ATPase Activity of the Cystic Fibrosis Transmembrane Conductance Regulator*  

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The gene mutated in cystic fibrosis codes for the cystic fibrosis transmembrane conductance regulator (CFTR), a cyclic AMP-activated chloride channel thought to be critical for salt and water transport by epithelial cells. Plausible models exist to describe a role for ATP hydrolysis in CFTR channel activity; however, biochemical evidence that CFTR possesses intrinsic ATPase activity is lacking. In this study, we report the first measurements of the rate of ATP hydrolysis by purified, reconstituted CFTR. The mutation CFTRG551D resides within a motif conserved in many nucleotidases and is known to cause severe human disease. Following reconstitution the mutant protein exhibited both defective ATP hydrolysis and channel gating, providing direct evidence that CFTR utilizes ATP to gate its channel activity.

The cystic fibrosis transmembrane conductance regulator (CFTR) is an integral membrane protein that resides on the apical membrane of several types of epithelial cells, and its absence or dysfunction in cystic fibrosis is thought to impair severely the salt and water transport capacity of these cells (1). CFTR shares structural similarities with other members of the ATP-binding cassette (ABC) superfamily of traffic ATPases; namely, it possesses two repeats, each consisting of a membrane-spanning domain followed by a nucleotide-binding domain (2, 3). Furthermore, CFTR possesses a unique cytoplasmic domain, the R domain, which possesses several putative sites for PKA and PKC phosphorylation and links the two halves of the molecule (2).

Unlike other members of the ABC superfamily, CFTR exhibits chloride channel activity. The chloride channel function of CFTR was implicated in mutagenesis studies in which substitution of certain charged residues within the putative transmembrane-spanning domains caused changes in single channel conductance or anion selectivity (4, 5). Further convincing evidence of the chloride channel activity of CFTR came from our electrophysiological studies of purified, reconstituted CFTR (6). Fusion of liposomes containing purified CFTR with planar lipid bilayers resulted in the appearance of chloride-selective channels that exhibited biophysical features identical to those associated with CFTR expression in epithelial cell membranes (7, 8).

Phosphorylation by PKA is required but not sufficient to activate CFTR chloride channel function. Electrophysiological evidence supports the hypothesis that hydrolysis of ATP is essential for normal opening and closing or gating of the channel (9–12). First, the addition of Mg-ATP, but not nonhydrolyzable ATP analogs, to PKA-phosphorylated CFTR causes channel gating (10). Second, the subsequent addition of nonhydrolyzable ATP analogs to ATP-activated CFTR channels interferes with normal channel gating kinetics (13). Third, chelation of magnesium ion, an essential cofactor in most reactions involving nucleotide binding and/or hydrolysis, causes alterations in the rates of channel opening and closing (14, 15). However, other research groups have reported electrophysiological studies with conflicting results, namely that nonhydrolyzable ATP analogs fail to compete with the opening or closing of CFTR channels (16) and that gating persists, albeit at a slower rate, after removal of magnesium (17, 18). As it has been widely hypothesized that CFTR is functionally coupled to other membrane proteins through direct protein interactions (19), it is possible that the sensitivity of CFTR channel gating to ATP is conferred or modulated by associated membrane proteins. Hence, contradictory reports such as those described above may reflect variabilities in the membrane environment for CFTR.

To eliminate possible modulatory effects of associated membrane proteins, we examined the role of ATP hydrolysis in CFTR channel gating using purified, reconstituted CFTR protein. We present novel biochemical evidence that the CFTR protein possesses intrinsic ATPase activity and that this catalytic activity is coupled directly to the channel function of this protein.

