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Cystic Fibrosis: A Disease of Altered Protein Folding

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Cystic fibrosis (CF) is caused by mutations in the gene that encodes the cystic fibrosis transmembrane conductance regulator, CFTR. Previously we demonstrated that the common ΔF508 mutation in the first nucleotide binding domain (NBD1) alters the ability of the domain to fold into a functional three-dimensional structure, providing a molecular explanation for the observation that the mutant CFTR is retained in the endoplasmic reticulum and does not traffic to the apical membrane of affected epithelial cells. Notably, when conditions are altered to promote folding of the mutant protein, it can assume a functional conformation. Correcting the folding defect may have therapeutic benefit for the treatment of cystic fibrosis. Here we summarize these results and discuss the implications *in vitro* folding studies have for understanding the pathobiology of CF.

KEY WORDS: Protein folding; protein stability; genetic disease; cystic fibrosis; CFTR.

INTRODUCTION

How does a protein fold into a functional three-dimensional structure? This is a question of fundamental importance to both cell biology and biophysics. The fact that alterations of the folding process have been implicated in the pathobiology of a growing number of human diseases makes it a question of great medical relevance.

Anfinsen and his coworkers[(1)] demonstrated more than 30 years ago that the information necessary for folding soluble proteins into their native structures is present in the primary amino acid sequence. Formation of transmembrane helices from peptide fragments of membrane proteins[(2,3)] indicates that this truism is not limited to soluble proteins. Thus, study of the physical properties of purified proteins and peptides has provided fundamental information about the energetics, kinetics, and pathways of protein folding. *In vivo,* proteins called molecular chaperones are intimately involved in this process, interacting with polypeptides until they achieve the fully folded native state. Here the CFTR (cystic fibrosis transmembrane conductance regulator), an integral membrane glycoprotein of 1480 amino acids, will be introduced as a paradigm for understanding the cell biology and biophysics of membrane protein folding and its alteration in disease.

Cystic fibrosis is a severe autosomal recessive disorder of fatal consequence. Over six hundred mutations in the gene encoding the CFTR have been associated with the disease (CF Mutation Data Base, Cystic Fibrosis Genetic Analysis Consortium). Patients exhibit a thick mucous layer and increased bacterial infection of pulmonary epithelia, as well as aberrant pancreatic secretion.[(4–6)] Underlying this pathology is a reduction in a regulated chloride conductance across the apical membrane of affected epithelia.[(7,8)] Heterologous expression studies indicate that CFTR itself functions as a chloride channel which is regulated by cAMP-dependent protein kinase (PKA) catalyzed phosphorylation of the CFTR regulatory domain (R) and the interaction of the two CFTR nucleotide binding domains (NBDs) with adenine nucleotides.[(9,10)] Presumably, the anion selective pore is formed by the two CFTR transmembrane domains (TMDs), which are

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predicted to span the apical membrane six times each.[11] In addition to serving as a Cl^- channel itself, CFTR has an emerging role in regulating other channels.[12]

The most common disease-causing mutation in CFTR is a 3bp deletion resulting in an in-frame loss of the phenylalanine residue at position 508 (ΔF508) in NBD1.[13] Cheng and coworkers[14] made the important observation that the ΔF508 mutant CFTR did not attain the mature glycosylation state because it was retained in the endoplasmic reticulum and, thus, did not transit though the Golgi system to the plasma membrane of expressing cells. The trafficking defect is evident in tissues from CF patients.[15] Studies on a set of chemically synthesized polypeptides demonstrated that the physical basis of the ΔF508 mutation was a destabilization of the polypeptide conformation and, thus, that this form of cystic fibrosis is a protein folding disease.[16] This work predicted that conditions which would stabilize the crucial conformer would reverse the disease phenotype[16,17]—a prediction that was subsequently borne out by the finding that the efficiency of maturation of the ΔF508 CFTR is increased at reduced growth temperature.[18] Notably, altered protein folding appears to be the basis of a growing number of human pathologies.[19] The benefit of correcting the folding/maturation defect for the CF patient is clear from the demonstration that the ΔF508 CFTR retains at least some ability to function when it reaches its native conformation.[16,18,20,21]

