The Disassembly and Reassembly of Mutants of *Escherichia Coli* Heat-Labile Enterotoxin: Replacement of Proline 93 Does Not Abolish the Reassembly-Competent and Reassembly-Incompetent States†

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ABSTRACT: The carrier moiety of heat-labile enterotoxin of *Escherichia coli* (EtxB) is formed by the noncovalent association of identical monomeric subunits, which assemble, in vivo and in vitro, into exceptionally stable pentameric complexes. In vitro, acid disassembly followed by neutralization results in reassembly yields of between 20% and 60% depending on the identity of the salts present during the acid denaturation process. Loss of reassembly competence has been attributed to isomerization of the native *cis*-proline residue at position 93. To characterize this phenomenon further, two mutants of EtxB at proline 93 (P93G and P93A) were generated and purified. The proline variants reveal only minor differences in their biophysical and biochemical properties relative to wild-type protein, but major changes were observed in the kinetics of pentamer disassembly and reassembly. Additionally, a loss of assembly competence was observed following longer term acid treatment, which was even more marked than that of the wild-type protein. We present evidence that the loss of assembly competence of these mutants is best explained by a cis/trans peptidyl isomerization of the unfolded mutant subunits in acid conditions; this limited reassembly competence and the biophysical properties of the native P93 mutant pentamers imply the retention of the native cis conformation in the nonproline peptide bond between residues 92 and 93 in the mutated proteins.

Heat-labile enterotoxin (Etx)[1] from *Escherichia coli* is a heterooligomeric protein comprising one A subunit (EtxA, $M_r = 27\,000$) and five B subunits, (EtxB, $M_r = 11\,700$) (*1*−*3*). The toxic A subunit possesses ADP-ribosyltransferase activity, while the B pentamer acts as a carrier system and binds to ubiquitous G_{m1} ganglioside receptors on the surfaces of vertebrate epithelial cells (*4*−*6*). During biosynthesis, the monomeric B subunits translocate across the bacterial cytoplasmic membrane by means of the Sec pathway and, upon release in the periplasm, rapidly fold and assemble into stable pentamers even in the absence of the A subunit (*7*). Each B subunit is composed of 103 amino acids with a single disulfide bond (C9−C86), a single *cis*-prolyl residue (P93), and a single tryptophan (W88), which acts as a useful spectroscopic marker. The properties of Etx make it a good model protein for studying the rate-limiting steps in protein folding, disulfide bond formation, cis/trans prolyl isomerization, and homo- and heterooligomerization.

Acid-mediated disassembly (*8*) and subsequent reassembly of monomeric EtxB (*9*) to the native pentameric structure have been well characterized in vitro. Reassembly yields approaching 80% could only be achieved in "double-jump" experiments, where the protein was disassembled at pH 1.0 and then neutralized after a few seconds. We showed that longer exposure to strong acid conditions initiated a process with the kinetic and equilibrium properties of cis/trans prolyl isomerization, which limited reassembly yields of wild-type EtxB to 20% from KCl−HCl (*9*) or 60% from H_3PO_4 (*10*). To gain better insights into the proposed molecular basis of reassembly, we sought to study the refolding and assembly properties of EtxB without the complications of cis/trans isomerization by the use of Pro93 mutants. Such a system would allow the study of other cellular factors that may contribute to the folding and assembly process in vivo.

The proline residue at position 93 was mutated to a glycine and to an alanine. The two EtxB variants were expressed and purified from *Vibrio* sp 60 to levels comparable with that of the wild-type protein, and the purified proteins were analyzed for any structural, functional, or stability changes. The results of all these analyses are strongly indicative of the proline mutants having biochemical and biophysical properties closely analogous to wild-type EtxB. The disassembly and reassembly properties of the P93 mutants were then characterized. In denaturing conditions, both mutants showed a time-dependent loss of reassembly competence; the rates and yields of reassembly were lower than those observed for wild-type EtxB after denaturation in comparable conditions. Together, the results suggest that the mutated proteins are secreted by *Vibrio* sp 60 with a nonproline cis peptide bond preceding residue 93, that this isomerizes to the preferred trans configuration in acid denaturing conditions

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[1] Abbreviations: Etx, *E. coli* heat-labile enterotoxin; EtxA, A-subunit of *E. coli* heat-labile enterotoxin; EtxB, B-subunit of *E. coli* heat-labile enterotoxin; wt, wild-type.

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and that this isomerization restricts the reassembly competence of the mutant subunits.

EXPERIMENTAL PROCEDURES

All methods were as described previously (*10*) except where specified below.

Generation of Mutant Proteins. Mutants of EtxB were generated using the Sculptor Mutagenesis kit (Amersham Pharmacia Biotech) and the plasmid vector pMMB68 (*11*), which encodes for EtxB as a template. Plasmids were authenticated by sequencing. A rifampicin-resistant derivative of marine *Vibrio* sp 60 (*12*) was used as a host for the plasmids pMMB68, pLR17, and pLR20 expressing wild-type (wt) EtxB and the proline variants P93A and P93G, respectively.

