Alteration of the Cystic Fibrosis Transmembrane Conductance Regulator Folding Pathway

EFFECTS OF THE ΔF508 MUTATION ON THE THERMODYNAMIC STABILITY AND FOLDING YIELD OF NBD1*

Bao-He Qu and Philip J. Thomas‡

From the Department of Physiology, University of Texas Southwestern Medical Center, Dallas, Texas 75235-9040.

The cellular phenotype of the most common cystic fibrosis-causing mutation, deletion of phenylalanine 508 (ΔF508) in the amino-terminal nucleotide binding domain (NBD1) of the cystic fibrosis transmembrane conductance regulator (CFTR), is the inability of the mutant protein to fold and transit to the apical membrane (13). Second, when cells expressing the ΔF508 protein are grown at reduced temperature, the maturation defect is partially corrected (14). Along with the finding that the ΔF508 mutant protein is functional when it reaches the native state (13–15), this information suggests that correcting the maturation defect may ameliorate this form of the disease.

Recent studies suggest that the structural maturation defect in the ΔF508 mutant occurs at an early step in vivo (16, 17). In addition, these studies indicate that the ΔF508 mutation affects the rate of CFTR maturation. Moreover, CF mutations cluster in the NBDs (2, 3), and many CF mutations identified as maturation-defective (18), are located in NBD1, implicating defective folding of this domain in the vast majority of CF cases. To quantitatively investigate the effect of the ΔF508 mutation on the folding of this domain in greater detail, we have developed an in vitro folding system. In the present study, we use the system to examine the effects of this common CF-causing mutation on the thermodynamic stability and folding pathway of NBD1.

EXPERIMENTAL PROCEDURES

Construction of the Expression Vectors Containing CFTR NBD1 and NBD1ΔF cDNAs—Expression cassette polymerase chain reaction (EC-PCR) was employed to synthesize the cDNA fragments of CFTR NBD1 and NBD1ΔF containing a 5′ NdeI site, a 3′ XhoI site, and a stop codon. The sequence of the primers used to define and amplify NBD1 and NBD1ΔF from Gly-404 to Ser-589 are as follows: G404 primer, 5′-GAAGATCGGGCATATGGGATTTGGGGAATTATTTGAG-3′ (37 bases); S589 primer, 5′-GAATTCGGCCTGAGTATGCTCTACATATTTTTTCG-3′ (41 bases). The plasmids pBQ2-4 and pCOF508 containing CFTR cDNA were used as templates to amplify NBD1 and NBD1ΔF, respectively. Digested PCR products were ligated into the NdeI and XhoI sites of the pET28a plasmid (Novagen). Correct recombinants were identified by restriction digestion and sequenced to confirm the fidelity of the PCR step and formation of the proper construct.

Overexpression and Purification of NBD1 and NBD1ΔF Proteins—Single colonies of BL21 (DE3) Escherichia coli transformed with pET28a/NBD1 or pET28a/NBD1ΔF were used to inoculate 100 ml of LB media containing 30 μg/ml kanamycin and grown at 37 °C with vigorous shaking until the absorbance at 600 nm reached 0.6 unit. Cells were then induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside and incubated for an additional 3 h at which time the cells were harvested by centrifugation at 3,200 × g for 20 min at 4 °C. The cell pellet was resuspended in 40 ml of 20 mM Tris-HCl, pH 7.9, containing 500 mM NaCl, 5 mM imidazole (buffer A) and sonicated on ice three times for 30 s to break the cell wall and shear the DNA. The lysate was centrifuged at 15,000 × g for 30 min to collect the inclusion bodies and cellular debris. The pellet was then resuspended in 5 ml of buffer A containing 6 M GdnHCl and incubated on ice for 1 h to solubilize the proteins. The sample was centrifuged at 39,000 × g for 20 min to remove any remaining insoluble material. The supernatant was then applied to a Hi-5 Bind resin column (2.5 ml of resin per 100 ml of culture) that was previously charged with 50 mM NiSO4 and equilibrated with buffer A. The column was washed with 10 volumes of buffer A containing 6 M GdnHCl and 6 volumes of buffer B containing 20 mM imidazole and 6 M GdnHCl. Purified NBD1s were eluted with 6 volumes of buffer A containing 400 mM imidazole and 6 M GdnHCl. The eluate was dialyzed twice against 100 volumes of 100 mM Tris-HCl, pH 7.4, 2 mM EDTA for 12 h at 4 °C. Slow removal of the denaturant led to precipitation of the recombinant proteins. After dialysis, the samples were centrifuged at 16,000 × g for 20 min at 4 °C. The precipitated proteins were collected, lyophilized, and stored at −70 °C. Tricine SDS-PAGE
Mutational Effects on CFTR Folding