In the last several years a growing body of work has built upon these original observations by identifying: the kinetic nature of the defect,[22] the accessory proteins involved in folding *in vivo*,[23,24] additional disease-causing mutations which are maturation defective,[25,26] the fate of the incompletely folded CFTR,[27,28] and osmolytes which correct the maturation defect.[29,30] Here, we review these results and discuss their implications for possible strategies to circumvent the CF phenotype and for the fundamental processes the cell uses to achieve efficient membrane protein folding.

DISEASE-CAUSING FOLDING/MATURATION MUTATIONS

The spectrum of CF-causing mutations can be divided into three groups, those that affect its expression, those in which native structure is achieved but nonfunctional, and those that affect its ability to fold into a native state. Currently, no disease-causing mutations have been identified in the promoter nor in the putative PKA phosphorylation sites. Notably, there is a clustering of known disease-causing mutations in the amino terminal nucleotide binding domain (NBD1) of CFTR.[31] All of the originally identified maturation-defective mutations (Fig. 1A) reside in NBD1.[14,25,26] More recently, mutations in the fourth intracellular loop (ICL4) have also been shown to be protein maturation defective.[32,33] The ultimate fate of mutant proteins unable to transit this complex, multi-step pathway is proteolysis (Fig. 1B). It is likely that mutations of critical residues throughout the CFTR protein cause CF by promoting defective folding. However, it is interesting to note that NBD1 is particularly sensitive to folding mutations, since mutation of residues in NBD2 homologous to those critical for folding in NBD1 does not affect maturation.[25]

Work from our laboratory has focused on reducing the complexity of the CFTR folding problem to allow a mechanistic description of the process and the step(s) affected by the ΔF508 and other disease-causing folding mutations. In addition, pathologically selected mutational data provides insights into the fundamental mechanisms at work in directing the efficient folding of a protein domain.

The initial demonstration that the ΔF508 mutation altered the folding of the CFTR came from studies of synthetic polypeptide models of NBD1. A sixty-seven amino acid fragment of NBD1 (P67) containing the Walker A consensus and a region of homology around F508 was shown to tightly and specifically bind adenine nucleotides.[34] Deletion of the F508 residue (P66) had no significant effect on the ability of the polypeptide to bind nucleotide, indicating that this portion of the ΔF508 CFTR was functional when folded.[16] However, the mutation had a dramatic effect on the conformational stability of the polypeptide. The urea concentration required to denature 0.5 mole fraction of the polypeptide[16] and the melting temperature were dramatically reduced by the mutation, indicating the mutation shifted the conformational equilibrium toward the denatured state.

When a portion of CFTR predicted to correspond to the entire NBD1 was studied, quite different behavior was observed. Residues G404 to S589 were expressed at high yield in *E. coli* and refolded into a soluble monomer capable of binding nucleotide.[22] To promote the folding of NBD1 and to maintain the expressed domain as a soluble monomer it was necessary to include the amphipathic osmolyte arginine in