Polyclonal Antibody Production. A polyclonal antibody was generated to provide a tool for the simultaneous monitoring of both the pentameric and monomeric species of EtxB and its proline variants in solution. A host rabbit was immunized with 0.1 mg of purified native EtxB, and after three months, a 1/1000 dilution of the final bleed produced a very strong signal; preliminary characterization showed that the antiserum recognized both monomer and pentamer EtxB and cross-reacted with both of the mutant species. Western blot and electrochemiluminescence (ECL) detection were performed using Immobilon P transfer membrane (Millipore) and ECL western blotting reagents (Amersham Pharmacia Biotech). The polyclonal antibody (LWR-1) was used in the G_{m1}-ELISA assay to detect both monomeric and pentameric species of EtxB. LWR-1 also specifically recognizes secreted EtxB pentamers in cell-free media and in periplasmic extracts of isopropyl-β-D-thiogalactopyranoside (IPTG)-induced *Vibrio* sp 60 expressing the pentamers.

RESULTS

Protein Production and Characterization. Both wild-type and P93G and P93A variants of EtxB were expressed to high levels in the media of a rifampicin-resistant derivative of *Vibrio* sp 60 expressing a pMMB68-based plasmid. The yields of purified protein were 8.5, 2.9, and 5.8 mg/L of culture for wild-type, P93A, and P93G proteins, respectively. The purity of the recombinant protein samples was confirmed by silver staining of SDS−polyacrylamide gels, where only bands corresponding to native pentameric EtxB were apparent with no visible contaminants. Analyses by electrospray mass spectrometry confirmed the molecular weights predicted for all three recombinant proteins and N-terminal sequencing confirmed the first six amino acids to be identical to those of the wt EtxB.

Far-UV circular dichroism was used to screen for possible changes in the secondary structure of the mutant forms of EtxB (see Figure 1). Small spectral shifts were seen for both the P93A and P93G mutants with a shift in the position at which the ellipticity is zero and an increase in molar ellipticity between 210 and 230 nm. As previously reported, there is a very significant underrepresentation of α-helix in the wild-type spectrum compared with that predicted from the crystal structure (*8, 13*), and hence, interpretation of the spectral shifts seen in the mutants is difficult, though both are closer to what would be expected from the crystal

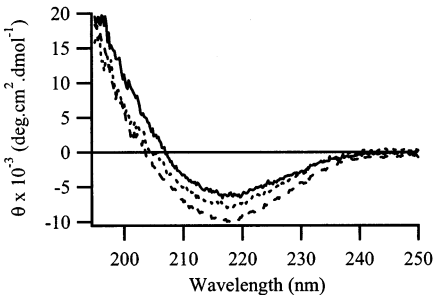

FIGURE 1: Far-UV CD spectra of wt, P93A, and P93G EtxB. Proteins were diluted in McIlvaine buffer, pH 7.0, and equilibrated at 25 °C prior to analysis. Final concentrations were determined by absorption at 280 nm. Each spectrum is the average of four scans. Solid line represents wt Etxb, dotted line represents P93A, and dashed line represents P93G.

structure of wild-type EtxB. However, the results do imply that both mutants contain significant amounts of regular secondary structure.

Sensitivity of the Mutant Proteins to Denaturation. Wild-type EtxB has been previously shown to exhibit a sharp pH threshold for disassembly. SDS−PAGE shows a transition from the protein running in its pentameric form (at an apparent molecular weight of 42 kDa) to its monomeric form (12 kDa) after exposure to 0.1 M KCl−HCl below pH 2.0 or to H_3PO_4 below pH 2.3 (*8, 10*). To compare their relative resistance to acid denaturation, samples of both mutants and wild-type hEtxB were incubated in acid buffers (H_3PO_4, pH 1.6−2.5, and McIlvaine buffer, pH 3.0−6.5), and the time and pH dependence of pentamer disassembly were determined directly by SDS−PAGE analyses. Following 15 min in 0.1 M H_3PO_4 at pH 2.0, the wild-type protein maintained its pentameric structure. At this pH however, the mutant proteins were unstable, and the threshold for pentameric disassembly was observed at the higher pH value of 2.5 (Figure 2A). Further analysis over the pH range 1.5−5.0 at incubation times of 5 min, 1 h, and 20 h revealed that under similar conditions in the pH range 2.2−3.0 the pentamers of both mutants were slightly more susceptible to disassembly than those of the wild-type protein with P93A relatively more unstable than P93G. However, following overnight acid treatment, all three proteins fully disassembled at pH values of 2.2 or below and were stable pentamers at pH 3.0 or above (data not shown).

A pH-dependent conformational change within the pentameric structure of EtxB has previously been demonstrated such that at pH 5.0 or below the pentamer becomes more susceptible to disassembly by denaturants such as the ionic detergent SDS or elevated temperatures (*13*). Analysis by SDS−PAGE revealed that both proline mutants of EtxB undergo this conformational change, becoming sensitive to SDS-mediated disassembly when subjected to mild acid treatment. However when compared with wild-type, where SDS-dependent disassembly was observed from pH 5.0 and below, the threshold pH for SDS-dependent disassembly of P93A and P93G was shifted by 0.5 units to pH 5.5 (Figure 2B) confirming the slightly lower stability of the mutant pentamers.

