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Spectroscopic Characterization of Two Soluble Transducers from the Archaeon *Halobacterium salinarum*

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In the present study, structural aspects of the two soluble transducers, HtrX and HtrXI, from the archaeon *H. salinarum* have been examined using UV circular dichroism and steady-state fluorescence spectroscopies. Circular dichroism (CD) data indicate that both HtrX and HtrXI exhibit salt-dependent protein folding. Under low-ionic-strength conditions (0.2 M NaCl or KCl) the CD spectra of HtrXI is similar to that of the Gdn-HCl- or urea-denatured forms and is indicative of random coil structure. In contrast, the CD spectrum of HtrX under low-ionic-strength conditions contains roughly 85% α-helical character, indicating a significant degree of folding. Addition of NaCl or KCl to solutions of HtrX or HtrXI results in CD features consistent with predominately α-helical character (>95%) for both proteins. In addition, the transition points (i.e., ionic strengths at which the protein converts from random coil to α-helical character) are quite distinct and dependent upon the type of salt present (i.e., either NaCl or KCl). Accessibility of tryptophan residues to the solvent was also examined for both HtrX and HtrXI in both folded and unfolded states using KI quenching. The Stern–Volmer constants obtained suggest that the tryptophans (Trp35 in HtrX and both Trp47 and Trp74 in HtrXI) are partially exposed to the solvent, indicating that they are located near the surface of the protein in all three cases. Furthermore, fluorescence quenching with the single Trp mutants Trp74AIa and Trp47AIa of HtrXI indicates different environments for these two residues.

KEY WORDS: Protein folding; chemotaxis; *H. salinarum;* circular dichroism.

1. INTRODUCTION

Of critical importance to cellular function is the ability to sense the environment surrounding the ell. Cells must continually sense this changing environment, interpret the sensation, and adapt to the new surroundings. In multicellular organisms, sensing is accomplished using specialized sense organs as well as complex mechanisms to communicate this information to other parts of the organism. The complexity of sensing and signal transduction in higher organisms makes studies of bacterial systems very attractive. The Archaeon *Halobacterium salinarum* provides an excellent system to study the mechanisms of signal sensing and transduction since these microorganisms not only sense light intensity and color (phototaxis), oxygen tension (aerotaxis), and chemical gradients (chemotaxis), but also constitute a third kingdom more closely related to eukaryotes than to bacteria.

Recently, we and others identified a 13-member transducer family in *H. salinarum* (Zhang *et al.,* 1996; Rudolph *et al.,* 1996). Analysis of the nucleotide sequences of these genes indicated that there are three distinct subfamilies: (A) eubacterial type transducer proteins (HtrII, HtrIII, HtrIV, HtrV, and HtrVI); (B) membrane-bound transducers lacking a periplasmic domain (HtrI, HtrVII, and HtrVIII); and (C) cytoplasmic (soluble) transducer proteins (HtrIX, HtrX, HtrXI, HtrXII, and HtrXIII).

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[5] Abbreviations: CD, circular dichroism; Trp, tryptophan; HtrX, Halobacterial transducer X; HtrXI, Halobacterial transducer XI; Arg, arginine.

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Members of subfamilies B and C do not have a periplasmic domain. We have identified the function of one of the soluble transducer HtrXI as a chemotransducer for Asp, Glu, and His (Brooun *et al.*, 1997). HtrXI senses Asp, Glu, and His without having a periplasmic receptor domain. It then transmits this information to the signaling domain without the intervention of transmembrane helices. Its methylation level changes, as expected, upon stimulation with those attractants. HtrXI also is unusual among prokaryotic chemosensors in being a soluble, cytoplasmic protein.