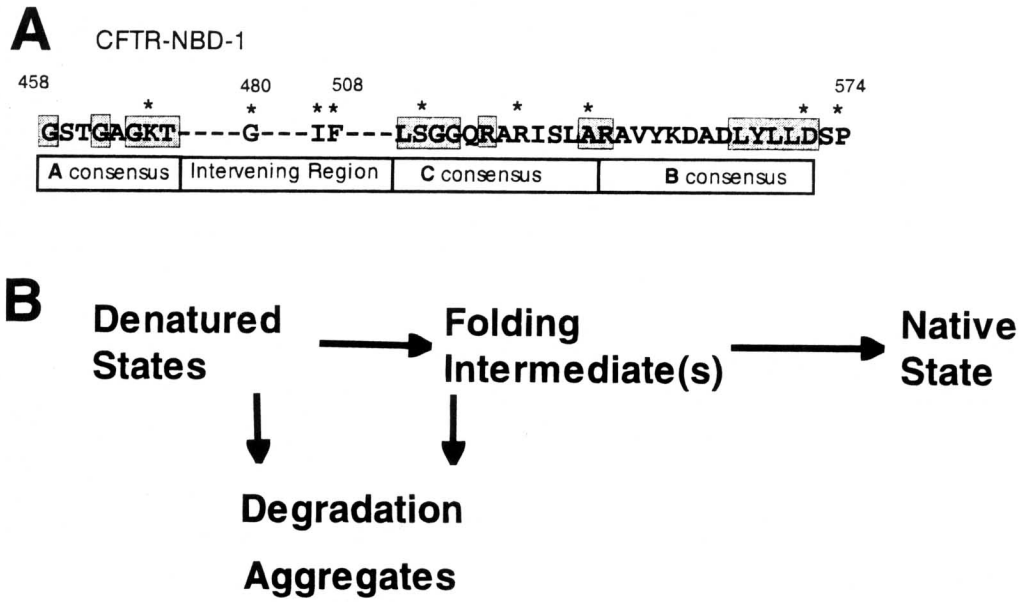

Fig. 1. CF-associated maturation mutations in NBD1. A. Residues in NBD1 indicated with an asterisk have been shown to be defective or promote maturation or folding of CFTR. References for the mutations are included in the text. B. Mutations may lead to defective folding *via* several effects. They could alter the equilibrium between the denatured and native states, resulting in a decreased free energy of stability of the functional conformer. They could alter the kinetics of the folding pathway or the associations that lead off the folding pathway.

the buffer. Again, the ΔF508 mutation had no effect on the nucleotide binding function of the expressed CFTR domain. However, in contrast to the synthetic P67 and P66 polypeptides, the thermodynamic stability of the NBD1 was not significantly altered by the ΔF508 mutation, indicating that F508 contributes little stability to the native conformer.[22] However, the mutation has a dramatic effect upon the temperature-dependent folding yield of the domain (Fig. 2), suggesting that the mutation affects a metastable intermediate of the folding pathway. For example, at 37°C, a temperature at which the native conformers of both wild type and ΔF508 NBD1 are thermally stable, the yield of the normal domain is nearly double that of the mutant.[22]

The altered folding yield is due to kinetic partitioning between the on-pathway first-order folding reaction (Fig. 2, inset) and the off-pathway higher-order self-association reaction that results in the formation of an insoluble aggregate. Thus, at elevated NBD1 concentrations the yield is reduced and the formation of aggregate is increased. The *in vitro* folding results demonstrate that ΔF508 is a kinetic folding mutation and are consistent with studies of CFTR folding *in vivo* which suggest that the defect occurs at an early step.[35,36] It may be useful to point out that the normal fate of malfolded CFTR *in vivo* also depends upon a second-order reaction with the proteasome.[27,28] In addition, when the activity of this protease is inhibited, a SDS-insoluble aggregate forms *in vivo,*[27] indicating that the crucial species present at the branch point is kinetically isolated from the proteolytic step. Therefore, proteasome inhibitors would appear to offer little hope of therapeutic benefit. At the present time a relationship between the aggregates formed *in vivo* and *in vitro* has not been established.