The pH-dependent conformational change in EtxB can also be monitored by fluorescence spectroscopy; the fluorescence intensity of wt EtxB decreases with decreasing pH with no change in λ_{max}, and this change fits to a single protonation

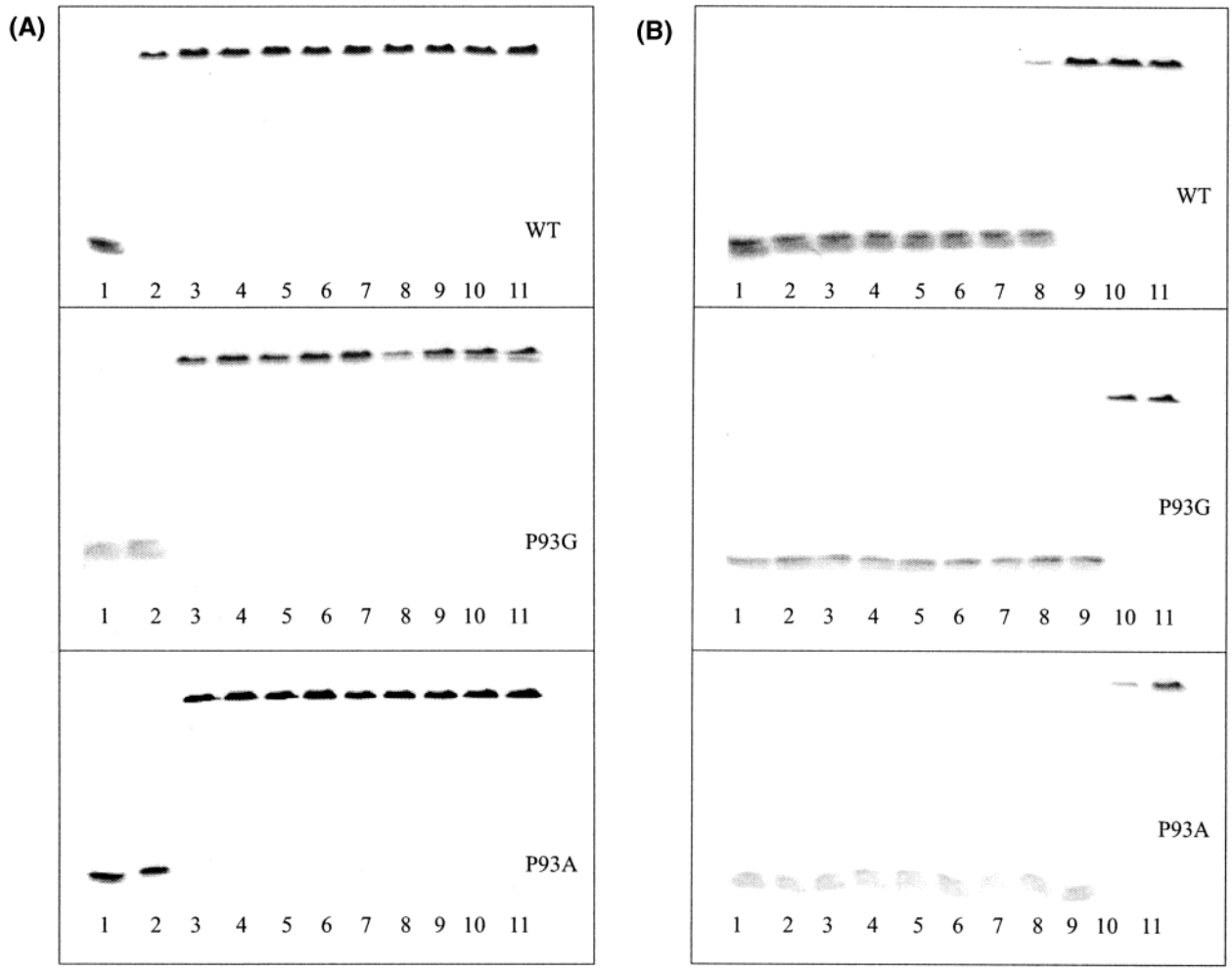

FIGURE 2: pH dependence of the stability of wild-type, P93A, and P93G EtxB. Samples were incubated for 15 min at ambient temperature. Representative gels from replicate experiments are shown. Panel A shows results in different pH buffers (0.1 M H_3PO_4, pH 1.6−2.5 and 0.1 M McIlvaine, pH 3.0−6.5): sample 1, pH 1.6; sample 2, pH 2.0; sample 3, pH 2.5; sample 4, pH 3.0; sample 5, pH 3.5; sample 6, pH 4.0; sample 7, pH 4.5; sample 8, pH 5.0; sample 9, pH 5.5; sample 10, pH 6.0; sample 11, pH 6.5. Panel B shows results in different pH buffers (same samples as those in panel A) in the presence of 1% SDS. The pH dependence of pentameric disassembly was determined by SDS−PAGE analyses after subsequent neutralization.