Of key interest is the mechanism by which the soluble transducers recognize ligands and transmit corresponding signals. Recent reports of a modified eubacterial Asp transducer (Tar) in which the transmembrane domain was eliminated indicate that the membrane domain may not be required for signal transduction (Ottemann and Koshland, 1997). We proposed that the N-terminal domain of the soluble transducers may contain a ligand-binding domain allowing these proteins to detect the ligand and transmit the binding information to the methylation domain of the protein (Brooun *et al.*, 1997). A first step in determining the mechanism of signal transduction in these proteins is to characterize structural aspects of the transducers. With this in mind we have begun an examination of the equilibrium structures of the two soluble transducers HtrX and HtrXI using circular dichroism and fluorescence spectroscopies. These studies will provide a foundation from which structure/function studies can performed to elucidate the signal transduction mechanism in these unique proteins.

2. MATERIALS AND METHODS

2.1. Chemicals and Electrophoresis Reagents

All chemicals were reagent grade unless specified. Urea and guanidinium HCl were purchased from Sigma (St. Louis, MO).

2.2. Determination of Protein Concentration

Protein concentration was determined using the BCA Protein Assay Reagent kit (Pierce, IL) according to the manufacturer's protocol.

2.3. Expression and Purification of Recombinant HtrX/XI in *E. coli*

Wild-type and mutated HtrXI (containing the W47A or W74A substitutions) were expressed under control of the T7 promoter using the pET expression system in BL21(DE3)pLysS *E. coli* host cells. Fast-flow perfusion chromatography was chosen for purification of the recombinant protein. A detailed description of the expression and purification of HtrX and HtrXI will be published elsewhere (Chen *et al.*, n.d.).

2.4. Optical Spectra

Circular dichroism spectra were obtained using a Jasco J600 spectrometer. Samples were placed in a 2-mm-pathlength quartz optical cuvette. Spectra are the average of three scans. Ionic strength dependence was determined by preparing protein solutions containing various concentrations of either NaCl or KCl. Protein solutions typically contained 30 μM protein in 10 mM potassium phosphate buffer (pH 6.5). Fits to the CD spectra were obtained using the K2d program (Andrade *et al.*, 1993). This program estimates the percentages of secondary structure from UV circular dichroism spectra using a Kohonen neural network algorithm.

Corresponding fluorescence spectra were obtained using an ISS K2 spectrofluorimeter equipped with a 300-W Xe arc lamp with 295-nm excitation wavelength. Integrated intensity was recorded by exciting at 295 nm and observing the emission through a Schott WG 320 filter. Potassium iodide quenching was performed by titrating KI from a 4.0 M stock solution (in the appropriate buffer/salt solution) into a quartz optical cuvette. (1 cm path length) containing approximately 10 μM protein. The corresponding integrated intensity was obtained and Stern–Volmer plots were constructed after correction of intensity data for dilution.

3. RESULTS AND DISCUSSION

3.1. Secondary Structure of Soluble Transducers HtrX and HtrXI

The CD spectra of HtrX and HtrXI under low- and high-ionic-strength conditions are displayed in Fig. 1. The corresponding spectral fits are displayed as open circles overlaid on each plot. In the case of HtrXI the fits predict ~40% random coil, ~30% β-sheet, and ~30% α-helical character under low-ionic-strength conditions, while at higher ionic strengths the prediction is for greater than ~90% α-helical character. In contrast, the predictions for HtrX give 85% α-helical and 15% random coil for the low-ionic-strength protein and greater than 95% α-helical character under high-ionic-strength conditions.

The effects of ionic strength on the CD spectra of both HtrX and HtrXI are shown in Fig. 2. The left panel