Protein folding mutants can be separated into two general classes: mutations that destabilize the final native state and mutations that alter the folding pathway.[37,38] In the first class, the folded protein is thermally labile; these mutations are called temperature sensitive (*ts*) mutants. Because it is an extensive state function that is affected, these defects can be described by equilibrium thermodynamics. In the second class, there may be no apparent decrease in the thermal lability of the mutant protein, as is the case for ΔF508; however, the process of folding is temperature sensitive. These mutants have been named temperature sensitive folding (*tsf*) mutants by Jonathan King.[37] In this case the folding pathway is altered and the process is under kinetic control, and, thus, requires a kinetic description. It is important to note that the existence of *tsf* mutants underlines the fact that proteins reach

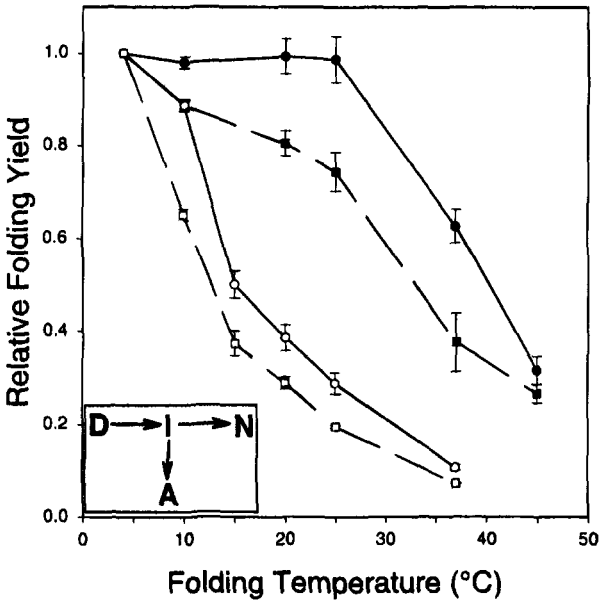

Fig. 2. Folding yield of CFTR-NBD1 and CFTR-NBD1ΔF508 as a function of temperature and protein concentration. Inset, under the conditions employed during *in vitro* folding a fraction of the denatured NBD1 proceeds to the functional native conformation and a fraction self-associates and precipitates from solution.[22] Results for wild type NBD1 at 2 μM (filled circle) and 18 μM (open circle) and ΔF508 NBD1 at 2 μM (filled square) and 18 μM (open square) are shown. The relative yield is the result of a balance between the first-order process of self-assembly into the native state and the higher-order process of aggregation. The final yield reflects the concentration of a transient conformer of NBD1 present during the folding process. The ΔF508 mutation affects the concentration of this branch point species.

their native state *via* a limited, if not unique, set of pathways as required for resolution of the Levinthal paradox.[39] The paradox states that the time required for a protein to indiscriminately search conformational space for the global energy minimum would be much longer than the present age of the universe, yet all information required for the native structure is present in the primary sequence. Clearly, the conformational search must be limited, and the primary sequence must specify the pathway(s) by which the kinetically accessible free energy minimum (native state structure) is reached. These issues have been reviewed in detail elsewhere.[40]

The most thorough studies of *tsf* mutants have been carried out on the tail spike endorhamnosidase from the *S. typhrimurium* phage P22 [37,38,41]. Notably, a large number of mutations of the P22 tailspike protein affect its folding, but not its native state structure, indicating that non-native state interactions may be important for directing the folding pathway.[41] Significantly, the *in vitro* pathway of P22 tailspike folding closely approximates the folding reaction in the cell. Furthermore, intragenic suppressor mutants have been isolated that correct the folding defect.[37] The *tsf* mutations reduce the rate of folding, while the suppressors increase the rate.[38] These results are consistent with *tsf* mutations destabilizing and suppressor mutations stabilizing a crucial intermediate state on the folding pathway.[38,41] Mutations which correct the maturation defect of CFTR NBD1 may function in a similar manner.[42]

The finding that the ΔF508 mutation affects only the folding yield of the NBD1, but dramatically alters the conformational equilibrium of the shorter P67 model, suggests that P67 and P66 may provide useful models of the metastable intermediate state. These polypeptides have several characteristics of the "molten globule" folding intermediate, including appreciable secondary structure[17] and exposed hydrophobic surfaces which bind the dye ANS. Thus, compounds that stabilize P66 without inhibiting the folding of NBD1-ΔF508 may be useful in promoting folding of mutant CFTR.

