Phosphorylation-induced Conformational Changes of Cystic Fibrosis Transmembrane Conductance Regulator Monitored by Attenuated Total Reflection-Fourier Transform IR Spectroscopy and Fluorescence Spectroscopy*

Received for publication, October 7, 2003, and in revised form, December 1, 2003
Published, JBC Papers in Press, December 2, 2003, DOI 10.1074/jbc.M311014200

Vinciane Grimard‡§, Canhui Li†, Mohabir Ramjeesingh‖, Christine E. Bear¶, Erik Goormaghtigh**+, and Jean-Marie Ruysschaert‡ ‡‡

From the ¥Center for Structural Biology and Bioinformatics, Free University of Brussels, Campus Plaine CP206/2, 1050 Brussels, Belgium and the Program in Structural Biology and Biochemistry, Research Institute, Hospital for Sick Children, Toronto, Ontario M5G 1X8, Canada

Cystic fibrosis transmembrane conductance regulator (CFTR) is a member of the ABC protein superfamily. Phosphorylation of a regulatory domain of this protein is a prerequisite for activity. We analyzed the effect of protein kinase A (PKA) phosphorylation on the structure of purified and reconstituted CFTR protein. $^3$H/$^2$H exchange monitored by attenuated total reflection Fourier transform IR spectroscopy demonstrates that CFTR is highly accessible to aqueous medium. Phosphorylation of the regulatory (R) domain by PKA further increases this accessibility. More specifically, fluorescence quenching of cytosolic tryptophan residues revealed that the accessibility of the cytoplasmic part of the protein is modified by phosphorylation. Moreover, the combination of polarized IR spectroscopy with $^3$H/$^2$H exchange suggested an increase of the accessibility of the transmembrane domains of CFTR. This suggests that CFTR phosphorylation can induce a large conformational change that could correspond either to a displacement of the R domain or to long range conformational changes transmitted from the phosphorylation sites to the nucleotide binding domains and the transmembrane segments. Such structural changes may provide better access for the solutes to the nucleotide binding domains and the ion binding site.

Cystic fibrosis is caused by a mutation in the membrane chloride channel CFTR\(^1\) (1). CFTR is a member of the ABC superfamily. As the other members of this family, CFTR contains two transmembrane domains and two nucleotide binding domains (NBD) responsible for ATP hydrolysis. In addition to the common ABC structure, CFTR possesses an R domain (regulatory domain) that contains several consensus phosphorylation sites. Phosphorylation of this domain followed by ATP binding and hydrolysis by the NBDs is necessary to induce chloride permeability (2, 3). Yet, an alternative activation mode has been proposed recently, where glutamate induces chloride channel activity in the absence of PKA and ATP (4), but it still needs further investigation.

CFTR contains 10 dibasic (R(R/K)X(S/T)) consensus sequences for PKA phosphorylation as well as several monobasic and low affinity sites, most of them located in the R domain (1). Although the overall sequence identity of CFTR R domains is low, the phosphorylation sites are remarkably conserved among species (5). It is believed that the R domain combines both inhibitory and stimulatory effects. Effectively, deletion of the residues 708–835 from the R domain (6, 7), and even of the residues 760–783 (8), generates constitutively active channels. Moreover, overexpression or addition of the unphosphorylated R domain, encompassing residues 590–858, inhibits chloride transport (9). PKA phosphorylation relieves this inhibition. On the other hand, it has been shown that exogenous phosphorylated R domain (either residues 590–858, 645–834, or 708–831) increases the open probability of a CFTR channel construct missing residues 708–835 from the R domain (7, 10, 11).

Nevertheless, the effect of the phosphorylation sites seems quite unspecific. Effectively, it has been shown that PKA phosphorylates the R domain with a stoichiometry of 5 mol/mol in vitro. Site-directed mutagenesis and tryptic phosphopeptide mapping showed the phosphorylation of 5 serines in vivo (Ser-660, Ser-700, Ser-737, Ser-795, and Ser-813) (12, 13). Mass spectrometry allows the detection of 8 phosphorylated serines (the same sites, plus Ser-712, Ser-768, and Ser-753) (14, 15).