EXPERIMENTAL PROCEDURES

CFTR Production and Purification—Two liters of Sf9 cells were infected with recombinant baculovirus containing the complete coding sequence for CFTR as described previously (6). CFTR purification was performed according to our published procedure (6) with the following modifications. Infected cells were harvested after 48 h, and the pellet was treated with a phosphate-buffered saline containing 2% Triton X-100, 2 units/ml DNase, 5 mM MgCl2, 2 mM dithiothreitol, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 mM benzamidine, and 10 µM E64. The mixture was stirred for 2 h at 15 °C after which the insoluble material was centrifuged at 100,000 × g for 2 h. The resulting pellets were treated with 180 ml of 2% SDS, 3% mercaptoethanol in 10 mM sodium phosphate, pH 7.2, and the mixture was stirred overnight at 4 °C. Insoluble material was centrifuged at 60,000 × g, and the supernatant was filtered through a 0.22-µm filter before being applied at 1 ml/min to
Purified CFTR was reconstituted into proteoliposomes containing a phospholipid mixture PE:PS:PC:ergosterol (5:2:1:1) by weight for bilayer studies. Nystatin (120 µg/ml) was introduced into the proteoliposomes for bilayer studies to promote proteoliposome fusion and to facilitate detection of these fusion events (24). Fusion of nystatin-containing liposomes was indicated by the appearance of transient “nystatin spikes” in bilayer conductance. As in our previous experiments, a 10 mg/ml solution of phospholipid (PE:PS at a ratio of 1:1, Avanti Polar Lipids) in n-decane was painted over a 200-µm aperture in a bilayer chamber to form bilayers. Bilayer formation was monitored electrically by observing the increase in membrane capacitance. In all experiments, bilayer capacitance was greater than 200 picofarads. Fusion of liposomes was potentiated with the establishment of an osmotic gradient across the lipid bilayer. The cis compartment of the bilayer chamber, defined as that compartment to which liposomes were added, contained 300 mM KCl; the trans compartment, connected to ground, contained 50 mM KCl. Single channel currents were monitored at a holding potential of −40 mV applied to the cis compartment and detected with a bilayer amplifier (custom made by M. Shen, Physics Laboratory, University of Alabama). Data were recorded and analyzed using pClamp 6.0.2 software (Axon Instruments, Inc.).

### RESULTS

The scheme for purification of recombinant CFTR from SF9 cells and reconstitution in phospholipid liposomes was published previously (6). We used a similar purification and reconstitution strategy in our present studies of CFTR ATPase activities. We confirmed the homogeneity of the purification products used in these experiments by evaluation of silver-stained gels overloaded with CFTR protein and by Western blot analysis of the reconstituted protein (Fig. 1A). In Fig. 1B we show that reconstituted, purified CFTR protein does possess intrinsic ATPase activity. The production of radiolabeled dinucleotide ([α-32P]ADP) from [α-32P]ATP was determined following separation of the nucleotides on thin layer chromatography plates, a technique commonly used for the measurement of GTPase activity of purified G proteins (27, 29). The rate of [α-32P]ADP production by liposomes containing purified, PKA-phosphorylated CFTR was linear over a 5-h time period (Fig. 1B). ATPase activity was determined by aspiration of the CFTR protein, as there was no detectable ATPase activity in suspensions of protein-free liposomes. In Fig. 1C we show that our measurements of CFTR ATPase activity are reproducible. The mean
ATPase activity exhibited by three distinct preparations of purified CFTR is significantly greater than that detected in preparations of protein free liposomes (p < 0.02).