event with a pK_a of 5.1 (*13*). At neutral pH, the fluorescence intensity at λ_{max} was lower in both mutants than in wt EtxB. The fluoresence data reflect the environment of the single tryptophan residue at position 88. Because Trp88 is spatially positioned close to Pro93, any small changes in the microenvironment around residue 93 will also result in changes in the microenvironment of Trp88 and hence changes in fluorescence. A pH-dependent decrease in fluorescence intensity with no change in λ_{max} was observed for all three proteins (data not shown). The midpoint of the transition for the wild-type protein was at pH 5.1 as previously observed; the mutant proteins showed midpoint values of 5.5 for P93A and 5.2 for P93G; however, the transitions for both P93A and P93G appear to be more complex than that for wild-type protein. The conformational change reported for wild-type EtxB has been linked to the protonation state of His57 (*13*), which is spatially close to Pro93. Hence mutation of Pro93 and minor changes in the microenvironment around residue 93 could be expected to result in alterations in the microenvironment of His57; the observed shifts in the pH dependence of fluorescence for both mutants could reflect this alteration.

Ligand Binding Properties of the Mutant Proteins. It has been reported that the G_{m1} binding site of EtxB is located near the subunit−subunit interfaces of the assembled pentamer and that only native pentamers will bind to G_{m1} (*7*). The binding properties of the mutant proteins were compared with those of wild-type protein and analyzed by G_{m1}-ELISA. Initially, the standard anti-EtxB monoclonal antibody (mAb118-87) was employed to detect bound EtxB; a plot of the resulting data revealed that the mutant proteins appeared to have a greater affinity for the ligand than the wild-type protein (Figure 3A). The G_{m1} binding site of EtxB is comprised of E51, Q56, H57, Q61, W88, N90, and K91 (*14*). Because the alanine and glycine substitutions at position 93 are in very close proximity to the binding site, an altered binding affinity of the mutant pentamers would not be surprising. However, the difference observed could also be due to a difference in recognition of the mutant pentamers by the monoclonal antibody 118-87. In an attempt to distinguish between these possibilities, a polyclonal antibody (pAb LWR-1) was generated and substituted for the monoclonal antibody (mAb118-87) in the G_{m1}-ELISA assay. Again the mutant proteins showed a greater affinity for the

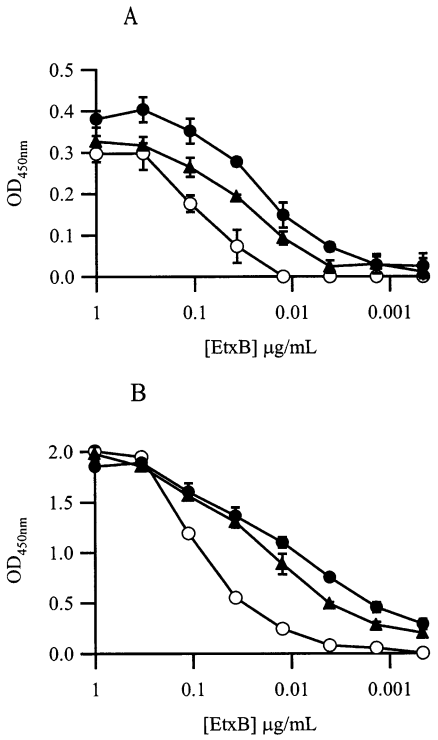

FIGURE 3: Comparison of the binding properties of native EtxB and mutants P93A and P93G to immobilized G_{m1}. Samples of wild-type EtxB (○), P93G (●), and P93A (▲) at 1 μg/mL were serially diluted 3-fold in PBS containing 0.1% bovine serum albumen. The concentration of bound protein was determined in triplicate by (A) monoclonal antibody 118-87 and (B) polyclonal antibody LWR-1.

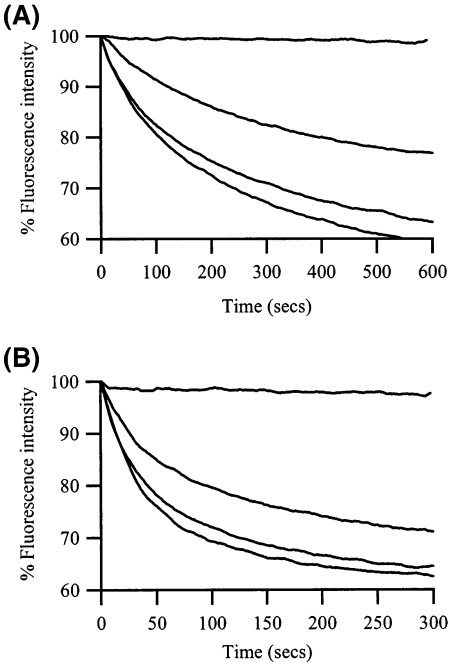

FIGURE 4: Fluorescence analysis of the acid-induced denaturation of the P93G and P93A mutants of EtxB as a function of pH. The proteins were denatured in 0.1 M phosphate buffers at 20 °C, and fluorescence was monitored with excitation at 280 nm, emission at 350 nm, and 5 nm band-pass slits. Panel A shows results for EtxB P93G; panel B shows results for EtxB P93A. From top to bottom, the traces represent pH 3.0, 2.0, 1.8, and 1.6.

ganglioside ligand (Figure 3B). Hence the observations are independent of the antibody used for detection and suggest that the substitution of proline by alanine and glycine results in assembled pentamers that have a slightly higher binding affinity for G_{m1} than the wild-type protein.

