Localization and Suppression of a Kinetic Defect in Cystic Fibrosis Transmembrane Conductance Regulator Folding*

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A growing body of evidence indicates that the most common cystic fibrosis-causing mutation, ΔF508, alters the ability of the cystic fibrosis transmembrane conductance regulator (CFTR) protein to fold and transit to the plasma membrane. Here we present evidence that ΔF508 mutation affects a step on the folding pathway prior to formation of the ATP binding site in the nucleotide binding domain (NBD). Notably, stabilization of the native state with 4 mM ATP does not alter the temperature-dependent folding yield of the mutant ΔF508 NBD1 in vitro. In contrast, glycerol, which promotes ΔF508-CFTR maturation in vivo, increases the folding yield of NBD1ΔF and reduces the off pathway rate in vitro, although it does not significantly alter the free energy of stability. Likewise a second site mutation, R553M, which corrects the maturation defect in vivo, is a superfolder which counters the effects of ΔF508 on the temperature-dependent folding yield in vitro, but does not significantly alter the free energy of stability. A disease-causing mutation, G551D, which does not alter the maturation of CFTR in vivo but rather its function as a chloride channel, and the S549R maturation mutation have no discernible effect on the folding of the domain. These results demonstrate that ΔF508 is a kinetic folding mutation that affects a step early in the process, and that there is a significant energy barrier between the native state and the step affected by the mutation precluding the use of native state ligands to promote folding. The implications for protein folding in general are that the primary sequence may not necessarily simply define the most stable native structure, but rather a stable structure that is kinetically accessible.

Cystic fibrosis (CF)1 is a disease of fatal consequence that, at the genetic level, is due to mutation of the gene that encodes the cystic fibrosis transmembrane conductance regulator (CFTR) (1). This protein, normally found in the apical membrane in secretory epithelia, is of central importance for the fluid secretion necessary for hydration of the pulmonary mucus and for secretion of digestive enzymes in the pancreas (2). The most common CF genotype is a 3-base pair in-frame deletion in exon 10 that results in the loss of a single phenylalanine residue at position 508 (ΔF508) in a nucleotide binding domain (NBD1) (1), which is important for the ATP dependence of CFTR function (3–9). The cellular phenotype of the ΔF508 mutation is a defect in protein maturation and transit to the plasma membrane (10). Several other CF-causing mutations known to be maturation-defective, including S549R, are located in NBD1 (11) as are mutations of critical functional residues such as G551D (12). Previously we suggested that the defect in ΔF508-CFTR intracellular trafficking was secondary to the diminished ability of the mutant NBD1 to achieve its native, functional conformation (13, 14). Studies on a 67-amino acid fragment of NBD1 (14) and intact NBD1 (5) are consistent with the notion that defective folding is responsible for the defective maturation of the mutant protein and, thus, the lack of functional protein.

Alteration of other residues in CFTR-NBD1 and of cellular conditions is known to ameliorate the defective maturation and trafficking defect caused by the ΔF508 mutation. In a German pancreas-insufficient patient homozygous for ΔF508 with typical gastrointestinal and pulmonary disease but sweat chloride in the normal range a second site mutation of R553Q was found on one ΔF508-CFTR allele (15). Two mutations at this position, R553M and R553Q, revert the mating phenotype of a ΔF508 STE6-CFTR chimera in yeast (16). Moreover, introduction of the second site mutations into human ΔF508-CFTR partially corrected the maturation defect, resulting in an increase of functional CFTR at the membrane (16). Recently two groups have demonstrated that when cells expressing ΔF508-CFTR are grown in the presence of glycerol the maturation and functional defects can be largely rescued (17, 18). Such a result had been predicted with the suggestion that conditions, which stabilize protein structure during the folding process, might restore wild-type function to the ΔF508-CFTR (13, 14).

Previously an in vitro folding system was developed to quantitatively investigate the effect of the ΔF508 mutation on the folding of NBD1 (5). These studies demonstrated that ΔF508 in NBD1 was a temperature-sensitive folding (tsf) mutation, suggesting the mutation altered the folding pathway of NBD1 rather than its native state thermodynamic stability. In the present study we use this system to examine the effects of the R553M second site mutation, the G551D functional mutation, the S549R maturation-defective mutation, glycerol, and ATP binding on the folding pathway and thermodynamic stability of NBD1. These studies provide direct evidence that the defect is on the folding pathway and that manipulations that correct the maturation defect of the full-length protein in vivo also correct the defective folding of the first nucleotide binding domain in vitro, establishing a causal link between the two observations.