(10% polyacrylamide) was carried out as described previously (19). Gels were stained with Coomassie Brilliant Blue R-250.

Folding of NBD1 and NBD1ΔF—Purified CFTR NBD1 and NBD1ΔF were dissolved in 6 M GdnHCl. The samples were diluted 30-fold with ice-cold 100 mM Tris-HCl, pH 8.0, containing 400 mM L-arginine-HCl, 2 mM EDTA, and 1 mM dithiothreitol (buffer B) to a final protein concentration of 18 μM and incubated at 4 °C overnight. Protein concentration was determined by absorbance at 280 nm using a molar extinction coefficient of 13,490 M⁻¹ cm⁻¹ calculated from the amino acid sequence (20).

HPLC Gel Filtration Chromatography—Folded proteins were analyzed on a Macrochrom GP C 300 7 U gel filtration column that was equilibrated previously with 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 400 mM L-arginine buffer. Elution profiles were monitored at 280 nm using a flow rate of 0.5 ml/min. Molecular size markers used for gel filtration chromatography were blue dextran, bovine serum albumin, carbonic anhydrase, and aprotinin, corresponding to 2000, 69, 29, and 6.5 kDa, respectively.

TNP-ATP Binding—Nucleotide binding was determined using fluorescence enhancement of the ATP analog TNP-ATP upon binding to NBD1 and NBD1ΔF (13, 21, 22). Samples containing 0.9 μM NBD1 or NBD1ΔF in buffer B at the indicated concentration of TNP-ATP were excited at 410 nm with a 2 nm band pass. The emission was then measured at 555 nm (with a 4 nm band pass).

Unfolding of NBD1 and NBD1ΔF by GdnHCl and Heat—Samples containing 1.8 μM folded NBD1 or NBD1ΔF in 20 mM Tris-HCl, pH 8.0, were incubated at room temperature for 2 h in the indicated concentration of GdnHCl. Intrinsic tryptophan fluorescence emission spectra were collected using 282 nm exciting light. Thermal unfolding of NBD1 and NBD1ΔF was monitored by light scattering at 400 nm. Scattered light due to aggregation of unfolded protein in the absence of a chemical denaturant was measured at a 90° angle. The temperature of samples containing 0.9 μM NBD1 or NBD1ΔF in buffer B was ramped from 6 to 75 °C at a rate of 0.5 °C/min.

Temperature Dependence of NBD1 and NBD1ΔF Folding—For studies of the temperature dependence of folding, NBD1 and NBD1ΔF were dissolved in 6 M GdnHCl at the desired protein concentration. Unfolded proteins were diluted 30-fold with buffer B to final concentrations of 18 μM or 2 μM and incubated at the indicated temperature overnight. The protein samples were centrifuged at 16,000 × g for 10 min to remove insoluble, misfolded protein. Folded, soluble protein was determined from the intrinsic tryptophan fluorescence at room temperature. All protein was soluble and folded after dilution of GdnHCl at 4 °C.

Aggregation of NBD1 and NBD1ΔF—For the off-folding pathway studies, NBD1 and NBD1ΔF were solubilized in 6 M GdnHCl, then were diluted 30-fold with buffer B to a final protein concentration of 18 μM and incubated at 23 °C. Scattered light at 400 nm was measured at a 90° angle to monitor the aggregation process.