Kinetics of Mutant Pentamer Disassembly. The time dependence of disassembly of P93A and P93G EtxB in H_3PO_4 over the pH range of 1.6−3.0 was determined by monitoring changes in the intrinsic fluorescence of the single tryptophan (W88) in the protein. At 20 °C and pH 3.0, no time-dependent changes were observed. However, between pH 1.6 and pH 2.0, a time-dependent decrease in fluorescence intensity was observed (Figure 4). The denaturation process in H_3PO_4 and KCl−HCl at pH 1.8 was examined in greater detail (Figure 5). The best fit for the denaturation process of P93A and P93G at pH 1.8 in both KCl−HCl and H_3PO_4 was to a two-step sequential process, which gave effectively random residuals. We have previously shown that denaturation of wt EtxB in the H_3PO_4 system was also via a biphasic process, but the rate at which denaturation of the wild-type protein occurred in the KCl−HCl system was too rapid to allow distinct phases to be resolved (*8, 10*). For the mutants in H_3PO_4, the rate constants for phase 1 were 2.38 ± 0.01 and 1.49 ± 0.01 min^{-1} for P93A and P93G, respectively. This phase was followed by a relatively slower second event with rate constants of 0.270 ± 0.001 (P93A) and 0.196 ± 0.001 min^{-1} (P93G).

To identify which of the events detected by fluorescence represents the process of pentamer disassembly, samples were incubated in H_3PO_4, pH 1.8, at 20 °C, and the rates of pentamer disassembly were determined by G_{m1}-ELISA.

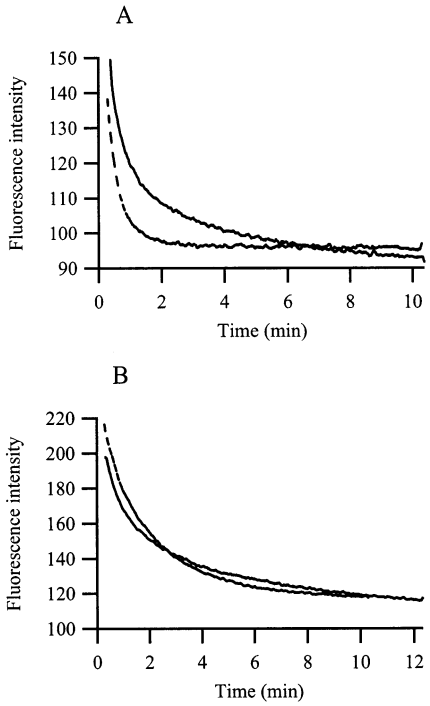

FIGURE 5: Fluorescence kinetics of denaturation at pH 1.8, 20 °C: (A) P93A; (B) P93G. Solid line is in H_3PO_4, while the dashed line is in KCl−HCl. In both cases, the best fit for the denaturation process for the mutated proteins was to a biphasic event (P93A, H_3PO_4, 2.38 ± 0.01 and 0.270 ± 0.001 min^{-1}; P93A, KCl−HCl, 3.20 ± 0.02 and 0.834 ± 0.011 min^{-1}; P93G, H_3PO_4, 1.49 ± 0.01 and 0.196 ± 0.001 min^{-1}; P93G, KCl−HCl, 1.69 ± 0.01 and 0.431 ± 0.001 min^{-1}).

(Figure 6). The rate constant for disassembly of P93A was 4.496 ± 0.087 min^{-1} ($t_{1/2}$ = 0.15 min) and that of P93G

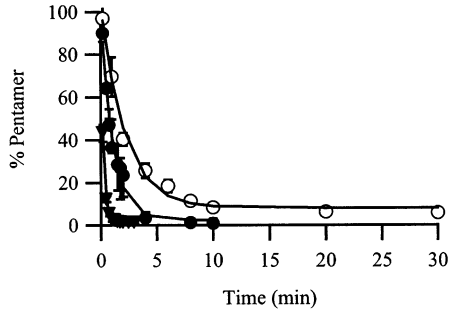

FIGURE 6: Kinetics of disassembly of native EtxB and mutants P93G and P93A in H_3PO_4 at pH 1.8. Samples of wild-type EtxB (○), P93G (●), and P93A (▲) were acid-incubated at 20 °C, and aliquots were diluted into neutralizing buffer at selected time points. The amount of pentamer remaining at each time point was determined in triplicate by G_{m1}-ELISA.

was 0.939 ± 0.107 min^{-1} ($t_{1/2}$ = 0.74 min). These values are in reasonable agreement with the rate constants from the fluorescence data for phase 1 (P93A $t_{1/2}$ = 0.29 min and P93G $t_{1/2}$ = 0.47 min) and suggest that, as for the wild-type protein, the overall process detected by fluorescence comprises disassembly of the mutant pentamers followed by a conformational change in the mutant subunits. All of the rate constants for disassembly/unfolding are considerably greater for the mutant species than for the wild-type one, implying that the mutants are destabilized relative to the wild-type protein. However, the relative magnitudes of the constants for the two phases in the mutants are in accord with those previously determined for the wild-type protein in H_3PO_4, pH 1.8, at 20 °C, initial rapid pentamer disassembly (k = 0.090 ± 0.009 min^{-1}) and subsequent monomer unfolding (k = 0.019 ± 0.004 min^{-1}; *10*). We therefore conclude that strong acid-induced disassembly and unfolding of the proline mutants occurs in a manner similar to that of the wild-type protein.