EXPERIMENTAL PROCEDURES

Expression, Purification, and Folding of CFTR NBD1s—Oligonucleotide-mediated mutagenesis (19) was used to generate R553M, G551D, and S549R mutations in plasmid pBQ2.4 containing CFTR cDNAs. The mutant primers are as follows: R553M primer, 5’-GAAATTCATGC-
Defective CFTR Folding

Expression, Purification, and Function—The cDNAs for wild-type and mutant NBD1s corresponding to exons 9–12 of the CFTR gene (Fig. 1) were cloned into pET-based expression vectors capable of directing the expression of this domain (Gly404 to Ser589) as a fusion with an amino-terminal polyhistidine tract. The expression levels of NBD1-R553M and NBD1-G551D, and NBD1-S549R containing a 5’ NdeI site, a 3’ XhoI site, and a stop codon as described previously for NBD1 and NBD1ΔF (5). Digested polymerase chain reaction products were ligated into the NdeI and XhoI sites of the pET28a plasmid. To construct the NBD1ΔF-R553M expression vector, pET28a NBD1-R553M and pET28a NBD1ΔF were digested with Spal. The larger fragment (513-base pairs) from the pET28a NBD1-R553M plasmid digestion and the small fragment (720 base pairs) from the pET28a NBD1ΔF plasmid digestion were purified and ligated with T4 ligase. The proper construct was verified by restriction digestion and sequencing.

The NBD1 proteins were purified from expressing BL21 (DE3) Escherichia coli strains and folded in vitro as described previously (5). The thermodynamic stability of the purified NBD1s was determined by extrapolation of the GdnHCl melting profiles (5).

The temperature dependence of the folding yield experiments was determined by monitoring the intrinsic tryptophan fluorescence at 2 μM final protein concentration (5). The fluorescence method allows not only the amount of protein to be quantitated but reports on the conformation as well. For the effect of ATP on the temperature-dependent folding of NBD1ΔF, unfolded protein was diluted with buffer B (100 mM Tris-HCl, pH 8.0, 400 mM l-arginine-HCl, 2 mM EDTA, and 1 mM diithiothreitol) containing 4 mM ATP. An excitation wavelength of 290 nm was used in the presence of ATP to avoid nucleotide-dependent absorption of the incident light.

ATP Binding—Nucleotide binding was determined from the quenching of protein tryptophan fluorescence upon ATP binding to the NBD1 proteins. Samples containing 1.8 μM NBD1s in buffer B at the indicated concentration of ATP were excited at 295 nm (2-nm band-pass). Uncorrected emission spectra were collected from 305 nm to 400 nm (4-nm band-pass). The dissociation constant (Kd) was calculated by nonlinear regression of the data according to the equation,

$$\Delta F = \frac{\Delta F_{\text{max}} \cdot [L]}{K_d + [L]} \quad (\text{Eq. 1})$$

where ΔF is fluorescence decrease and [L] is the concentration of ATP.

Off Pathway Kinetics—To determine the rates of formation of off folding pathway species, NBD1s were solubilized in 6 M GdnHCl and rapidly diluted 30-fold with buffer B to a final protein concentration of 18 μM at 23 °C or 2 μM at 37 °C. Under these conditions the folding yields of wild-type and NBD1ΔF are much less than at 4 °C (5). Scattered light at 400 nm was measured at a 90° angle or by turbidity to monitor the aggregation progress. In experiments containing glycerol, 10% yields of wild-type and NBD1 were verified by DNA sequencing. Expression cassette polymerase chain reaction products were purified and ligated with T4 ligase. The proper construct was verified by restriction digestion and sequencing.

