K464A Mutation Little Influences Binding at 0°C, but Diminishes Occlusion at 30°C, at Low [Nucleotide]**

Because the mutation K464A at the Walker A lysine in NBD1 impairs CFTR maturation in mammalian cells (e.g., Aleksandrov et al., 2001), we compared nucleotide interactions with WT and K464A CFTR expressed in oocytes, in which we also compared electrophysiological responses. Despite loading twice as much membrane protein in the K464A lanes as in the WT lanes (Fig. 8 A, right) to compensate for diminished K464A expression (Fig. 8 A, left), negligible occlusion of [α32P]8-N3ATP at 30°C was observed for K464A CFTR, either with or without Vi. In contrast, simple binding of 5 or 50 μM [α32P]8-N3ATP, assayed by photolabeling in oocyte membranes at 0°C, seemed little different for Flag-K464A than for Flag-WT CFTR (Fig. 8 B), even though that binding occurs predominantly at NBD1 (Fig. 3, above), the structurally altered catalytic site. The fact that opening of K464A CFTR channels is impaired at low μM [MgATP] (Vergani et al., 2003) suggests that channel opening and nucleotide occlusion at NBD1 share a common step that occurs after the simple association with nucleotide.

The K464A Mutation Virtually Eliminates the Vi-dependent Slowing of CFTR Channel Closing

Unlike the slow decline of roughly half of the WT CFTR current (Fig. 4, above), the macroscopic current flowing through K464A channels activated by PKA, MgATP, and Vi decayed relatively rapidly upon nucleotide withdrawal (Fig. 8 C). The current relaxation after exposure to Vi had a double exponential time course (smooth fit line, Fig. 8 C, center): most of the current decayed rapidly (τa = 0.9 ± 0.1 s, n = 10) with a time constant like that of closing (τb = 620 ± 58 ms, n = 24; Vergani et al., 2003) of K464A channels exposed to just PKA and 5 mM MgATP, indicating that the majority of K464A CFTR channels did not respond to the presence of Vi. The smaller current component after Vi (a/(a + b) = 0.32 ± 0.06, n = 10; Fig. 8 E, black bar) decayed somewhat more slowly (τa = 5.2 ± 0.5 s, n = 10; Fig. 8 D, black bar), but still ~5 times faster than the slower component of WT CFTR channel current after exposure to Vi (compare black bars, Fig. 8 D).

**DISCUSSION**

Our measurements of interactions of 8-N3-labeled nucleotides with CFTR confirm and substantially extend results from previous studies (Travis et al., 1993; Szabó et al., 1999; Aleksandrov et al., 2001, 2002). By comparing the kinetics of these nucleotide interactions with the kinetics of CFTR channel gating, we are able to derive new mechanistic insights. We find that nucleotide binds with relatively high affinity to NBD1 with or without Mg2+, before becoming occluded at NBD1 in a
Mg$^{2+}$-requiring reaction; once occluded, the nucleotide dissociates with a time constant of $\sim$15 min at 30°C, whether or not $V_i$ was present during the initial incubation. As this dissociation is orders of magnitude slower than channel opening and closing events, we conclude that the nucleotide remains bound at NBD1 throughout many CFTR channel gating cycles. This means that the nucleotide-dependent events that control the timing of normal channel gating (on the time scale of seconds) occur at NBD2. In addition, we find that the nucleotide remaining for several minutes at NBD1 at 30°C is largely ATP, not ADP, which leads us to conclude that NBD1 is incapable of rapidly hydrolyzing ATP. K464A CFTR, mutated at the Walker A lysine in NBD1, binds but does not occlude micromolar concentrations of 8-N$_3$ATP, in accord with our conclusion from analyses of channel gating that the mutation lowers the apparent affinity for nucleotide interactions required for the transition to the channel open-burst state. Our finding that phosphorylation of CFTR by PKA little alters nucleotide binding or occlusion suggests that phosphorylation might regulate not events at the NBDs, but transduction of those events to channel gates elsewhere. By comparing CFTR channel gating kinetics in Mg8-N$_3$ATP and in MgATP, we find that the slow step that rate limits channel opening at saturating [nucleotide] follows nucleotide binding to both NBDs.