Reassembly Properties of Mutant Monomers. We have previously demonstrated that the yield of pentamer upon reassembly of wt EtxB is dependent on the conditions used for disassembly; except for very short periods of acid exposure, yields of less than 20% are obtained from KCl−HCl but ca. 60% from H_3PO_4. We have ascribed the loss of reassembly competence to cis/trans isomerization of Pro93 in the denatured state and inferred that the position of the cis/trans equilibrium is sensitive to the acid denaturation conditions (*9, 10*). To ascertain whether similar effects would be obtained with the mutants, we examined the reassembly competence of both P93A and P93G after long periods of acid denaturation. Samples were denatured at pH 1.8, and aliquots were removed, checked for denaturation, and renatured. When analyzed by SDS−PAGE, reassembly of P93A from both KCl−HCl and H_3PO_4 denaturation systems was severely limited and gave very low yields of pentamer (Figure 7A). The final yield of P93G pentamer reassembly was greater than that for P93A but substantially lower than that obtained for wt EtxB (Figure 7A). These results show that the substitution of P93 has not generated mutants that reassemble readily, in contrast to what would be expected if the mutations had abolished all complexities due to cis/trans isomerization. To further investigate the reassembly process of the mutant forms of EtxB, the reassembly kinetics of P93A and P93G from both the KCl−HCl and H_3PO_4 denaturation

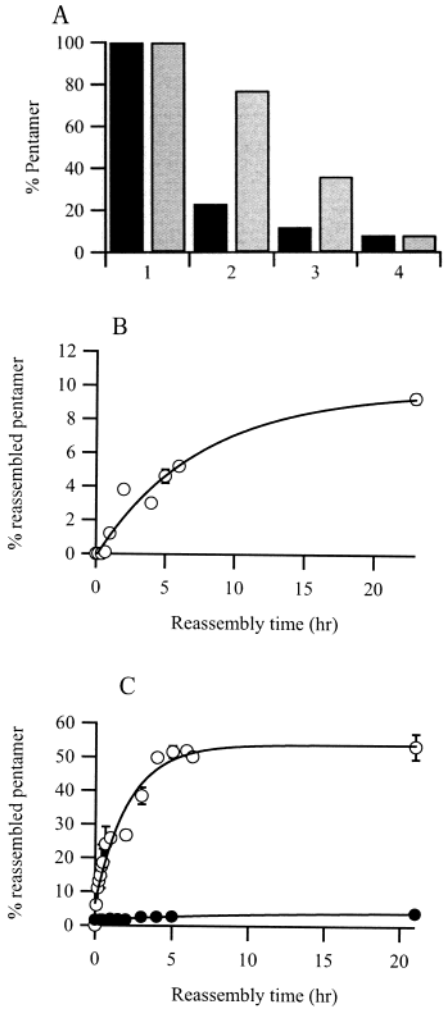

FIGURE 7: Analysis of reassembly competence. Panel A shows reassembly competence following acid denaturation at pH 1.7 in KCl−HCl (black) and H_3PO_4 (grey): sample 1, control; sample 2, wild-type EtxB; sample 3, P93G EtxB; sample 4, P93A EtxB. Fully denatured samples were diluted in McIlvaine buffer, pH 7.5, and allowed to reassemble for 2 h at ambient temperature. Analyses were by SDS−PAGE with the percentage of reassembled pentamer being determined by densitometric scanning of the polyacrylamide gels. Panel B shows time dependence of reassembly of P93G (○) fully denatured in KCl−HCl, and panel C shows that of P93A (●) and P93G (○) in H_3PO_4 at pH 1.8 and 25 °C. Samples were diluted into neutralizing buffer and allowed to reassemble for 21 h. At regular time points, aliquots were removed and the amount of reassembled pentamer was determined in triplicate by G_{m1}-ELISA.

systems were examined by G_{m1}-ELISA (Figure 7B,C). The reassembly process from H_3PO_4 was extremely slow for P93A, occurring with a rate constant of 0.124 ± 0.032 h^{-1} ($t_{1/2}$ = 5.6 h) and with a maximum pentamer yield of only 4%, while the maximum yield for P93G pentamer reassembly was 55% with a rate constant of 0.464 ± 0.093 h^{-1} ($t_{1/2}$ = 1.5 h). This is close to the 60% reassembled pentamer yield observed for wild-type protein, but the reassembly of the mutant is much slower since half-time for reassembly of the wt protein under these conditions was only 6.2 min. From the KCl−HCl system, no reassembly of the P93A mutant could be observed and P93G reassembly was severely limited with a maximum yield of 9% ($t_{1/2}$ = 5.3 h, cf. 18% and $t_{1/2}$ = 0.14 h for wt EtxB).