As in the case of CFTR chloride channel activity, CFTR ATPase activity is dependent upon phosphorylation (Fig. 2). Pretreatment of purified CFTR with the serine/threonine protein phosphatase 2A caused marked inhibition of ATP hydrolysis (Fig. 2A). Hence, purified CFTR likely remains partially phosphorylated throughout the purification procedure. Modulation of CFTR ATPase activity by phosphorylation is shown in further studies where CFTR is fully phosphorylated with PKA catalytic subunit. PKA pretreatment of CFTR caused a 2–3-fold activation of the rate of ATP hydrolysis. The substrate dependence for ATPase activity of both partially phosphorylated (not pretreated with PKA) and fully phosphorylated (PKA-treated) protein was determined and the data fitted by nonlinear regression analysis using the Hill equation, \( v = \frac{S^n V_{\text{max}}}{S^n + K_m^n} + y \), where \( v \) and \( V_{\text{max}} \) are the initial and maximum initial rates, respectively; \( S \) is the ATP concentration, and \( m \) is the Hill coefficient. The constant \( K_m \) is related to \( K_m \), by the equation \( K_m = (K_m)^m \). As apparent in Fig. 2B and Table I, PKA treatment modifies ATPase activity of CFTR by causing a reduction in apparent \( K_m \) from 1 mM to 0.3 mM, presumably by increasing the affinity of CFTR for ATP. The shape of the function describing ATP dependence of ATPase activity changed from hyperbolic, for the partially phosphorylated protein, to sigmoidal for the PKA-treated, fully phosphorylated protein. This change in shape was reflected by a change in the Hill coefficient from 1 to 1.7. Therefore, full phosphorylation of CFTR appears to induce positive cooperativity between two sites of ATPase activity. The \( V_{\text{max}} \) for CFTR ATPase activity, 50 nmol/mg of purified protein/min, was not altered by PKA treatment. It is probable that we have underestimated the ATPase activity of CFTR as it is likely that only a fraction of purified protein has been reconstituted completely to its active configuration. Previously, we estimated on the basis of reconstitution of single channel activity, that 20–40% of total purified protein was functional following fusion to planar lipid bilayers (6). Hence, the ATPase activity by CFTR may be as high as 125–250 nmol/mg/min, which corresponds to a turnover number of approximately 0.5–1 molecules of ATP hydrolyzed/s.

We have shown in previous publications that purified, reconstituted CFTR exhibits channel activity with biophysical properties identical to those observed for CFTR in cell membranes; namely, it is anion-selective, exhibits a low unitary conductance of approximately 10 picoSiemens, and requires phosphorylation by the addition of exogenous PKA plus Mg-ATP for activity (6, 28, 29, 30). Addition of Mg-ATP but not its nonhydrolyzable analog, Mg-AMPPNP, caused channel activity of phosphorylated CFTR reconstituted in planar lipid bilayers (data not shown), supporting the hypothesis that ATP hydrolysis rather than binding is required to open the CFTR channel.

**Fig. 1. Purified, reconstituted CFTR functions as an ATPase.** Panel A, Left, silver staining after SDS-polyacrylamide gel electrophoresis (6% acrylamide) of aliquots containing approximately 750 ng of purified CFTR protein. Right, immunoblot of reconstituted CFTR protein probed with monoclonal antibody M3A7. Panel B, ATP hydrolysis exhibited by proteoliposomes containing 75 ng of purified, PKA-phosphorylated CFTR (○) (from preparation assessed in panel A) but not by liposomes alone (▲). The rate of hydrolysis by purified CFTR was linear with time over 5 h. Panel C, three different preparations of proteoliposomes containing 75 ng of phosphorylated CFTR (+CFT) exhibiting significantly higher ATPase activity than that measured in paired protein-free, liposome preparations (−) (p < 0.01). ATPase activity was determined at 4 h after initiation of the reaction in the presence of 1 mM Mg-ATP. The mean value ± S.D. is shown.