**Nucleotide Occlusion at NBD1 at 30°C, and CFTR Channel Gating**

That [α$^{32}$P]8-N$_3$ nucleotide bound at NBD1, but not at NBD2, could survive washing with ice-cold buffer before photolabeling with UV light was recently reported (Szabó et al., 1999; Aleksandrov et al., 2001, 2002), but the dwell time of nucleotide at NBD1 at temperatures capable of supporting CFTR channel gating was unknown. We show here that, after incubation at 30°C in the presence of Mg$^{2+}$ (with or without $V_i$), 8-N$_3$ nucleotide not only remains at NBD1 throughout two washes with ice-cold buffer, but dissociates very slowly during subsequent postincubation at 30°C, with a time constant of $\sim$15 min (Fig. 5 B). 8-N$_3$ nucleotide similarly became occluded at 37°C, regardless of the presence of Mg$^{2+}$ (or of $V_i$), in NBD1 (and not in NBD2) of the ABC-C family relative SUR1 (Ueda et al., 1997), and that nucleotide also dissociated slowly during postincubation at 37°C (Ueda et al., 1999).

We also find that, on average, CFTR channels expressed to MgATP or Mg8-N$_3$ATP switch back and forth between the closed interburst state and the open burst
state at least once every few seconds at room temperature (Fig. 1), and gating is even faster at 30°C (Mathews et al., 1998; Aleksandrov and Riordan, 1998; Csanády et al., 2000). Together, these results imply that nucleotide remains tightly bound at NBD1 throughout many gating cycles. The nucleotide-dependent events that time CFTR channel opening and closing must therefore occur at NBD2 (compare Gunderson and Kopito, 1995). As NBD1 seems to stay nucleotide bound, this corroborates our recent conclusion, from analysis of the [MgATP] dependence of CFTR channel gating, that both NBDs must be occupied by nucleotide before a CFTR channel can open (Vergani et al., 2003). The higher apparent affinity for activation of CFTR channel opening by Mg8-N3ATP than by MgATP (Fig. 1 B) then suggests a higher affinity interaction of Mg8-N3ATP than of MgATP with NBD2.

The extremely slow release of nucleotide from NBD1, but rapid release from NBD2, satisfactorily explains the markedly asymmetric kinetics of inhibition by N-ethylmaleimide of CFTR channels with a single cysteine introduced into the phosphate-binding Walker A loop of NBD1 or NBD2 (Cotten and Welsh, 1998). Increasing concentrations of MgATP could protect against inhibition at either NBD, but, even with low [MgATP], inhibition at 35°C took many minutes when the cysteine was in NBD1 but only seconds when it was in NBD2, consistent with the different delays for nucleotide to leave the two binding sites predicted from our measurements at 30°C.

Our failure to observe photolabeling of NBD2 by [α32P]8-N3 nucleotide after extensive washing and postincubation with nucleotide-free solution at 30°C (Figs. 5 A and 6 B) agrees with Szabó et al. (1999) and Aleksandrov et al. (2001, 2002), who found that even a single ice-cold wash before UV irradiation abolished NBD2 labeling. Nor could we clearly discern a signal attributable to NBD2 after incubation with 5 or 50 μM [α32P]8-N3ATP at 0°C followed by UV irradiation without washing, although Aleksandrov et al. (2002) detected labeling of NBD2 under those conditions, at least at >25 μM [α32P]8-N3ATP. In our experiments, weak NBD2 signals could have been obscured by overlapping nonspecific signals that were also present in membranes from control oocytes not expressing CFTR (Fig. 3 A). The inability to clearly photolabel NBD2 cannot be attributed to a failure of nucleotide to bind to NBD2 in the split CFTR construct we used for distinguishing NBDs, for several reasons. First, the [MgATP] dependence of channel opening, interpreted above as reflecting effects at NBD2, showed an apparent affinity of this split channel, CFTR 3–835 + 837–1480 for MgATP ~2-fold higher than that of WT CFTR (Csanády et al., 2000). Second, nucleotide occlusion (at NBD1) in this split channel was consistently augmented in the presence of V, (Fig. 5 A), believed to temporarily trap ADP at NBD2 after hydrolysis of ATP there (discussed below). Finally, even a split CFTR channel lacking the entire R domain (CFTR 3–633 + 837–1480) showed markedly prolonged open bursts after mutation of its NBD2 Walker A lysine, K1250, suggesting nucleotide interaction at NBD2.

**NBD1 in Intact CFTR Fails to Hydrolyze Mg8-N3ATP**

Previous work suggested that 8-N3ATP might be more rapidly hydrolyzed at NBD2 than at NBD1 in CFTR (Aleksandrov et al., 2002), but the hydrolysis rate could not be estimated at either NBD, and kinase-mediated incorporation of 32P was not addressed. Here we show a strong UV-dependent component (i.e., not related to phosphorylation) of labeling by [γ32P]8-N3ATP of CFTR’s NBD1 in both HEK293T-cell and oocyte membranes after washing and a 5-min postincubation at 30°C (Fig. 6). That more than half of the occluded nucleotide at NBD1 remained in the form of N3ATP, rather than N3ADP, after 5 min at 30°C implies a time constant of N3ATP loss >430 s, and hence a hydrolysis rate <0.002 s⁻¹ under those conditions. This is at least two orders of magnitude lower than the rate of ATP hydrolysis by purified CFTR (Li et al., 1996), which may therefore essentially reflect hydrolysis at NBD2 alone.