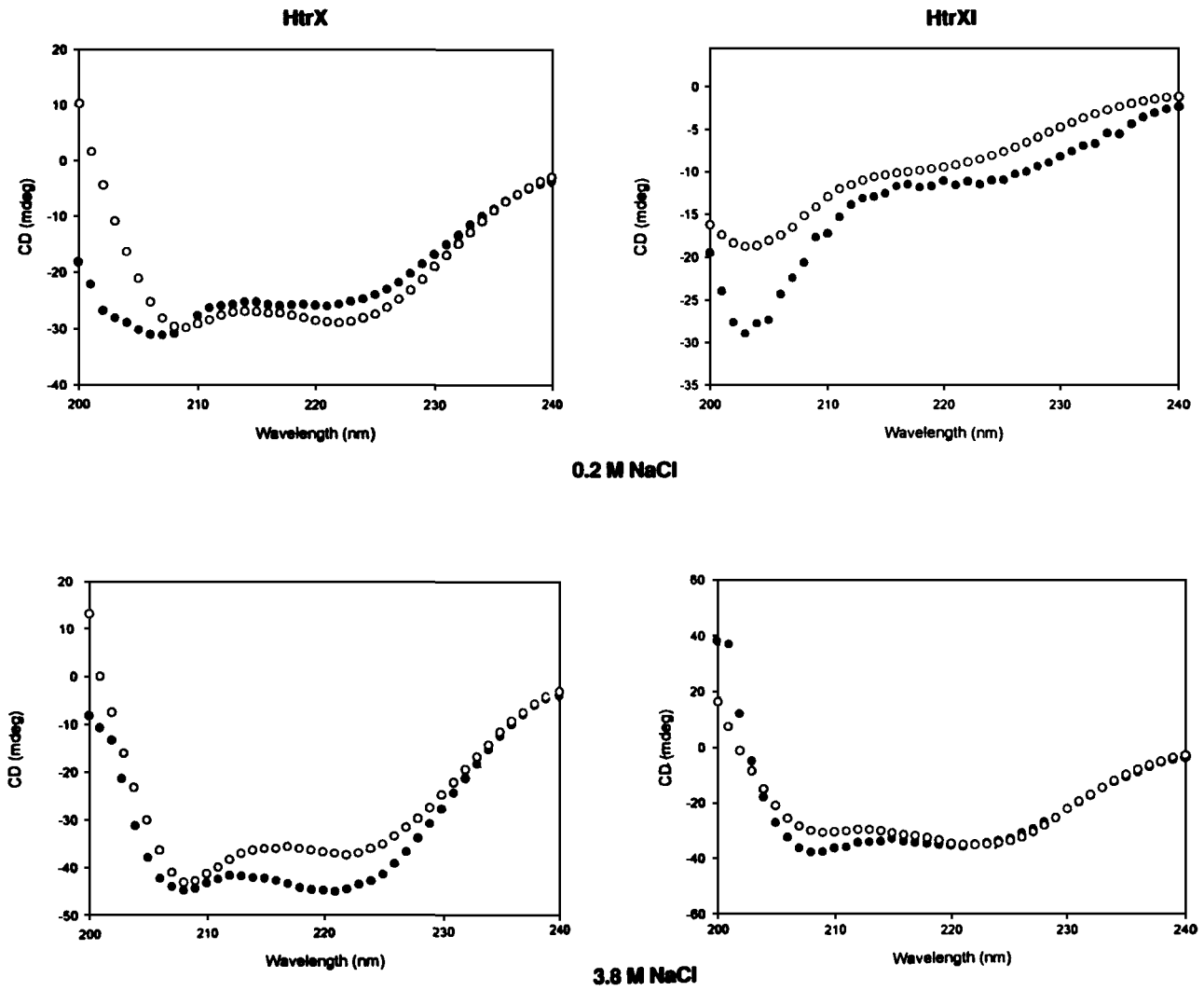

Fig. 1. Circular dichroism spectra of HtrX and HtrXl obtained under high-and low-ionic-strength conditions. Closed circles represent raw data, while open circles represent predicted CD spectra using K2D fitting software (available using ftp anonymous to *swift.embl-heidelberg.de*). Raw data were collected using approximately 30 μM protein (both HtrX and HtrXl) in 10 mM potassium phosphate buffer, pH 6.5, with either 0.2 M (low-ionic-strength data) or 3.6 M (high-ionic-strength data) NaCl. The spectrum is the sum of three scans obtained using a 2-mm quartz optical cell.