Taken together, the evidence for temperature sensitivity of CFTR-ΔF508 maturation,[18] the destabilization of a polypeptide that may model a folding intermediate,[16,43] and the lack of effect of ΔF508 on the equilibrium stability of the native-like NBD1,[22] all indicate that ΔF508 is a *tsf* mutation.

OTHER PROTEINS INVOLVED IN FOLDING

Since disease-causing mutations often alter protein folding and trafficking *in vivo*, an involvement of molecular chaperones in disease pathogenesis is a reasonable possibility.[43–45] Recently, putative roles for Hsp70 and calnexin in human diseases have been reported. Hsp70 was shown to interact with the mutant oncogene product p53val^{135}, but not wild-type p53 when the proteins were translated *in vitro*.[46] Both wild-type and ΔF508 forms of CFTR are coimmunoprecipitated with an Hsp70 antibody from CFPAC cells (a pancreatic tumor line with a ΔF508 phenotype that normally expresses little CFTR) infected with a CFTR adenovirus expression vector.[23] Calnexin has been found in association with both a secretion incompetent[47] and wild-type α_1-antitrypsin[48] as well as wild-type and ΔF508 forms of CFTR.[24] It has been postulated that these associations are required for retention of mutant proteins in the endoplasmic reticulum (ER)

and their ultimate degradation.[23,24,47] The requirement for accessory proteins in the maturation process is underscored by the fact that, *in vivo,* conformational maturation of CFTR is dependent on the availability of ATP,[36] invoking the involvement of an accessory protein which requires ATP to function. However, several observations raise the possibility that proteins other than Hsp70 and calnexin may be required. First, Hsp70 does not have a KDEL ER retention sequence and is not itself limited to this compartment. Second, the ΔF508 mutation occurs within a cytoplasmic domain of CFTR, on the opposite side of the ER membrane from the conserved regions of calnexin.[49] And third, studies of MHC class I biosynthesis indicate that phosphocalnexin does not play a role in the retention of unassembled complexes in the ER.[50] The role of chaperones in promoting the efficient folding and recognition of misfolded CFTR deserves further study.

STRATEGIES TO CIRCUMVENT THE FOLDING DEFECT

In light of the fact that ΔF508 mutant CFTR is functional once it reaches the native state, identification of a compound which corrects the ΔF508 CFTR folding defect would be of potential therapeutic benefit.[17] Although several general strategies are available that correct the folding defect, an agent specific for the CFTR folding process is desirable because the cellular machinery responsible for protein maturation and quality control exists in a very delicate balance. Generalized treatments such as reduced temperature to promote maturation of the mutant CFTR would likely have a plethora of effects and, in the end, may do more harm than good. The growing list of general ways to increase the folding yield of mutant CFTR includes reduced temperature, the addition of osmolytes such as arginine, glycerol, trimethylamine oxide (TMAO), and possibly compounds such as phenylbutyrate.

A variety of osmolytes affect the folding and maturation of CFTR. Arginine promotes the folding of NBD1 and maintains it in a soluble conformation *in vitro,*[22] presumably by its interactions with surfaces of the domain which are prone to self-association or by ordering solvent interactions with the domain. The mechanism by which glycerol[29,30] and TMAO[29] encourage folding *in vivo* has not been established. They may act either by upregulating a cellular process designed to promote folding, or they may act directly on the CFTR molecule. The cell type dependence of glycerol in promoting the folding of mutants of the CFTR homologue P-glycoprotein argues for an indirect effect.[51] If glycerol and TMAO exert their action *via* direct effects on CFTR, they may stabilize the protein by ordering the solvent environment.[52-54] It is important to note that these osmolytes may not exert their stabilizing effects on the final native state, but during the critical folding transition; or by increasing the viscosity, thereby retarding the higher-order processes such as recognition by quality control proteins and self-association.