This apparent failure of NBD1 in full-length CFTR to hydrolyze ATP seemed independent of phosphorylation status, as [γ32P]8-N3ATP was occluded (presumably at NBD1) both with and without PKA prephosphorylation. But the failure might reflect sequence asymmetry between CFTR’s NBD1 and NBD2 that implies the presence of unorthodox residues at key positions in the NBD1 catalytic site. These include a serine (S573) instead of the conserved glutamate that normally follows the Walker B aspartate (D572 in NBD1), and an another serine (S605) in place of the conserved histidine that in HisP interacts with the γ-phosphate and is believed to fulfill a switch function (Hung et al., 1998; Karpowich et al., 2001). Mutational analysis of the Walker B glutamate has shown that even the most conservative charge-removing substitution (glutamine) abolishes steady-state ATP hydrolysis by full-length P-gp (Urbatsch et al., 2000; Sauna et al., 2002), as well as by isolated bacterial NBDs (Moody et al., 2002). In addition, given recent compelling evidence for a head-to-tail dimeric organization of NBDs of Rad50 (Hopfner et al., 2000) and MutS (Junop et al., 2001), of bacterial ABC transporters (Fetsch and Davidson, 2002; Locher et al., 2002; Moody et al., 2002; Smith et al., 2002), and of human P-gp (Loo et al., 2002), the unorthodox signature sequence (LSHG, instead of the ABC consensus, LSGGGQ) from the NBD2 polypeptide is anticipated to complete the structure of the NBD1 catalytic site in intact CFTR (compare Jones and George, 1999). Inter-
estingly, the NBDs of other ABC-C family members show asymmetric sequence abnormalities like those in CFTR, possibly accounting for the similar suggestions that in SUR1, SUR2A, SUR2B (Ueda et al., 1997; Matsuo et al., 1999, 2000), and in MRPI (Hou et al., 2000), ATP hydrolysis occurs predominantly at NBD2 and far less (if at all) at NBD1. These findings contrast with the nearly symmetrical contributions to nucleotide binding and hydrolysis made by the two structurally similar NBDs of ABC-B family member P-gp (Senior et al., 1995; Urbatsch et al., 1995a,b; but cf. Hrycyna et al., 1999).

The conclusion that NBD1 in CFTR hydrolyzes ATP at a negligibly low rate is at odds with two kinds of earlier results. First, isolated NBD1 constructs, comprising the entire functional domain (at least residues 433–633; Chan et al., 2000) purified from E. coli as His-tagged (Duffieux et al., 2000) or tandem GST-NBD1-R domain (Howell et al., 2000) preparations, reportedly hydrolyzed MgATP at rates of 0.2 s⁻¹ and 0.4 s⁻¹ (at 30°C), with Kₐ₅₄ values for [MgATP] of ~250 and ~60 μM, respectively, comparable to values (0.2 s⁻¹) found for intact CFTR (Li et al., 1996). However, the mutation does substantially shorten prolonged (locked) open bursts ascribed to gating (Carson et al., 1995; Gunderson and Kopito, 1999). Unless the specific activity of that K464A CFTR was greatly underestimated (due to unrecognizable nonfunctional K464A protein), if NBD1 is essentially catalytically inactive in intact CFTR as we propose, a possible explanation for the reduced hydrolysis by K464A CFTR is that this mutation somehow impairs MgATP hydrolysis at the NBD2 catalytic site. Gating measurements offer no support for this possibility, as the K464A mutation little affects normal CFTR-channel gating (Carson et al., 1995; Gunderson and Kopito, 1995; Powe et al., 2002; Vergani et al., 2003) other than reducing the apparent affinity for MgATP (Vergani et al., 2003). However, the mutation does substantially shorten prolonged (locked) open bursts ascribed to NBD2 catalytic-site occupancy by the equivalent of nonhydrolyzed nucleotide, due to the K1250A mutation or to exposure of wild-type CFTR to MgATP plus nonhydrolyzable ATP analogs (Powe et al., 2002; Vergani et al., 2003) or to MgATP plus Vi₂ (Fig. 4 vs. Fig. 8, C and D). Although this destabilization could reflect loss of an allosteric influence of the wild-type NBD1 catalytic site on the NBD2 catalytic site, if transition to the open burst state involves formation of an NBD1/NBD2 dimer with interfacial catalytic sites (Vergani et al., 2003), then the K464A mutation could act locally within the interface to reduce stability of the NBD dimer.