**Fig. 2. Phosphorylation modulates ATPase activity of purified CFTR.** Panel A, regulation of CFTR ATPase activity by phosphorylation. ATPase activity of PKA-treated preparations was expressed as a percentage of the activity measured in a paired, untreated control preparation. The bar graph shows the mean percentage increase ± S.D. caused by PKA phosphorylation relative to control for four different purified protein preparations. The change in ATPase activity induced by phosphorylation was significant, p < 0.02. Purified CFTR was dephosphorylated using protein phosphatase 2A. This phosphatase caused marked inhibition of CFTR ATPase activity relative to untreated control preparations in both experiments. Panel B, untreated (▲) or PKA-treated (○) CFTR protein was assessed for ATPase activity at varying Mg-ATP concentrations to determine the substrate dependence of this reaction. Each reaction mixture contained 25 ng of protein and was incubated for 4 h before quenching. One-μl aliquots of each reaction mixture were analyzed for ADP formation. Each point shows the mean ± S.D. of triplicate experiments. The curves were fitted by nonlinear regression analysis using the Hill equation. The best curve fit was assessed as the fit that corresponded to the lowest S.D. The software program for regression analysis was Regression, version M1.11 (Blackwell Scientific Publications, Oxford, UK). The inset in the lower right corner shows an expansion of the curve fit for the relationship between low ATP concentrations (0.05–1.0 μM ATP) and ATPase activity for phosphorylated CFTR (○).

**Table I**

| PKA modification of CFTR ATPase activity | Kinetic parameters |
|---------------------------------------|--------------------|
|                                       | \( V_{\text{max}} \) | \( K_m \) | Hill coefficient |
| CFTR                                  | 51.3               | 969.7   | 1               |
| CFTR-P\(^c\)                          | 53.8               | 303     | 1.73            |

\( ^c \) Calculated from \( K_m = (K_m)^m \).

\( ^c \) Obtained by nonlinear regression analysis to the Hill equation.

Phosphorylated CFTR.
It has been postulated that ATP is utilized by CFTR to cause gating of its chloride channel activity. Hence, the rate of ATP hydrolysis by CFTR should be comparable to the rate of channel gating. As we have quantitated ATP hydrolysis by purified CFTR we can examine this hypothesis directly in the same proteins specimen. Our planar bilayer studies of purified protein verified patch-clamp studies that showed CFTR exhibits a bursting pattern of gating in the presence of 1 mM Mg-ATP (Fig. 3A). As shown in Fig. 3B, there is a single population of channel-open times and two populations of channel-closed times, a short and a long closed time. Only the longer of the two closed times exhibits ATP dependence, suggesting that only the transition from the long duration closed time to the channel-open state is substrate-dependent. For subsequent burst analysis, we used a value of 60 ms as the burst delimiter. 

The relationship between channel long closed times and Mg-ATP concentration can be fit using a single exponential decay function and predicts half-maximal inhibition of long closed times at 445 μM ATP. Panel C, burst analysis was performed as described under “Experimental Procedures.” Burst and interburst time histograms (shown left and right, respectively) are fit by single exponential distributions. 

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Briefly, tc was derived from the function describing the closed dwell time histogram as the nadir between the peak values that define the mean short closed (intraburst) times and the long closed (interburst) times. Following analysis of the ATP dependence of burst and interburst duration histograms (Fig. 3C), we found that both the transition rate to the open burst (1/between burst duration) and the transition rate to the interburst, closed state (1/burst duration) were altered by increasing ATP concentration. The effective bursting rate increased with increasing ATP concentration, and this relationship was fit by nonlinear regression analysis using the Hill equation to yield a K_m of 584 μM, maximal opening rate of 10/s, and a Hill coefficient of 2. The Hill coefficient of 2 suggests that there is positive cooperativity between two sites through which ATP can act to promote channel bursting. The effective closing rate from an open burst decreased slightly with increasing ATP concentrations, and these data were fit using an exponential decay function to derive an estimate of the ATP concentration required for half-maximal inhibition of closing, 434 μM. Assuming that the overall transition rate between the two conductance states of CFTR channel is limited by the slowest rate, i.e. bursting or closing rate, we predict that at 1 mM ATP, the slow closing rate will limit gating of CFTR to 1–2 transitions/s. Our quantitation of the rate of CFTR gating is comparable to the rate of ATP hydrolysis by CFTR, namely, 0.5–1 molecules of ATP hydrolyzed/s. Hence, we argue on the basis of our kinetic analyses that CFTR channel activity is coupled to ATP turnover.