Phenylbutyrate, a drug used clinically to treat patients with urea cycle disorders, increases the amount of mature, functional ΔF508 CFTR channels observed in the plasma membrane in cell culture.[55] It is presently unknown if phenylbutyrate promotes the folding of CFTR directly or acts by inducing the expression of other proteins that promote its folding. The later possibility is supported by phenylbutyrate's ability to increase the level of fetal hemoglobin in sickle cell anemia patients.[56]

Strategies that are specific for promoting CFTR folding would be less likely to alter the folding of other essential proteins. One possibility is to stabilize the native state by addition of a ligand. This method has successfully overcome folding problems for several other proteins. Kelly and his co-workers[57] used the ligand thyroxine to stabilize the native state of transthyretin *in vitro,* shifting the overall equilibrium away from an intermediate prone to nonproductive self-association. Loo and Clarke[51] demonstrated that substrates of the P-glycoprotein enable transit of mutant proteins which are otherwise retained in the ER. However, ligand-dependent stabilization of the native state would be expected to increase the folding yield of ΔF508 CFTR only if the branch point intermediate is not kinetically isolated from formation of the native binding pocket (Fig. 3).

Strategies which interfere with the normal cellular machinery for protein folding may not provide sufficient specificity for the design of therapeutic strategies to correct the CFTR folding defect. For this reason, an increased understanding of the precise CFTR folding pathway and its intermediates is essential. In particular, it will be critical to understand which of the steps on the folding pathway are affected by the mutation. The reduction of the mutational defect to a single protein domain *in vitro* and the ability to control conditions which favor either folding or misfolding of NBD1[22] provides a means of both defining the steps on the