Influence of Orthovanadate on Gating and Photolabeling of CFTR Channels

In several ATPases, upon ATP hydrolysis the analogue, Vi₂, can replace the dissociated phosphate and form a stable Mg–ADP–Vi₂ complex that traps ADP in the catalytic site (Chabre, 1990). This property of Vi₂ was exploited, together with 8-N₃ nucleotide, to demonstrate obligate catalytic cooperativity between P-gp’s two similar NBDs (Urbatsch et al., 1995a,b). In P-gp, no nucleotide trapping or photolabeling occurred without Vi₂ (Urbatsch et al., 1995a). In contrast, even without Vi₂, CFTR tightly bound 8-N₃ nucleotide at 30°C exclusively in NBD1 (Fig. 5 A), but the photolabeling was enhanced (on average by ~30%) by the initial presence of Vi₂ (Figs. 5, 7, and 8; cf. Szabó et al., 1999; Aleksandrov et al., 2001), although Vi₂ little influenced dissociation of the occluded nucleotide from NBD1 (τ ~15 min, Fig. 5 B). In addition, exposure of CFTR channels to Vi₂ in either MgATP or Mg8-N₃ATP resulted in marked prolongation of open bursts (compare Bauskrowitz et al., 1994; Gunderson and Kopito, 1994), evident as a slow relaxation of macroscopic current after nucleotide withdrawal from patches with many channels (Fig. 4). But, the time constant of that slow current decay, a measure of the mean duration of Vi₂-locked open bursts, presumably reflecting slow loss from catalytic sites of Vi₂-trapped ADP (or 8-N₃ADP) after hydrolysis, was only ~30 s (Fig. 4 D). A consistent interpretation of these seemingly disparate time courses is made possible by the observation that Vi₂ elicited comparable slow current relaxations whether it was applied before, or shortly after, exposure to MgATP alone (Fig. 4, C, F, and G). This allows us to conclude that Vi₂ locked the channels open by acting at NBD2, because the ~2-min interval between exposures in Fig. 4 F could permit dissociation of only ≤12% of tightly bound nucleotide from NBD1 (assuming dissociation τ ≥15 min at room temperature), whereas 46% of open channels were in locked-open bursts at the time of nucleotide withdrawal. Similarly, in MRPI, Vi₂-trapped ADP was found predominantly at NBD2 (Gao et al., 2000; Hou et al., 2000). The ~30-s time constant we observed for current relaxation (Fig. 4) suggests a mean dwell time of ~30 s for the Vi₂-trapped ADP at NBD2 of CFTR, readily accounting for the absence of photolabel at NBD2 (Fig. 5) after our extensive washing procedure, but its presence if the wash is omitted (Aleksandrov et al., 2001, 2002). In accord with this interpretation, NBD2 does
appear to hydrolyze ATP (Aleksandrov et al., 2002; cf. Ramjeesingh et al., 1999) and is where the hydrolysisabolishing mutation, K1250A, similarly (like $V_i$) prolongs bursts (Carson et al., 1995; Gunderson and Kopito, 1995; Ramjeesingh et al., 1999; Zeltwanger et al., 1995; Vergani et al., 2003).

Most of the nucleotide that dissociates slowly from NBD1 can bind tightly there even without $V_i$. If, as Fig. 1999; Vergani et al., 2003). Thus, nucleotide occluded in the presence or absence of $V_i$ dissociates at roughly the same rate, this would imply that in either case the nucleotide is released from the same CFTR conformation, one that can be reached without $V_i$. Presumably, therefore, $V_i$ action at NBD2 somehow increases the steady-state fraction of CFTR channels in that tight nucleotide-binding conformation, resulting in increased photolabeling after the same period of incubation at 30°C. The alternative explanation, that the $V_i$-induced increment in occluded nucleotide reflects $V_i$-trapped ADP at NBD1, is less likely because the nucleotide occluded without $V_i$ would have to dissociate as slowly as $V_i$-trapped nucleotide, the explanation would not account for $V_i$-locked open bursts of ~50-s duration, and it would require some hydrolysis at NBD1.