Further evidence for coupling between CFTR channel and ATPase functions is found in our studies of the sensitivity of these two activities for magnesium ion and sodium azide. In Fig. 4A we show that chelation of magnesium ion with EDTA inhibits channel opening as well as ATPase activity. Phosphorylated CFTR protein reconstituted in planar lipid bilayers...
The chloride channel activity was observed in the presence of MgCl₂ (10 mM) and Na-ATP (100 μM) at a holding potential of −40 mV. This record shows activity of three CFTR channels with openings indicated as O₁–O₆. Mean channel-open probability was 0.3 in this particular experiment. Addition of EDTA (1 mM) as indicated completely abolished the CFTR channel activity, where the free Mg²⁺ concentration was estimated at 4 mM. This experiment is representative of four studies. ii, effect of magnesium on ATPase activity. In the left lane (−Mg), CFTR (75 ng) was phosphorylated as described previously and dialyzed against a buffer containing 50 mM Tris, 50 mM NaCl, and 5 mM EDTA for several hours prior to the ATPase assay. ATPase activity was determined 4 h after the addition of Na-ATP, in the absence of added Mg²⁺ (n = 3). In the right lane (+Mg), ATPase activity of phosphorylated CFTR was determined in the presence of 5 mM MgCl₂ (n = 3). Bars indicate the mean ± S.D. for ATPase measurements. Panel B, i, chloride channel activity exhibited by purified, phosphorylated CFTR, MgCl₂ (100 μM) was inhibited by the addition of NaN₃ (1 mM) to both cis and trans compartments of the bilayer chamber (n = 5). ii, the bar graph shows that ATPase activity of phosphorylated CFTR (75 ng) was inhibited by pretreatment with 5 mM NaN₃ (n = 3). The mean ± S.D. is shown.

ATPase Activity of CFTR

Fig. 5. CFTRG551D exhibits defective ATPase and channel activities. Panel A, channel activity of a single molecule of reconstituted CFTRG551D is observed following fusion of proteoliposomes with the lipid bilayer at a holding potential of −40 mV. Fusion was detected as a nystatin conductance spike. The CFTRG551D channel opens infrequently and for a brief period of time. Panel B, relationships between ATP concentration and ATPase activity of reconstituted CFTR (○, n = 3) and CFTRG551D (□, n = 3) were fitted by nonlinear regression analysis using the Hill equation. The mean ± S.D. is shown. Each sample of CFTR and CFTRG551D contained 75 ng of purified protein.

In these studies of purified, reconstituted CFTR protein we have provided the first direct biochemical evidence that this protein functions as an ATPase. We measured the ATPase activity of purified CFTR as 50 nmol/mg/min and estimated that this activity may be as high as 125–250 nmol/mg/min. Significantly, Ko and Pedersen (37) recently reported that a fusion protein containing the first nucleotide-binding fold of CFTR was also capable of hydrolyzing ATP, albeit at a somewhat slower rate of 30 nmol/mg/min. Our estimate of CFTR ATPase activity is less than that measured for P-glycoprotein, a closely related member of the ABC superfamily of transporters, with hydrolytic rates measured as 300 (nmol/mg/min) (38) and 1,650 (nmol/mg/min) (39) and less than rates determined for purified P-type ATPases; Na⁺,K⁺-ATPase (800 nmol/mg/min) (40) and Ca²⁺-ATPase (600 nmol/mg/min) (40). On the other hand, CFTR ATPase activity is similar to that reported for the intrinsic GTPase rates of dynamins (41) and much higher than intrinsic rates reported for the low molecular weight GTPases such as ras (42). The low rate of ATP hydrolysis by CFTR may explain why it cannot be measured in native cell membranes nor in some preparations of fusion proteins containing the first nucleotide-binding fold of CFTR (43).