Thus, after exposure to acid denaturing conditions, the mutants of EtxB generate a mix of reassembly-competent

Table 1: The Change in Gibbs Free Energy Associated with Cis/Trans Isomerization for Native and Mutant EtxB and $\Delta\Delta G$ Values Relative to wt EtxB[a]

	KCl−HCl				H_3PO_4			
	cis/trans	K_{eq}	ΔG (kJ/mol/M)	$\Delta\Delta G$ (kJ/mol/M)	cis/trans	K_{eq}	ΔG (kJ/mol/M)	$\Delta\Delta G$ (kJ/mol/M)
wt	0.16:0.84	0.19	4.1		0.64:0.36	1.8	−1.4	
P93A	<0.02:>0.98	0.02	>9.7	>5.6	0.04:0.96	0.04	8.0	9.4
P93G	0.09:0.91	0.10	5.7	1.6	0.54:0.46	1.17	−0.39	1.0

[a] The values were obtained from pseudo-equilibrium constants converted by the equation $\Delta G = -RT \ln K_{eq}$, where $R = 8.314$ J K^{-1} mol^{-1} and T = temperature in K. No reassembly was seen for P93A from KCl−HCl; however, it is known that reassembly of wild-type protein does not occur below 2 μg/mL (*9*); hence, all that can be said is that <2% of the 100 μg/mL reassembly mix is in the cis form.

and reassembly-defective subunits, as for the wild-type protein. However, reassembly is much less successful for the mutants compared to the wild-type protein with marked effects both on yields and rates.

DISCUSSION

Conformation of the Native Mutant Pentamers. The mutant forms of EtxB were expressed to high levels and could be purified in yields comparable to that for the wild-type protein. By spectroscopic, biophysical, and immunological methods, we have been unable to detect any major differences in the biophysical or biochemical properties of wt EtxB and its P93A and P93G variants. All of the methods used revealed only moderate differences, all of which were consistent with the suggestion that substitution of Pro93 by alanine or glycine results in a local minor conformational alteration. We conclude that the mutants are well-folded, stable proteins and that the mutations have not introduced any global structural changes.

Disassembly and Reassembly Properties of the Mutants. The most obvious difference between the Pro93 mutants and the wild-type EtxB was in their kinetics of disassembly/unfolding and kinetics and yields of refolding/reassembly. The mutants showed the same two-phase behavior as the wild-type protein for disassembly/unfolding but with greatly increased rate constants. Conversely, the rate constants for reassembly of the mutants were much lower than those for the wild-type protein. The yields of reassembly were also lower than that for the wild-type protein under comparable conditions, especially for P93A. Independent of the protein, the proportion of reassembly-competent species is greater in H_3PO_4 than in HCl−KCl; independent of the denaturing conditions, the proportion of reassembly-competent species decreases in the order wild-type protein > P93G > P93A.

Additional differences were observed in disassembly behavior between the mutants and the wild-type protein; the pH threshold for acid denaturation was slightly higher (pH 2.2 rather than 2.0), as was the pH threshold for SDS-indcued denaturation (pH 5.5 rather than 5.0). None of these studies of disassembly was carried out under conditions of thermodynamic equilibrium, but all of these observations imply that the mutations slightly destabilize the native structure.

We had previously suggested that the low yield of reassembled pentamer for wild-type EtxB following extended periods in acid and the absence of a "slow" reassembly process indicated the formation of unfolded monomeric species with a trans configuration at position 93, which are assembly-defective; they misfold upon neutralization to a state that prevents isomerization and subsequent assembly (*9*). This suggestion has been substantiated by our studies of wild-type EtxB reassembled from various denaturing systems (*10*). If the native state of the Pro93 mutant forms of EtxB studied here were to include a trans peptide bond preceding residue 93, the disassembly/unfolding of the mutants in acid conditions would yield only trans species, would not lead to a peptidyl isomerization, and would be expected to generate only reassembly-competent subunits. The time-dependent irreversibility of acid denaturation attributed to a prolyl cis to trans isomeration should not be observed in the absence of a cis bond and a putative trans conformation at position 93 in the native state would be expected to result in higher yields of reassembled material. This, however, was not observed. Our experimental results indicate that, with increasing time in strong acid denaturing conditions, disassembly of P93A and P93G also leads to an equilibrium where the majority of the protein enters a state from which disassembly is irreversible. The reassembly behavior of the variants is similar to that of wild-type protein and can be explained on the basis of the isomerization model for wild-type reassembly where the native states of the mutants retain the cis configuration found in the wild-type protein and pentamer yield is restricted by cis/trans isomerization in the denatured state.

The Cis/Trans Equilibrium. Under denaturing conditions, an apparent equilibrium is established between the cis and trans states of monomeric EtxB, which results in the generation of assembly-competent (cis) and assembly-incompetent (trans) forms. We have shown that this equilibrium is altered by the salts present in the denaturation buffer (*10*), and the data above suggest that this equilibrium can also be shifted by mutagenesis of proline 93 to alanine or glycine.