RESULTS

Expression, Purification, and Function—The cDNAs for wild-type and mutant NBD1s corresponding to exons 9–12 of the CFTR gene (Fig. 1) were cloned into pET-based expression vectors capable of directing the expression of this domain (Gly404 to Ser589) as a fusion with an amino-terminal polyhistidine tract. The expression levels of NBD1-R553M and NBD1ΔF-R553M are similar to NBD1 and NBD1ΔF (5). The expression levels of NBD1-G551D and NBD1-S549R are approximately one-half of the wild-type NBD1 level (data not shown).

The wild-type and all mutant proteins form inclusion bodies under these growth conditions in E. coli. The inclusion bodies were isolated, dissolved in GdnHCl, and purified on His-tag affinity resin under denaturing conditions. Densitometry of the proteins resolved by 10% Tricine SDS-polyacrylamide gel electrophoresis (20) and stained with Coomassie Blue indicated that all samples are greater than 95% pure.

All denatured mutant NBD1s can be folded into functional nucleotide binding conformations in vitro in buffer B by the cooperative burial of the Trp496 residue, solubility, and the ability to bind nucleotide. The saturable binding of ATP to the wild-type NBD1 is shown in Fig. 2. Upon interaction with the nucleotide the domain changes to a conformation in which Trp496 fluorescence is partially quenched. The fact that the binding is greatly attenuated in the presence of GdnHCl indicates that high order structure is required for interaction with the nucleotide. None of the mutations has a dramatic effect on the affinity of the isolated domain for ATP. The apparent Kd for ATP binding to the other NBD1s determined in similar experiments (data not shown) are presented in Table I.

Temperature-dependent Folding Yield and Off Pathway Kinetics of NBD1s—Fig. 3A shows the temperature dependence of the folding processes of each of the NBD1s. The data for wild-type and NBD1ΔF were previously published (5) and are presented here for comparison. At 37 °C, 63% of the wild-type
ATP binding to NBD1s was measured by the decrease of intrinsic tryptophan fluorescence (Fig. 2). The $K_d$ values were calculated by nonlinear regression of the data. The standard errors are $\pm 10 \mu M$.

| Protein       | NBD1 | NBD1F | NBD1-R553M | NBD1ΔF-R553M | NBD1-S549R | NBD1-G551D |
|---------------|------|-------|------------|--------------|-------------|-------------|
| $K_d$ (μM)    | 91   | 88    | 89         | 87           | 81          | 61          |

NBD1 and only 38% of the NBD1F fold into the soluble conformation, whereas 96% of NBD1-R553M assumes the folded conformation at this temperature (Fig. 3A). Thus, the R553M mutation significantly enhances the folding yield of NBD1. For the double mutant NBD1ΔF-R553M the folding yield is indistinguishable from that of the wild-type. Thus, the second site mutation R553M effectively suppresses the $\Delta F_{508}$ mutation defective folding yield in vivo.

The rate of formation of the off pathway conformer was assessed by light scattering at 18 $\mu M$ protein concentration and 23 °C. A lag phase followed by an increase in light intensity was observed (Fig. 3B). Significantly the R553M mutation increases the length of the lag phase and decreases the rate of change in light scattering, indicating that the rate of formation of the off pathway conformer is dramatically reduced in this mutant. Once again the double mutant NBD1ΔF-R553M dramatically increases the lag time and decreases the rate change in light scattering in comparison with NBD1ΔF.

Two other mutations, S549R and G551D, do not affect the domain folding yield compared with the wild-type NBD1 in vitro (Fig. 4A). In vivo studies indicate that the S549R mutation but not G551D is maturation-defective in the full-length protein (11); however, the NBD1-S549R folding yield is not decreased in vitro indicating that it does not act by altering the ability of the domain itself to fold. Moreover, neither mutation affects the rate of formation of the off pathway conformer of this domain (Fig. 4B).