RESULTS AND DISCUSSION

NBD1 and NBD1ΔF Expression, Purification, and Folding in Vitro—A pET based expression vector capable of directing the expression of exons 9 through 12 of CFTR (Gly-404 to Ser-589) as a fusion with a polyhistidine sequence was constructed. A 1-liter culture of BL21 (DE3) E. coli transformed with the plasmid expressed 120 mg of NBD1 in 3 h in response to isopropyl-1-thio-β-D-galactopyranoside. This high rate of expression leads to the formation of insoluble NBD1 inclusions which can be isolated and dissolved in 6 M GdnHCl prior to further purification on a nickel chelate affinity column. The resulting denatured NBD1 is greater than 95% pure as assessed by laser densitometry of Coomassie-stained SDS-PAGE (Fig. 1A).

Denatured NBD1 and NBD1ΔF were folded into nucleotide binding monomers in vitro. The key to the success of the folding was the inclusion of L-arginine in the folding buffer (23, 24). Possibly, the guanidinium group of arginine increases the solubility of exposed polar side chains in the denatured NBD1 and the amphipathic character of the amino acid protects exposed hydrophobic interaction surfaces in the folded domains. In this regard, it is important to note that NBD1 has been removed from the context of the large multidomain membrane protein in which it normally resides. Thus, surfaces that are normally involved in domain-domain interactions in the intact protein, and which may be responsible for the tendency of NBD1 to form polymers (21) or interact with membranes in vitro (25), may be shielded by arginine. However, in the presence of 400 mM arginine, NBD1 can be folded and maintained as a soluble, functional monomer as assessed by intrinsic tryptophan fluorescence, size exclusion chromatography, and TNP-ATP binding (Fig. 1B–D).

Fluorescence emission spectra of the wild type and ΔF508 NBD1s reveal a pronounced blue shift in the peak position and an increase in fluorescence intensity upon removal of the chemical denaturant, consistent with burial of the single tryptophan at position 496 in a hydrophobic environment as the domain folds (Fig. 1B). Four additional lines of evidence argue that both NBD1s are folded. First, they are soluble in the absence of denaturant. Second, both elute from a molecular sizing column intermediate to carbonic anhydrase (29 kDa) and aprotinin (6.5 kDa), a position consistent with a globular monomer with a predicted molecular weight of 22,000 (Fig. 1C). Third, as has been described previously for expressed soluble NBD1 (15, 21), in vitro folded NBD1 and NBD1ΔF bind the nucleotide TNP-ATP (Fig. 1D). The apparent Kₐ of both NBD1s for TNP-ATP is 3 μM under these conditions in agreement with previous results (21). Finally, denaturation of the folded NBD1s is highly cooperative, indicating disruption of an ordered structure (Fig. 2A, inset).

Thermodynamic Stability of NBD1 and NBD1ΔF—Notably, the concentration of GdnHCl required to induce cooperative unfolding is similar for the two domains. The C₅₀ values (half-maximum denaturation concentration for unfolding) are 1.5 M for NBD1 and 1.3 M for NBD1ΔF (Fig. 2A). Comparison of the
thermodynamic stabilities of NBD1 and NBD1ΔF. The native structures of folded NBD1 and NBD1ΔF were denatured by addition of the chemical denaturant GdnHCl or by increasing the temperature. In the absence of GdnHCl, the denatured domains form insoluble associations that scatter 400 nm light. A, denaturation of NBD1 resulted in decreased tryptophan fluorescence and a red shift in the emission maximum as the single tryptophan, Trp-496, is exposed to solvent (Fig. 1B). B, the single tryptophan, Trp-496, is exposed to solvent (see Fig. 1B). 1.8 mM of wild type NBD1 (solid line) and NBD1ΔF (dashed line) in buffer B were incubated with GdnHCl at the indicated concentration for 2 h. The sample was excited at 282 nm and fluorescence emission peaks were collected. The equilibrium dependence of the fluorescence emission peak position on the denaturant concentration (inset) reveals cooperative unfolding of the domain. As this is a reversible process which approximates a two-state conversion, the free energy of denaturation (ΔG_D) can be calculated from the fraction folded over the transition region. Extrapolation to the absence of denaturant indicates that ΔG_D between NBD1 and NBD1ΔF is 1.1 kJ/mol. B, thermal denaturation of folded NBD1 and NBD1ΔF. NBD1 (0.9 μM, solid line) and NBD1ΔF (0.9 μM, dashed line) in buffer B were heated from 6 to 75 °C at a rate of 0.5 °C/min. Scattered light was measured at 400 nm at an angle of 90°.