Nevertheless, the mutation of all these serines as well as the mutation of the 10 dibasic consensus sites, did not prevent PKA-dependent opening of the channel (16), suggesting that phosphorylation of monobasic and low affinity phosphorylation sites can relieve, partly at least, the inhibitory effect of the R domain. Furthermore, the substitution of serines by negatively charged aspartates relieves R domain inhibition (17), suggesting that the negative charges of the phosphate groups play a major role in this process.

Even though the role of phosphorylation on CFTR activity has been widely studied, the only structural information regarding CFTR phosphorylation that has been obtained so far concerned soluble R domains (11, 18). In the present study, we reconstituted active CFTR in proteoliposomes and analyzed the
effect of PKA phosphorylation both on the entire protein and on specific regions of CFTR distinct from the R domain. \(^{1}H/H\) exchange combined with polarized and unpolarized ATR-FTIR spectroscopy, as well as tryptophan fluorescence quenching by acrylamide were used to detect modifications of solvent accessibility occurring specifically in the transmembrane or cytosolic domains of CFTR upon PKA phosphorylation of the R domain.

**EXPERIMENTAL PROCEDURES**

**Materials—**\(D_{2}O\) was from Merck, nickel-nitrioltriacetic acid resin was from Qiagen, m3a7 CFTR primary antibody and anti-mouse secondary antibody were from Chemicon, ECL+ Western blot detection kit was from Amersham Biosciences, pentadecafluorooctanoic acid (PFO) was from Fluorochem, SM2 Bio-Beads were from Bio-Rad, bovine c-AMP-dependent protein kinase A catalytic domain was from Promega, and bovine alkaline phosphatase was from Sigma. All other reagents were from Merck and Sigma. Homemade software programs necessary for the analysis of IR spectra were written in Matlab (The Mathworks).

**Production and Purification of CFTR-His Proteins—**Procedures describing production and purification of CFTR-His proteins were published previously (19, 20). Briefly, SF9-baculovirus expression system was used for large scale production of wild type CFTR. Crude plasma membranes from frozen SF9 cell pellets expressing recombinant CFTR-His proteins were solubilized in 8% PFO, 25 mM phosphate, pH 8.0. Purification of CFTR-His protein was performed using nickel affinity chromatography. The solubilized sample was applied to a freshly generated nickel column at a rate of 1 ml/min. A pH gradient of 8.0–6.0 was then applied to the column using a liquid chromatography pump and gradient former from Bio-Rad. 5-m fractions were collected. Dot blot was used for CFTR detection in the fractions eluted from the column. Immunopositive fractions were selected and further analyzed by silver-stained 6% SDS/polyacrylamide gel electrophoresis and Western blotting. Fractions of high purity were then concentrated in a Centricon YM-100 concentrator from Millipore.

**Reconstitution of CFTR-His Proteins—**A film of asolectin was formed by evaporation of chloroform under a stream of \(N_{2}\) followed by overnight drying under vacuum. Liposomes were prepared by sonication of the lipid film (7 min, on a 250-watt Vibra Cell Sonifier) in 6.5 ml of reconstitution buffer (20 mM Tris-HCl, 75 mM NaCl, 0.5 mM EDTA, and 1 mM dithiothreitol, pH 7.2). The sonicated phospholipid solution containing 1 mg of lipids was mixed with 50 µl of PFO 3%. The mixture was stirred continuously for 20 min at room temperature. Purified protein, dissolved in 4% PFO, was added to lipid/detergent-mixed micelles, at a 1/20 (w/v) protein/lipid ratio, and the volume was adjusted to 1 ml with reconstitution buffer. The detergent/protein/phospholipid mixture was stirred for 30 min at room temperature, and the detergent was removed by six incubations with SM2 Bio-Beads (previously washed with methanol and MilliQ water) at 4 °C.

The supernatant collected from SM2 Bio-Beads was mixed with an equal volume of 80% sucrose and overlaid with a 30 to 10% sucrose linear gradient. After an overnight centrifugation at 120,000 \(\times\) g, the gradient was fractionated from the bottom of the tube. The phospholipids and protein distribution were measured, respectively, by the enzymatic colorimetric assay for phosphatidylcholine (Roche Applied Science) and by tryptophan fluorescence (excitation = 290 nm, and emission = 330 nm).