**Influence of Phosphorylation by PKA on Nucleotide Interactions with CFTR**

Though CFTR channels require phosphorylation by PKA before they can be opened by nucleotides (for reviews see e.g., Gadsby and Nairn, 1999; Sheppard and Welsh, 1999), PKA had relatively little influence on either nucleotide binding at 0°C (compare Travis et al., 1999) or occlusion at 30°C (Fig. 8, A–C). But, at the low [8-N3ATP] used, both assays predominantly monitor NBD1, whereas the nucleotide events that control channel gating occur at NBD2 (above; Vergani et al., 2003). However, Aleksandrov et al. (2002) omitted washing to also observe photolabeling at NBD2 and noted that “none of the major features of the nucleotide interactions at each NBD...were strongly influenced by PKA phosphorylation”. So, it appears that PKA phosphorylation might not be required for nucleotide binding, occlusion, or hydrolysis in intact CFTR in cell membranes, as suggested by ATPase measurements on purified CFTR, in which phosphorylation merely increased apparent affinity for ATP ~3-fold (Li et al., 1996).

Could exogenous PKA have had little effect in all the above studies because CFTR was already phosphorylated by endogenous kinases? Although some phosphorylation sites on CFTR might have remained phosphorylated (indeed, phosphatases did reduce ATPase activity at fixed [MgATP]; Li et al., 1996), it is unlikely that phosphorylation was sufficient to support channel gating because, for both CFTR purified from insect cells (Li et al., 1996) and CFTR in patches excised from insect cells (Bear et al., 1992), channels were not activated by MgATP until PKA was also added. Similarly, dephosphorylation by endogenous membrane-bound phosphatases of oocytes (e.g., Csanády et al., 2000), and of the BHK cells (Linsdell and Hanrahan, 1998) used by Aleksandrov et al. (2002), prevented activation of CFTR channels in excised patches until the channels were exposed to PKA. So these results suggest that CFTR channels can occlude and hydrolyze ATP under conditions in which channel gating does not occur. This conclusion does not contradict the proposed requirement for MgATP hydrolysis at the NBD2 catalytic site to normally terminate an open burst, but it does imply the existence of a mechanism, analogous to an automobile clutch, which links NBD function to the gating machinery and is modulated by phosphorylation.

**The Rate-limiting Step for CFTR Channel Opening Follows Both Nucleotide Binding Steps**

The rate at which CFTR channels leave the closed interburst state (of average duration, $\tau_b$) and initiate open bursts, the opening rate ($r_{CO}$ given by $1/\tau_b$), is a saturating function of both MgATP and Mg8-N3ATP concentration (Fig. 1 B). At low concentrations of either nucleotide, opening is rate limited by infrequent binding. Because $r_{CO}$ does not increase linearly with [nucleotide], channel opening cannot be a single-step reaction, $C\rightarrow O$, but must involve at least two closed states, and two distinct steps, e.g., $C_1\rightarrow C_2\rightarrow O$. If $C_1\rightarrow C_2$ is the nucleotide-binding reaction, and at saturating [nucleotide] it occurs rapidly compared with the channel-opening step, $C_2\rightarrow O$, then $r_{CO}$ is expected to display Michaelis-Menten dependence on [nucleotide] as observed (Fig. 1 B), with maximal opening rate reflecting $C_2\rightarrow O$. However, in principle, the step that limits opening rate at saturating [nucleotide] could precede the binding step (e.g., it could reflect a slow isomerization among closed, nucleotide-free, states). This second possibility is ruled out, however, by our finding that the maximal opening rate is slowed ~2-fold by the azido (N3) group substitution in the adenosine ring of ATP (which does not preclude its hydrolysis by ABC proteins; e.g., Urbatsch et al., 1994), because no step that precedes nucleotide binding could respond to modification of the nucleotide structure.

We therefore conclude that, for both MgATP and Mg8-N3ATP, CFTR channel opening is rate limited at saturating [nucleotide] by a relatively slow step that follows nucleotide binding, that is strongly temperature dependent (Aleksandrov and Riordan, 1998; Mathews et al., 1998) and Mg$^{2+}$ dependent (Dousmanis et al., 2002), and is sensitive to the detailed structure of the $\beta$ and $\gamma$ phosphate groups in the activating Mg$^{2+}$–nucleo-
side triphosphate complex (Vergani et al., 2003). We have proposed (Vergani et al., 2003) that this slow step might be related to formation of an NBD1/NBD2 dimer stabilized by the two Mg$^{2+}$–nucleotide complexes in the catalytic sites at the dimer interface (e.g., Hopfner et al., 2000; Smith et al., 2002) and, in particular, might correspond to formation of a prehydrolysis complex (Dousmanis et al., 2002), most likely at the NBD2 catalytic site.

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