Equilibrium constants can be calculated from the concentrations of reaction components at equilibrium; thus ΔG between the cis and trans states under different denaturing conditions can be calculated for both wild-type and mutant proteins and a value for $\Delta\Delta G$ can be determined. The method of detecting the cis/trans equilibrium is not a direct measurement; rather, the yield of reassembled pentamers is quantified by G_{m1}-ELISA to give a measure of the proportion of assembly-competent (cis) monomers and by inference the proportion of assembly-incompetent (trans) monomers. From these equilibrium values, pseudo-K_{eq} have been calculated so that the change in Gibbs free energy associated with cis/trans isomerization for each protein species could be calculated and the $\Delta\Delta G$ for the proline variants relative to wild-type protein determined (Table 1). Given the indirect method for determining equilibrium concentrations, the

absolute values for ΔG should be treated with caution, but the methodology does provide a way of calculating relative values thereby allowing the determination of $\Delta\Delta G$ values for different denaturation systems or for different proteins in the same system (Table 1).

For wild-type EtxB, the values for ΔG are considerably lower in H_3PO_4 than in KCl−HCl, consistent with the shift in equilibrium toward the assembly-competent cis state in H_3PO_4. The $\Delta\Delta G$ value between the two denaturing systems is 5.55 kJ mol^{-1}; hence, phosphoric acid appears to stabilize the cis denatured conformer by 5.55 kJ mol^{-1} relative to HCl−KCl. When compared to the wild-type protein, there is only a minor shift in the cis/trans equilibrium toward trans for P93G with differences in ΔG of 1.59 and 1.04 kJ mol^{-1} in KCl−HCl and H_3PO_4, respectively. For P93G, the $\Delta\Delta G$ value between the two different denaturation conditions is 6.1 kJ/mol.

In contrast to the P93G mutant, the cis/trans equilibrium for P93A mutant strongly favors the trans state. The $\Delta\Delta G$ value between P93A and wt EtxB in H_3PO_4 is 9.41 kJ mol^{-1}. For KCl−HCl, the value for $\Delta\Delta G$ cannot be determined for P93A but must be >5.58 kJ mol^{-1} (Table 1). These larger values for the P93A mutant are consistent with the cis configuration being very unfavored for the alanine mutant in the denatured state. The glycine mutant is more energetically favored than the alanine mutant to form the cis state, probably due to the lower steric repulsion resulting from its smaller side chain. These destabilizations of the cis state in the mutants are comparable to the reported destabilization by 11.3 kJ mol^{-1} relative to wt protein for a nonproline cis P93A mutant of ribonuclease A (*15*). However, these reported values were for the native state of ribonuclease A, whereas the values reported here are for differences in the "denatured" state.

In folded proteins, cis isomers not only occur at peptide bonds preceding proline residues but have also been found at other peptide bonds. Nonprolyl cis peptide bonds are rare however because the trans state is energetically favored 100−1000-fold over cis (*16*, *17*) whereas, in contrast, the generally accepted prolyl cis/trans equilibrium distribution for unfolded proteins is 0.2:0.8. Although nonprolyl cis peptide bonds are rare, there is growing evidence for their existence in the literature. In a nonredundant set of 571 proteins from the Brookhaven Protein Data Base, a total of 43 nonproline cis peptide bonds have been identified, and it has been suggested that, using higher resolution structure determinations, these bonds will be found to exist in greater abundance than previously thought (*18*). Most of the known nonprolyl cis bonds have been found to occur in functionally important regions close to the active sites of proteins, and some have been shown to play significant roles in positioning crucial residues to carry out ligand binding or catalysis or both, for example, in α-like scorpion toxins (*19*), bacterial luciferase (which has a cis prolyl bond and a conserved cis alanine or cis glycine, *20*), and human cellular factor XIII zymogen, which contains two nonprolyl cis peptide bonds (*21*).

Our findings suggest that the substitutions of alanine or glycine for Pro93 have not in fact resulted in a trans peptide bond between amino acids 92 and 93 in the assembled pentamer but rather that the cis peptide bond has been maintained in both the P93G and P93A mutants. This is not unprecedented; there are other examples in the literature of proline substitutions in which the native cis peptide bond has been retained with the nonproline cis bond having only a small destabilizing effect on the recombinantly expressed protein. When the cis proline residue of carbonic anhydrase is replaced by alanine, the native cis conformation is retained with the mutant about 5 kcal/mol less stable than the wild-type protein (*22*). Similar results have been reported for the P93A mutant of ribonuclease A, which is destabilized by 2.7 kcal/mol relative to the wild-type protein (*15*). A strong destabilization of about 5 kcal/mol for the P39A mutant of ribonuclease T1 has also been reported, but it is not clear whether the mutated protein still contained the peptide bond in the cis conformation (*23*).

Both proline varients of hEtxB were expressed and secreted as assembled pentamers by *Vibrio* sp 60 with yields of up to 5.8 mg/L (68% of wt EtxB). In vivo, all peptide bonds are believed to be synthesized in the same conformation, that is, trans. If the bond between residues 92 and 93 in the assembled pentamers of the mutated proteins is present in the cis form, as our findings would suggest, then presumably adoption of the cis conformation is prerequisite for the efficient production and secretion of these recombinant proteins in *Vibrio* sp 60 and a peptidyl isomerase or other factor must exist in vivo to successfully effect this isomerization. The identity of any factor or factors involved in effecting isomerization of nonprolyl peptide bonds has yet to be determined, but the Pro93 mutants of EtxB reported here may be a useful reporter system for the identification of such factors.

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