**Phospholipid and Dephosphorylation of CFTR-His Proteins—**Half of the reconstituted CFTR protein batch was phosphorylated by the catalytic subunit of PKA for 1 h at room temperature (20). Phosphorylation reaction mixture contained 250 mM catalytic subunit of PKA, 1 mM MgCl\(_2\), and 500 µM ATP in 50 mM Tris-Cl, 50 mM NaCl, pH 7.5. The other half of the reconstituted protein was dephosphorylated in the same reaction mixture with alkaline phosphatase in place of PKA. PKA and alkaline phosphatase were removed by ultracentrifugation at 180,000 \(\times\) g for 2 h. The pellets containing the proteoliposomes were resuspended in 2 mM Hepes, 150 mM NaCl, pH 7.2, and the absence of residual kinase and phosphatase was assessed by 6% SDS/polyacrylamide gel electrophoresis.

**Uptake of Chloride by Proteoliposomes—**A concentrated tracer uptake assay, described previously (20), was used to measure \(^{36}\text{Cl}^{-}\) flux into proteoliposomes containing purified and reconstituted CFTR protein. Intravesicular \(^{36}\text{Cl}^{-}\) was assayed after incubation of proteoliposomes with \(^{36}\text{Cl}^{-}\) for 30 min, in the presence of MgATP, for phosphorylated and dephosphorylated samples.

**Attenuated Total Reflection Fourier Transform IR Spectroscopy—**ATR-FTIR spectra were recorded, at room temperature, on a Bruker IFS55 FTIR spectrophotometer equipped with a liquid nitrogen-cooled mercury-cadmium-telluride detector at a nominal resolution of 2 cm\(^{-1}\) and encoded every 1 cm\(^{-1}\). The spectrophotometer was continuously purged with air dried on a FTIR purge gas generator (75-62 Balston, Maidstone, UK). The internal reflection element (ATR) was a germanium plate (50 \(\times\) 20 \(\times\) 2 mm) with an apertur e angle of 45°, yielding 25 internal reflections (21).

**Secondary Structure Analysis—**512 scans were averaged for each measurement. The spectra were corrected for atmospheric water absorption as described previously (21, 24). Secondary structure content was determined by comparison with the spectra of 50 proteins from a reference data base. For all spectra, a baseline was subtracted between 1700, 1600, and 1500 cm\(^{-1}\), and the spectra were normalized between 1700 and 1500 cm\(^{-1}\). A linear model containing two wavenumbers was constructed from the reference data base for each secondary structure and applied to the CFTR spectra. Both the protein data base and the mathematical model have been described previously in the case of circular dichroism (25).

**Kinetics of Deuteration—**Films containing 20 µg of reconstituted phosphorylated or dephosphorylated CFTR were prepared on a germanium plate as described above. Before starting the deuteration, 10 spectra of the sample were recorded to test the stability of the measurements and the reproducibility of the area determination. At zero time, a \(^{2}H_{2}O\)-saturated \(N_{2}\) flux, at a flow rate of 100 ml/min (controlled by a Brooks flow meter), was applied to the sample. For each kinetic time point, 24 spectra were recorded and averaged at a resolution of 2 cm\(^{-1}\). The resulting spectra were corrected for atmospheric water absorption and side-chain contribution as described previously (23, 24).

The area of Amide II, characteristic of the \(\delta(N-H)\) vibration, was obtained by integration between 1575 and 1505 cm\(^{-1}\). For each spectrum, the area of Amide II was divided by the area of the lipid ester vibration band integrated between 1780 and 1700 cm\(^{-1}\), to correct for any change in the phospholipid spectrum. **FIG. 1.** 6% silver-stained SDS/PAGE of purified CFTR. Lane A shows the molecular weight reference proteins. The left arrows indicate the position of the molecular weight markers. Lane B shows the result of CFTR purification. Lane C displays a Western blot of CFTR using m3a7 monoclonal antibody.
in total intensity of the spectra during the deuteration process. This ratio, expressed as a percentage, was plotted versus deuteration time. The 100% value is defined by the Amide II/lipid ratio obtained before deuteration. The 0% value corresponds to a zero absorption in the Amide II region, observed for the full deuteration of a protein (27).