The results presented in this paper support the hypothesis that CFTR ATPase and channel activities are coupled. Both CFTR ATPase activity and channel gating are regulated by PKA phosphorylation. Our kinetic analyses suggest that PKA phosphorylation enhances CFTR ATPase activity by increasing affinity for ATP, possibly through exposure of a second catalytic site. Although this hypothesis must be examined directly in future studies, we speculate that the phosphorylated R domain may function to coordinate the activities of the two nucleotide-binding folds of CFTR. It is well known that CFTR channel gating requires phosphorylation, and a role of the R domain in the coordination of ATP-dependent channel gating has also been implicated (12). Further, we determined that the rates of ATP turnover and channel gating by phosphorylated, CFTRG551D protein exhibits altered ATPase activity (Fig. 5B). At 1 mM Mg-ATP the rate of ATP hydrolysis by the mutant protein occurs at approximately 10% that of the wild type (2.5 nmol/mg/min versus 20 nmol/mg/min). Hence, we have shown that a mutation within a sequence conserved among NTPases causes a reduction both in channel gating and ATPase activity.

DISCUSSION

Finally, we assessed coupling between the ATPase and channel functions of CFTR by determining the effect of a site-directed mutation on both activities. The relatively common cystic fibrosis mutation, CFTRG551D, leads to severe disease (33). Glycine 551 lies within a sequence in the first nucleotide-binding fold, D-X-[G/A]-G-Q, which shows remarkable conservation in ABC transporters and in G proteins and has been implicated in NTP-binding and hydrolysis (34–36). In the present studies, we found that purified CFTRG551D exhibits altered chloride channel activity when reconstituted in lipid bilayers (Fig. 5A). Although the unitary conductance of CFTRG551D is comparable to that of the wild type protein, approximately 10 picosiemens, arguing against global changes in protein structure, the open probability of the mutant protein was much lower than that determined in the wild type protein, 0.011 versus 0.48, respectively. Similarly, reconstituted
purified CFTR are similar, approximately 1/s, attesting to the proposal that these activities are coupled.

The requirement for CFTR ATPase activity in channel gating was assessed by inhibition of ATPase activity by site-directed mutagenesis. Glycine 551 of CFTR lies within a motif that is conserved in nucleotidases, and mutation of this residue to aspartic acid caused a marked reduction in both ATPase activity and channel gating. Hence, the mutation CFTRG551D likely causes altered channel activity (44) and human disease (33) because it possesses defective ATPase activity. Further, chemical modulation of CFTR ATPase activity caused comparable inhibition of the rate of channel gating. In contrast to some electrophysiological studies of CFTR in biological membranes (17, 18), we could observe a clear dependence of channel activation on magnesium ion. This difference may relate to an improved ability to regulate free magnesium ion concentrations in bilayer studies of purified protein.

To understand how CFTR functions in cells it is important to determine which of its putative activities are intrinsic or dependent upon the context of other membrane proteins. This issue is of particular relevance to CFTR and other members of the ABC superfamily of membrane proteins as it has been hypothesized that they may mediate cellular transport through direct protein-protein interactions within the membrane (19). Studies of purified, reconstituted CFTR can be used to verify or to argue against putative activities of CFTR (6, 30). For example, the chloride channel activity of CFTR was originally verified in planar lipid bilayer studies of purified protein (30). Conversely, the putative ATP-channel function of CFTR could not be confirmed in our recent studies using purified protein (30), leading us to speculate that CFTR-mediated cellular efflux of ATP reported in some studies (45) may be due to an effect of CFTR on associated membrane proteins.

Finally, the results in the present paper clearly show that CFTR possesses intrinsic ATPase activity and support the hypothesis that CFTR utilizes ATP to gate its channel function. Hence, the ATP dependence of channel activity is unlikely to be mediated by neighboring membrane proteins. These results pave the way for more detailed investigations of the mechanism through which ATP is utilized to drive CFTR channel gating. Furthermore, other putative activities for this protein, such as its possible function as a pump for organic substrates, can now be tested directly (3).

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