Concentrations, the intramolecular interactions responsible for folding of NBD1 into soluble, functional, monomers predominate. At elevated temperatures and protein concentrations, intermolecular interactions occur that result in the formation of an insoluble conformer that scatters 400 nm light. Thus, at 2 μM final NBD1 concentration and 37 °C, 63% of the wild type polypeptide folds into the soluble conformation, while only 38% of the ΔF508 assumes the folded conformation. At 18 μM final polypeptide concentration and 25 °C, 29% of the wild type domain reaches the native state in contrast to 19% of the ΔF508 mutant.

Rate of Aggregation—When the rate of formation of the off-
pathway conformer is assessed at 18 μM final domain concentration and 23 °C, conditions under which the folding yield approximates the 30% efficiency observed in vivo (17), a lag phase followed by an increase in light scattering is observed. Significantly, the ΔF508 mutation decreases the length of the lag phase and increases the rate of change in light scattering indicating that the rate of formation of the off-pathway conformer is enhanced by the disease-causing mutation (Fig. 3B). However, the altered kinetics may not be due to a direct effect of the mutation on the rate constant for an off-pathway step. Rather, they may reflect an increased concentration of a folding intermediate which is prone to self-association at high concentration. In this regard, the cell the proteasome is responsible for degradation of both wild type and ΔF508 CFTR (26, 27). However, while proteolysis is inhibited in vivo, the efficiency of maturation is not enhanced as misfolded full-length CFTR simply aggregates into a detergent-insoluble form (26). Thus, in the cell, intermediate(s) on the folding pathway can meet three potential fates: they can continue to the native state, they can self-associate forming insoluble inclusions, or they can undergo proteolytic degradation. It is possible that all three processes are in kinetic competition for the same intermediate state substrates and that molecular chaperones may be responsible for presenting the intermediate(s) to one or all of these processes. It is important to note that the in vitro system described here is limited to the folding of the first nucleotide binding domain and cannot address the role of domain-domain interactions on the folding of the full-length protein.

Previous results demonstrated that a peptide fragment of NBD1, P67, containing the A consensus region and a region of homology around Phe-508, capable of binding ATP, is destabilized by the ΔF508 mutation (13). These results suggested that conditions counteracting the destabilizing effects may allow maturation of the mutant CFTR and rescue of the disease phenotype (13, 28). It was subsequently observed that reduction of the temperature at which expressing cells were grown increased the efficiency of CFTR maturation (14). Moreover, functional CFTR chloride channels could be observed in the plasma membrane at the sensitive temperature of 35 °C after growth at the permissive temperature of 26 °C. Thus, these findings are consistent with the current results which indicate that a step on the folding pathway, rather than the stability of the native state is affected by the mutation. Thus, destabilization of the peptide P67 by the ΔF508 mutation suggests it may provide a model of a kinetically trapped folding intermediate (28).

The fact that Phe-508 is critical for interactions that direct folding, but makes little contribution to the stability of the native state is not unique. For example, a large number of mutations of the P22 tail spike protein affect its folding but not its native state stability, indicating that non-native state interactions may be important for directing the folding pathway (29, 30). These mutations are, thus, temperature-sensitive for folding (tsf), as is ΔF508. Interestingly, global second site suppressor mutants of these tsf mutations have been isolated (30). It remains an intriguing possibility that intragenic suppressors of the ΔF508 phenotype (31, 32) may act by correcting the folding defect.

Understanding the interactions that take place on the CFTR folding pathway may have profound importance for describing the mechanisms of both the disease process and how primary sequence determines the final native structure of proteins. Therapies directed at correcting the folding defect deserve further consideration as treatments for cystic fibrosis. Potentially, alteration of molecular chaperone expression or of cellular conditions to increase the on-pathway rate or decrease the off-pathway rate may prove useful. However, it is important to remember that simply inhibiting the final proteolytic off-pathway step is apparently not adequate to correct the disease phenotype (26, 27) as might be expected if the steps prior to proteolysis were in equilibrium with the native state. More likely, positive impact on the disease state will require intervention at the initial off-pathway steps.

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