Polarized ATR-FTIR Spectroscopy—Films containing 20 μg of reconstituted phosphorylated or dephosphorylated CFTR were prepared on a germanium plate as described above. Spectra were recorded with the incident light polarized parallel and perpendicular with respect to the incidence plane before and after 12-h deuteration. For each spectrum, 512 scans were recorded and averaged, and the resulting spectra were corrected for atmospheric water absorption (23, 24). Dichroism spectra were computed by subtracting the perpendicular polarized spectrum from the parallel polarized spectrum. The subtraction coefficient was chosen so that the area of the lipid ester band equals zero on the dichroism spectrum, to take into account the difference in the relative power of the evanescent field for each polarization as described before (29).

Fluorescence Quenching Experiments—Acrylamide quenching experiments were carried out on a SLM Aminco 8000 fluorometer at an excitation wavelength of 290 nm to reduce the absorbance of acrylamide. Acrylamide aliquots were added from a 3 M solution to the proteoliposome suspension containing 5 μg of reconstituted phosphorylated or dephosphorylated CFTR. Fluorescence intensities were measured at 330 nm after each addition of quencher. All measurements were carried out at 25°C. Acrylamide quenching data were analyzed according to the Stern-Volmer equation for collisional quenching (30).

RESULTS

Purification and Active Reconstitution of CFTR—The use of biophysical techniques such as ATR-FTIR and fluorescence spectroscopy requires purified and reconstituted proteoliposomes with a relatively high protein to lipid ratio (21, 30).

Purification of His-tagged CFTR was performed as previously described (19) and yielded a well-purified protein as shown on a silver-stained polyacrylamide gel and by Western blot (see Fig. 1). The diffuse band is due to the variation of glycosylation considering the expression in Sf9 insect cells (31). To reconstitute CFTR, Bio-Beads were used to remove the detergent (32). After several incubations with the Bio-Beads, the sample was applied to a sucrose gradient (Fig. 2). A homogeneous population of proteoliposomes migrated at a density of 1.10 g/cm³. No protein was observed elsewhere in the gradient. The diameter of these proteoliposomes evaluated by photon correlation spectroscopy was found to be 148.0 ± 0.8 nm (results not shown). These proteoliposomes appeared to be suitable for spectroscopic purposes, with a protein to lipid ratio of 1/30 (w/w).

Extensive phosphorylation by PKA or dephosphorylation by alkaline phosphatase was performed as previously described (20). The chloride channel activity of this sample was evaluated by monitoring the uptake of radioactive chloride ions. Fig. 3 shows that phosphorylated CFTR was effectively able to transport chloride ions at a rate of 145 nmol/μg of protein per hour. When CFTR was dephosphorylated, the chloride flux drops significantly to a rate of 80 nmol/μg of protein.

Phosphorylation-induced Conformational Changes—The reconstituted sample was used for ATR-FTIR experiments. The IR spectrum of a protein is mainly characterized by two bands, the Amide I and Amide II, due mainly to the absorption of the amide υ(C=O) and δ(N–H) vibrations, respectively (21). The Amide I is located between 1700 and 1600 cm⁻¹, and its shape is particularly sensitive to the secondary structure (33). The comparison of the Amide I shape of phosphorylated and dephosphorylated CFTR shown in Fig. 4 reveals no significant change. Secondary structure content was further determined...
for the two samples (Table I) by comparison with the spectra of a 50-protein data base (25). Due to the proximity of $\beta$-strands and random coil absorption in the Amide I band (33), a small underestimation of the $\beta$-strands content is possible. However, we can see that no significant change of secondary structure occurs upon CFTR phosphorylation.