**Thermodynamic Stability of NBD1s**—The GdnHCl-induced cooperative unfolding of the domains is shown in Fig. 5. The concentration of GdnHCl required is similar for all NBD1s. The $C_m$ values are between 1.2 M and 1.5 M (Table II). The $\Delta G_{D}$ of the NBD1s, indicating the domains’ thermodynamic stability, can be calculated from this reversible two-state transition (Fig. 5, inset). Compared with the wild-type NBD1, the maximum mutant $\Delta G_{D}$ is only 1.4 kJ/mol (Table II). In comparison, the $\Delta G_{D}$ of the native state wild-type NBD1 is 15.5 kJ/mol. Thus, the effect of the $\Delta F_{508}$ mutation on the stability of NBD1 under these conditions is minimal. The cooperativity of the transition is reflected in the $m_{GdnHCl}$ values (the slope of the natural log of the fraction folded versus the denaturant concentration) (Fig. 5, inset). The $m_{GdnHCl}$ values are similar for the NBD1s except for S549R, which has a somewhat larger value indicating that the change in solvent-accessible surface area upon folding is appropriate for a protein of this size. These results indicate that the inability of the $\Delta F_{508}$ and S549R CFTR to transit to the apical membrane and the effect of the R553M suppressor cannot be explained simply by a reduction or enhancement in the free energy of stability of the mutant proteins.

**Off Pathway Kinetics of NBD1ΔF in the Presence of Glycerol**—As shown in Fig. 6, the formation of off pathway species is inhibited by the presence of 10% glycerol. In addition, the temperature-dependent folding yield is increased by this osmolyte (data not shown). These results provide an in vitro explanation for the observation that when glycerol is added to cultured cells expressing CFTR the amount of mutant protein that is successfully trafficked to the plasma membrane is increased. The in vitro results here suggest that the mechanism by which glycerol works is to assist the mutant nucleotide binding domain in its folding. GdnHCl unfolding of NBD1ΔF in the presence of 10% glycerol (data not shown) yields a $\Delta G_{D,0}$ of 14.3 kJ/mol representing a $\Delta G_{D,0}$ of 0.1 kJ/mol compared with the $\Delta G_{D,0}$ value reported for the mutant in the absence of glycerol (5). Therefore, the presence of 10% glycerol does not...
have a significant effect on the Gibb's free energy of stability of the domain.

Folding of NBD1ΔF in the Presence of ATP—Since the ΔF508 mutant form of the protein is functional when folded, it had been suggested that native state ligands might serve to stabilize this conformation and overcome the folding defect (13, 14). Recently such an approach has been effective in promoting the folding of mutant forms of transthyretin and the CFTR homologue P-glycoprotein (21, 22). To test this possibility we examined the effect of the natural ligand ATP on the folding yield and off pathway rate of NBD1ΔF. The folding reaction and off pathway reaction were performed in the presence of 4 mM ATP. This concentration is at least 40 times the $K_D$ of NBD1ΔF for ATP. Although the binary complex is more stable than NBD1ΔF alone, the presence of the nucleotide did not increase the folding yield or decrease the off pathway rate (Fig. 7).

DISCUSSION

An emerging body of evidence indicates that many genetic diseases have their basis in the defective folding of the protein product of the mutant gene (23). For example, in the case of cystic fibrosis, deletion of a single phenylalanine residue at position 508 in the 1480-amino acid CFTR protein leads to a...
reduction in the folding yield in vitro (5) and of the efficiency of maturation and transit to the plasma membrane in vivo (10). Understanding the molecular pathology of this disease should provide not only a means of developing therapeutic strategies, but also aid in elucidating the relationships between primary structure and the process of folding a protein sequence into its functional three-dimensional structure.

The results presented here support our earlier studies which suggested that the defective folding of ΔF508 CFTR is due to alteration of the process of folding rather than to destabilization of the native state of NBD1 (5). First, the R553M suppressor mutation effectively corrects the ΔF508 folding defect in vitro but does not significantly alter the free energy of stability. Like ΔF508, R553M may exert its effect on an intermediate formed during the process of folding. In both cases, the results indicate that the mutations are kinetic in nature but with opposite characteristics; R553M is a superfolder, whereas ΔF508 is an ineffective folder. Results in vivo indicate that the R553M/ΔF508 CFTR double mutant only partially corrects the ΔF508 maturation defect, and the fully mature mutant protein is only partially functional (16). The current results indicate that the diminished function of R553M observed in vivo is not due to a loss of the ability of NBD1 to bind ATP. Altered function may then be due to deficient catalysis or coupling between the nucleotide site and other parts of the protein. The fact that in the in vitro folding system the ATP binding function of the double mutant is normal and the suppression is complete, as opposed to the partial function and suppression in vivo (16), indicates that although NBD1 folding reliably reports the fundamental effects of the mutations the situation is more complex in the cell, and subtle secondary effects may not always be evident in the in vitro system. For example, the reduction of the folding yield by the ΔF508 mutation is not as pronounced as in vivo, perhaps indicating that cellular mechanisms for identifying malfolded conformers are more efficient than in vitro aggregation.