of Fig. 2 displays the CD spectra of HtrX as a function of NaCl concentration, while the right panel shows the corresponding NaCl dependence for HtrXI. Figure 3 displays a plot of the CD amplitude at 220 nm to that at 207 nm providing a measure of the change in α-helical content of HtrX/HtrXI as a function of solution ionic strength. In the case of HtrXI, a gradual transition between random coil and α-helix is observed with a midpoint near 1.5 M NaCl. The random coil to α-helical transition is much sharper for HtrX and occurs at a significantly higher NaCl concentration (~2.5 M). Similar effects are observed for KCl (physiologically relevant salt) with the transition points slight shifted (~1 M KCl for HtrXI and ~3.5 M for HtrX). In addition, the CD spectra of both HtrX and HtrXI under both high- and low-ionic-strength conditions are the same regardless of the nature of the salt (Fig. 4).

The secondary structure estimated from the CD of both HtrX and HtrXI under high-ionic-strength conditions is consistent with that predicted from the amino acid sequences of each protein (i.e., >90% α-helix in each case). Interestingly, the CD spectra of HtrX and HtrXI differ significantly at lower ionic strengths. The CD spectra of HtrXI at 0.2 M NaCl/KCl is consistent with predominately random coil, while the CD spectra of HtrX predicts roughly 85% α-helical character. These data suggest that HtrXI is predominately unfolded under