Because the tertiary structure could change in the absence of significant secondary structure modification, we monitored $\text{H}_2\text{H}$ exchange kinetics for the phosphorylated and the dephosphorylated samples. After exposure to $\text{H}_2\text{O}$, the labile hydrogens from the amide bonds are exchanged by deuterium. This results in the decrease of the area of the Amide II band located between 1575 and 1505 cm$^{-1}$. This decrease can be related to the solvent accessibility and the secondary structures stability (Fig. 5) (27). The exchange observed here is fast in comparison with other proteins, because 70% of the amide hydrogens are already exchanged after 20 min. The phosphorylation induces an increase of the $\text{H}_2\text{H}$ exchange, giving rise to 90% of exchange in the same time interval (Fig. 6). These exchange curves can be fitted by a multiexponential decay corresponding to the different groups of amide protons exchanging at various rates (27). Table II shows that the population of the fast exchanging protons is enhanced by 32% upon phosphorylation. A corresponding decrease is observed in the two others proton populations.

**Location of the Phosphorylation-induced Conformational Changes**—To locate more specifically the conformational changes induced by phosphorylation, two approaches were used to detect structural changes occurring specifically in the cytosolic and membrane domains, respectively, upon phosphorylation of the R domain. The first one takes advantage of the intrinsic fluorescence of tryptophan residues. Tryptophan residues give rise to a fluorescence emission with a maximum wavelength at 330 nm. The aqueous quencher acrylamide induces a decrease of the fluorescence, which depends on the accessibility of the cytosolic tryptophans to the quencher (34). Because no tryptophans are located in the R domain, the measure will reflect changes occurring outside this domain. Quenching was observed for the phosphorylated as well as for the dephosphorylated samples, but the intensity of the effect was more pronounced for the phosphorylated CFTR, demonstrating that the tryptophans are more accessible to acrylamide in this conformational state (Fig. 7).

The second method was developed previously in our laboratory (24, 36). It is based on the use of polarized IR light. The IR light absorbed by a chemical bond will be maximal if its dipole transition moment is parallel to the electric field component of the incident beam. The dichroic spectrum results from the difference between spectra obtained, respectively, using light polarized parallel and perpendicular to the incidence plane (21). Monitoring the evolution of the dichroic spectrum in the course of $\text{H}_2\text{H}$ exchange allows focusing

|          | $\alpha$-Helices | $\beta$-Strands | $\beta$-Turns | Random coil |
|----------|-----------------|----------------|---------------|-------------|
| CFTR-dP  | 45 ± 4          | 1 ± 1          | 17 ± 2        | 37 ± 3      |
| CFTR-P   | 47 ± 2          | 2 ± 2          | 16 ± 2        | 35 ± 2      |

**Fig. 4.** Secondary structure of actively reconstituted CFTR. Amide I spectra of 20 $\mu$g of phosphorylated (solid line) and dephosphorylated (dashed line) CFTR have been superimposed. The spectra were corrected for atmospheric water absorption and side chains contributions and smoothed at 4 cm$^{-1}$.}

**TABLE I**

**Secondary structure of CFTR**

Secondary structures were determined as described under “Experimental Procedures” for dephosphorylated (CFTR-dP) and phosphorylated (CFTR-P) CFTR. Numbers are given in percentages. Values are the mean of three experiments and are given with indication of the standard deviation.
specifically on the exchange of oriented domains of a protein. The positive deviation in the Amide I region that can be observed at 1650 cm$^{-1}$ in the resulting spectrum (Fig. 8) is characteristic of the presence of $\alpha$ helices with an orientation close to perpendicular to the membrane plane, as are most transmembrane helices. Although the Amide I contribution is mainly due to the vibration of the amide $\nu$(C=O), its shape is influenced by the deuteration, because there is some coupling between the $\nu$(C=O) and the N−$^3$H or N−$^2$H bonds. This results in a shift of the Amide I contribution maximum of the dichroic spectrum during $^1$H/$^2$H exchange (33). The importance of this shift has been shown to be related to the extent of the exchange (33). For the dephosphorylated sample, a shift of 8 cm$^{-1}$ was detected upon deuteration. In contrast the phosphorylated CFTR gives rise to a considerably larger shift of 15 cm$^{-1}$, corresponding to a markedly increased $^1$H/$^2$H exchange of the transmembrane segments of CFTR after phosphorylation of the R domain (Fig. 9).
DISCUSSION