A second line of evidence that the ΔF508 folding defect is kinetic in nature is that addition of the native state ligand has no discernible effect on the folding yield or on the rate of formation of off pathway conformers (Fig. 7). Although not all of the binding energy contributes to the stabilization of the native state, as some is expended to affect the conformational change, the binary complex would be expected to be significantly more stable than the native state in the absence of nucleotide. The fact that the addition of ATP does not increase the folding yield is, thus, consistent with the notion that the intermediate affected by the mutation is kinetically isolated from the native state by a significant energy barrier during the process of folding.

The other CF-associated mutations tested here have no significant effects on either the temperature-dependent folding yield or the off pathway kinetics under our experimental conditions. The G551D mutation, which has no observable effect on the maturation of CFTR in vivo (11) but rather alters the

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**Fig. 6.** Glycerol affects the rate of formation of off pathway conformers. The formation of off pathway species of NBD1ΔF in the absence (dashed line) or presence (solid line) of 10% glycerol in buffer B was determined at 37 °C and 2 μM final protein concentration by turbidity at 400 nm. Data shown are an average of three experiments.

**Fig. 7.** Folding yield and formation of off pathway species of NBD1ΔF in the presence of ATP. A, the effects of ATP binding on the folding yield of NBD1ΔF were determined in vitro in the presence of 4 mM ATP in buffer B (▼). The data for temperature-sensitive folding of NBD1 (●) and NBD1ΔF (○) were previously published (5) and are presented here for comparison. B, formation of off pathway species at 37 °C was followed by turbidity at 400 nm. NBD1ΔF was present at 2 μM, and the ATP concentration was 4 mM (solid line). Data shown are an average of three experiments.
ability of the mature protein to function (7), has no effect on the ability of NBD1 to fold as would be expected. In addition, G551D does not significantly alter the binding affinity of NBD1-G551D to hydrolyze ATP (7). Similarly, the S549R mutation, which like ΔF508 inhibits the formation of mature functional CFTR in vivo (11), has no discernible effect on the ability of NBD1 to fold (Fig. 4). The S549R mutation may alter a surface important for interaction with other CFTR domains or other proteins during the process of conformational maturation.

We suggested previously that alcohols and other compounds that are known to stabilize protein and peptide structures might restore wild-type like folding to the ΔF508-CFTR (13, 14). In this regard, it has been demonstrated recently that such a compound, glycerol, corrects the defective maturation of ΔF508 in vivo (17, 18). In vitro, 10% glycerol inhibits the off pathway reaction and significantly enhances the temperature-dependent folding yield of ΔF508 NBD1. Glycerol does not significantly affect the free energy of stability of the native state. Therefore, its effects may be to stabilize the putative mutational sensitive intermediate or transition state proposed here, or to inhibit the off pathway association reaction of partially folded chains. Notably, this intermediate is not significantly populated under equilibrium conditions as the denaturation curves fit a two-state approximation. With these results in mind, our previous suggestion for therapeutic ligands should be modified to either the special case of compounds that lower a kinetic barrier by stabilizing a crucial transition or those that specifically interfere with the formation of off pathway species.

The results presented here indicate that mutational and environmental effects on the folding of NBD1 of CFTR in vitro in many ways parallel their effects on the folding and maturation of the full-length protein in vivo. As such, this study demonstrates that a defect in protein folding is the biochemical basis of CF. Thus, although commonly referred to as a trafficking defect, the ΔF508 mutation is primarily a protein folding defect with altered protein trafficking or kinesis secondary to altered folding. The distillation of the pathology of CF to a single soluble protein domain is a step toward generating a molecular description of the disease process. In addition, since most cases of CF are due to defective folding and maturation, this information is necessary for the logical design of novel therapeutics directed at ameliorating the disease in humans.

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