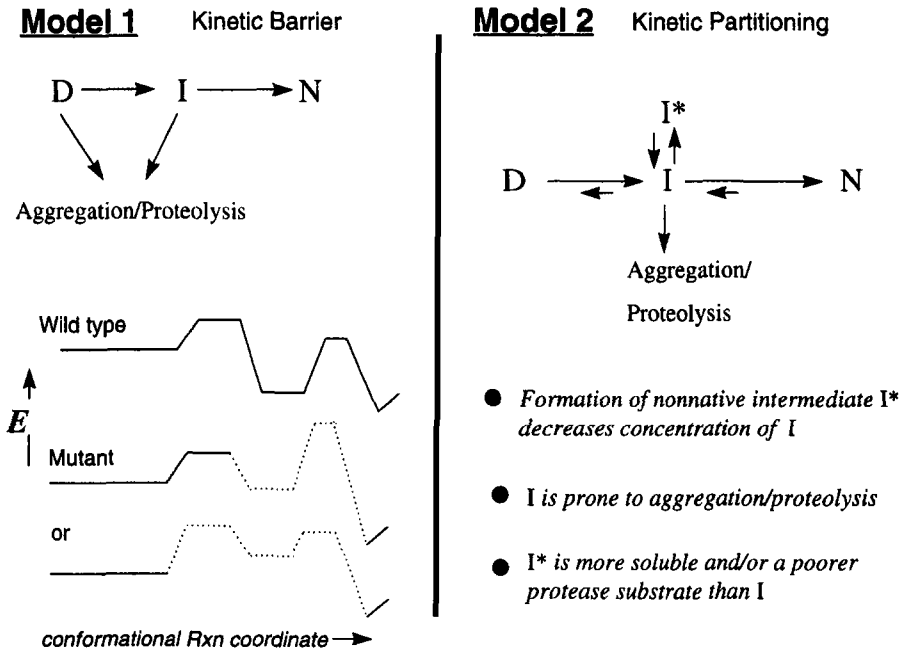

Fig. 3. Kinetic defects in protein-folding reactions. At least two mechanisms can account for an inability of a mutant protein to fold at high yield in the absence of any significant effects upon the native state stability. *Model 1:* the mutation raises an energy barrier on the folding pathway, slowing the rate of folding and effectively raising the concentration of a transient intermediate which is prone to association with itself or cellular quality control proteins. The cellular proteins could kinetically edit the folding reactions by interacting with those intermediate species that exist at high enough concentration for a binding reaction to occur at an appreciable rate. *Model 2:* Control of the partitioning of a reaction could be achieved in another manner. An off-pathway species, which does not directly lead to the native state, when in a rapidly achieved equilibrium with the association-prone on-pathway intermediate, would effectively reduce the concentration of this intermediate. Low I concentration favors the first-order folding reaction over the higher-order misassociations. The I* species would be dictated by the primary sequence which has been selected to fold efficiently. In a similar manner, I* could be a binary complex with cellular chaperones also reducing the concentration of I available for self-association or proteolysis.

folding pathway and of developing methods for screening compounds that specifically promote folding of the diseased CFTR. Characterization of steps on the folding pathway affected by the ΔF508 mutation may aide the design of a drug capable of serving as a structural template during acquisition of CFTR tertiary structure, thus functionally complementing the loss of phenylalanine 508. A specific screening assay could provide a means for discovering a specific, therapeutic, protein folding drug. An *in vitro* assay for folding which is amenable to high throughput is desirable to efficiently screen large libraries for compounds that rescue the misfolding of the mutant protein. The *in vitro* NBD1 folding system described in Fig. 2 provides such an assay.[22] When the temperature and protein concentrations are adjusted so that the NBD1 folding yield is low, the ΔF508 mutation has a dramatic and measurable effect on the apparent lag phase and the rate of formation of the off-pathway aggregate.[22] (Fig. 4). Notably, the formation of these off-pathway species is easily monitored as increased light scattering or turbidity, making the assay appropriate for use in a multiwell plate format.

The ΔF508 mutation subtly upsets the delicate balance between correct and errant steps of the CFTR folding pathway. It does not, however, distort the CFTR structure so drastically that the native state is unobtainable, as is underscored by intragenic second-site mutations which correct the folding defect.[42,58] Therefore, a subtle alteration of folding kinetics may be all that will be required of a drug. The results summarized here should further encourage the search for strategies to circumvent the folding defect of CFTR and treat the disease.

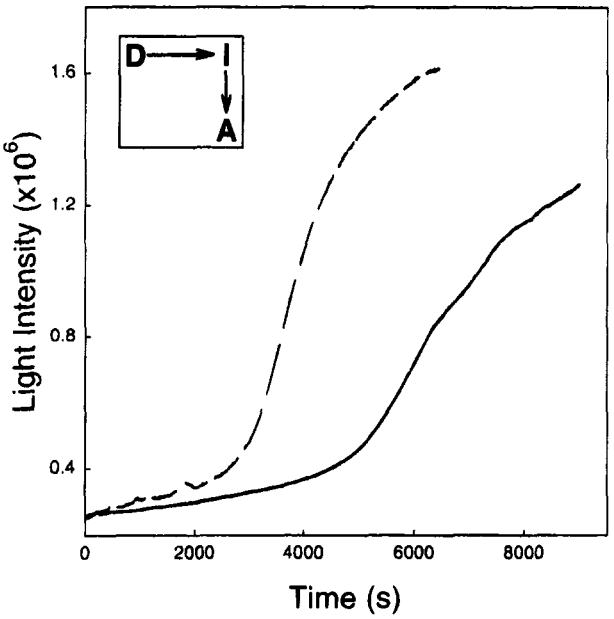

Fig. 4. The ΔF508 mutation decreases the lag phase and increases the rate at which off-pathway conformers are formed. A simple light-scattering assay demonstrates the dramatic difference between the wild type (solid line) and the ΔF508 NBD1 (dashed line).[22] The assay may have utility as a simple screen for a first generation of specific drugs for the treatment of CF-causing folding defects.

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