This report focuses on the effect of c-AMP-dependent PKA phosphorylation on the structure of CFTR. We have shown that a severe structural change occurs upon phosphorylation of the R domain, suggesting long range conformational changes transmitted to regions distal from the R domain. To achieve this, the use of purified and reconstituted protein was necessary to get more insight in the molecular mechanism of CFTR. In this report, we developed a reconstitution procedure of CFTR that used the hydrophobic Bio-Beads as a detergent remover. This method has already been widely used for membrane proteins (32), including some ABC transporters (37, 38). It presents several advantages: it is faster than the methods previously used for CFTR, and it allows reconstitution at high protein to lipid ratio. The tendency of CFTR to form aggregates has already been reported. The sucrose gradient profile showed in Fig. 2 clearly demonstrates the absence of such aggregates. This is most probably due to the fact that the hydrophobic beads interact with the aggregates, because protein assay on the beads reveals the presence of proteins on their surface (results not shown). This new procedure is thus efficient for the reconstitution of active CFTR proteins and is promising for future studies of this protein by spectroscopic approaches.

Previously, circular dichroism spectra of the isolated R domain revealed that it was mostly unstructured and that phosphorylation didn’t have any effect on its secondary structure (11). But the conformation of the entire protein was not yet examined. ATR-FTIR was used as a tool to detect global secondary or tertiary structural changes of the protein upon phosphorylation. Although it demonstrates that PKA phosphorylation has a large impact on the conformation of CFTR, and more specifically on its accessibility to the aqueous medium, these conformational changes appeared to be mainly limited to tertiary structure changes, because no major secondary structure modifications were detected. Previously, circular dichroism spectra of a soluble protein corresponding to an extended R domain revealed a decrease in helical content upon phosphorylation (18). Even though these results were obtained on a fragment of the protein, we cannot exclude the possibility that this modification would be too small to be detected on an entire protein, considering the accuracy of secondary structure determination by IR spectroscopy or circular dichroism. Because the limit of reproducibility determined by our secondary structure determination is of 3%, a change in secondary structure involving less than 45 residues of CFTR would not be detected.
ATR-FTIR spectroscopy is also a good tool to study conformational changes that do not involve the secondary structure but the tertiary structure of a protein. This means changes that will modify the arrangements of the secondary structure elements with respect to each other, or the intrinsic stability of these elements. The $^1\text{H}/^2\text{H}$ exchange is a measure of this arrangement, because it determines the solvent accessibility of the different structures. We demonstrate a high accessibility of dephosphorylated CFTR as 70% of the amide hydrogens are exchanged to deuterium after a few minutes. This indicates that more than 1000 residues are accessible to the aqueous medium. This accessibility is further enhanced upon phosphorylation giving rise to 90% of exchange. Presently, such a high exchange rate has been shown only for a few membrane proteins like, for example, 90–95% of exchange for the lactose permease (39), and 81% for the human erythrocyte glucose exchanger (40) after deuteration. These proteins present an exchange behavior clearly distinct from that encountered for most of the membrane proteins where almost no exchange occurs in the membrane domain (41–44). These fast exchanging proteins share the common function of transporting relatively large hydrophilic molecules, and consequently it is expected that they should possess a large hydrophilic pore. Because CFTR has been shown to be permeant not only to chloride but also to polyatomic anions (45) and to glutathione (46, 47), it certainly shares the same properties. Moreover, the modification of the exchange rate upon phosphorylation implies that it induces a large conformational change that modifies the accessibility of CFTR. This large modification can also mean that the stability of defined secondary structure domains has been altered. A detailed analysis of the exchange curves for phosphorylated and dephosphorylated CFTR revealed that more than 450 amino acids that were partially or completely buried in the structure of CFTR become accessible to the solvent upon phosphorylation. Such a large conformational change implies that it does not concern the R domain exclusively but that other domains of the protein are affected by phosphorylation of the latter.

We therefore analyzed the quenching of cytosolic tryptophan residues by the aqueous quencher acrylamide. CFTR contains 23 tryptophans. None of them are located in the R domain. The increase of accessibility of some of these tryptophans upon phosphorylation of the R domain can thus be related to an increased accessibility of the NBD domains or/and the intracellular loops.

Polarized ATR-FTIR demonstrates a large increase of accessibility of the $\alpha$ helices oriented perpendicular to the membrane plane upon phosphorylation of the R domain. We believe that these helices are most probably the transmembrane segments of the protein. Effectively, a detailed analysis performed on another ABC transporter, LmrA, shows that only the residues from the membrane-spanning segments of LmrA contribute significantly to the dichroic spectrum (24). Crystal structures of other ABC transporters MsbA and BtuCD (48–50) present also oriented $\alpha$-helices in the membrane. In MsbA, but not in BtuCD, other perpendicularly oriented $\alpha$-helices were observed in the intracellular loops. Thus, we cannot rule out the existence of oriented $\alpha$-helices in these loops in CFTR. The presence of such structures in the R domain seems unlikely considering that previous structure determination on an isolated R domain by circular dichroism demonstrates that this domain is predominantly unstructured (11). In conclusion, we demonstrated that a large change in CFTR conformation occurs upon PKA phosphorylation of the R domain, modifying the accessibility of residues located outside...
this domain, in the nucleotide binding domains, the intracellular loops, and the transmembrane segments.

The use of isolated fragments of CFTR constructs permits assessment of possible domain-domain interactions. For example, the R domain has been shown to physically interact with other domains of the protein (51), and more specifically with the amino-terminal tail (26) and each of the NBDs of CFTR (28). The “loose” structure of the R domain likely permits its

Fig. 9. Dichroic spectra of actively reconstituted CFTR. Polarized spectra of 20 μg of dephosphorylated (A) and phosphorylated (B) CFTR were recorded before (solid line) and after 12 h (dashed line) of deuteration. The arrows indicate the maximum of each band.
interaction via its multiple phosphoserine residues with multiple regions of the protein simultaneously. Moreover, the substitution of serines by the negatively charged aspartates relieves R domain inhibition (17). It has also been shown that phosphorylation can enhance the ATPase activity of CFTR (2). Consequently, the increase of accessibility observed in CFTR upon phosphorylation of the R domain is consistent with the hypothesis that the unphosphorylated R domain is responsible for steric inhibition, preventing access of the solutes to the NBDs and to the CFTR pore. The negative charges of the phosphates could induce electrostatic repulsion, promoting a displacement of the R domain, relieving this steric inhibition, and allowing solvent accessibility to residues surrounding the cytoplasmic entrance of the membrane pore of CFTR, i.e., allowing access for chloride ions to the ion binding site and for ATP to the NBDs, inducing ATP hydrolysis and chloride permeability. However, this does not take into account the stimulatory effect of the R domain phosphorylation on CFTR activity (11).

Another possibility is that the R domain phosphorylation could induce long range conformational changes transmitted from the phosphorylation sites to the nucleotide binding domains, the intracellular loops, and/or the transmembrane segments. This could implicate the interdomain interactions mentioned above (26, 28, 51). It has been suggested that R domain phosphorylation could regulate the interaction of the NBDs with ATP (10), as well as dimerization of the NBDs (35). Because a secondary structural change was observed on an extended R domain construct (18), although no changes were observed on the soluble R domain alone (11), we can suggest that this additional segment connecting the R domain to the first nucleotide binding domain may be one of the segments responsible for the transmission of these conformational changes and for the increase of ATPase activity measured upon PKA phosphorylation (2). Another possible stimulatory effect of CFTR could be the modification of the orientation of the α-helices forming the channel, triggering the formation of the active ion-binding site. This is supported by the modification of the accessibility of the ion pore, as well as the observation of different helix orientations in the different crystal structures of MscL.

The stimulatory effect of CFTR clearly favors the second hypothesis. However, we cannot rule out the possibility that PKA phosphorylation could both relieve a steric inhibition due to the unphosphorylated R domain and induce concomitant long range conformational changes responsible for stimulation of activity. Additional experiments should be performed to determine precisely the residues playing a major role in the conformational changes. This would give a deeper insight on the mechanism of CFTR R domain inhibition and activation.

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