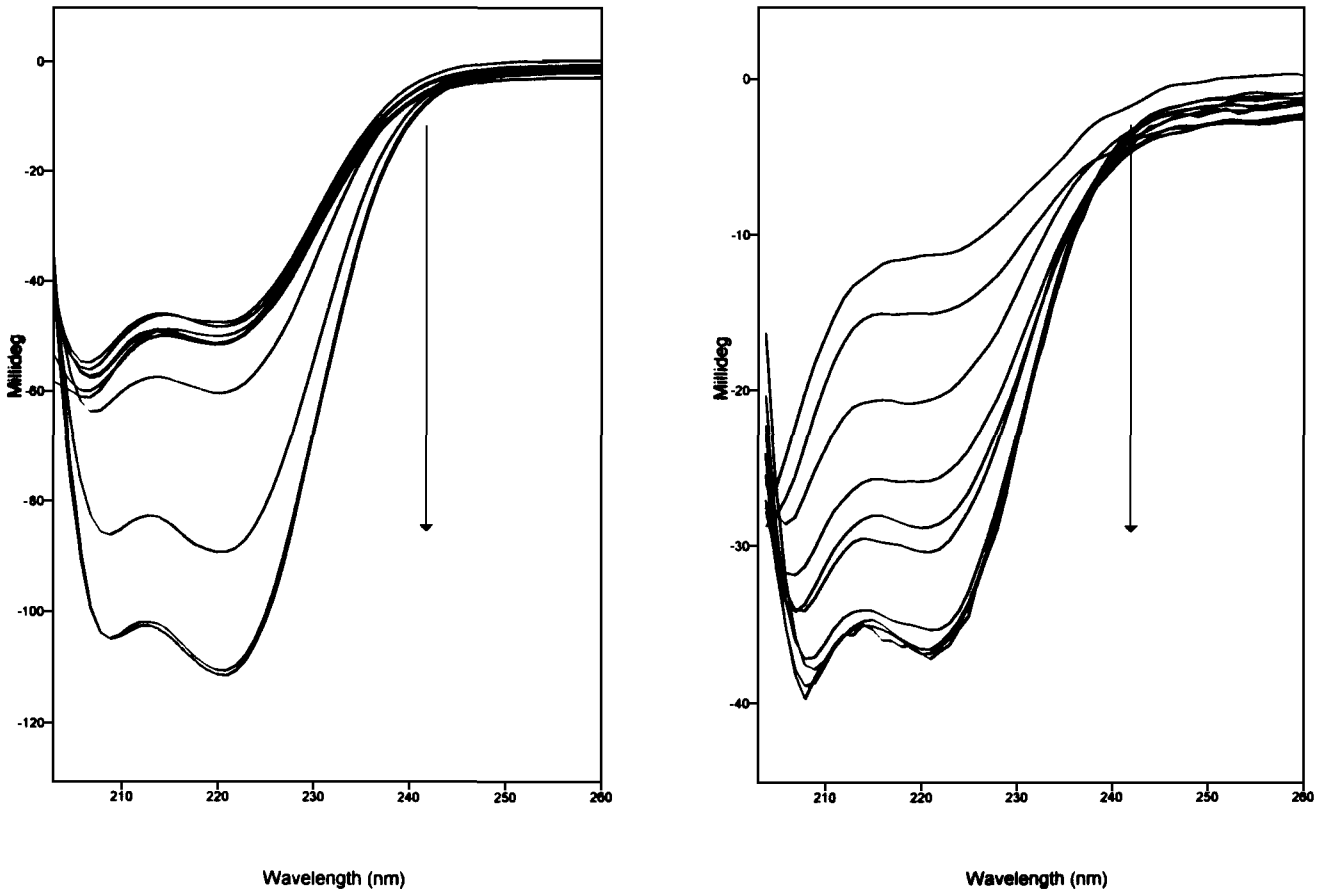

Fig. 2. Circular dichroism spectra of HtrX (left panel) and HtrXl (right panel) as a function of NaCl concentration. The arrow indicates the direction of increasing NaCl concentration. Spectra were obtained a 0.2, 0.58, 0.96, 1.34, 1.72, 2.10, 2.48, 2.86, 3.24, and 3.62 M NaCl. Sample conditions are as described in Fig. 1.

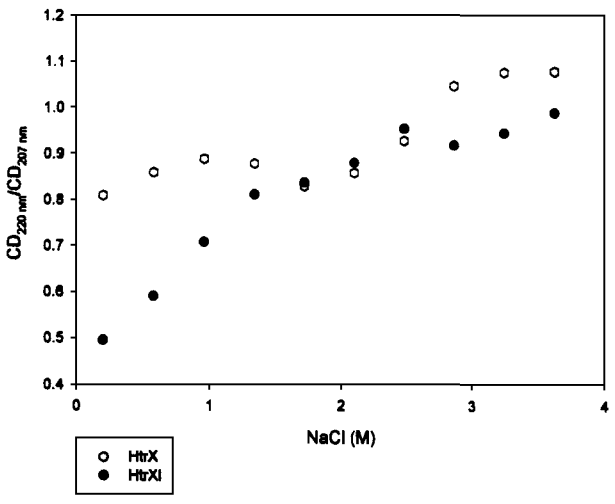

Fig. 3. Plot of the CD obtained at 220 nm to that obtained at 207 nm for both HtrX (open circles) and HtrXl (closed circles) as a function of NaCl concentration.

low-ionic-strength conditions and exhibits salt-dependent folding with a midpoint transition at roughly 1.5 M NaCl. In the case of HtrX, two possible scenarios could account for the observed CD spectra. In one case the protein adopts a low-ionic-strength conformation in which one or more domains of the protein are unfolded giving rise to the ~15% random coil structure. These domains then fold into predominately α-helical structure upon increase in solution ionic strength. Alternatively, the protein preparation may contain more than one isoform and different isoforms may exhibit different folding characteristics. Thus, at low ionic strengths, one isoform remains predominately unfolded while other isoforms remain folded. Increasing the ionic strength then causes the unfolded isoforms to adopt α-helical secondary structure. Although we cannot rule out either possibility at this point, preliminary immunoassays suggest only a single isoform.

It is also of interest to note that although the degree of α-helical character is relatively consent over a wide

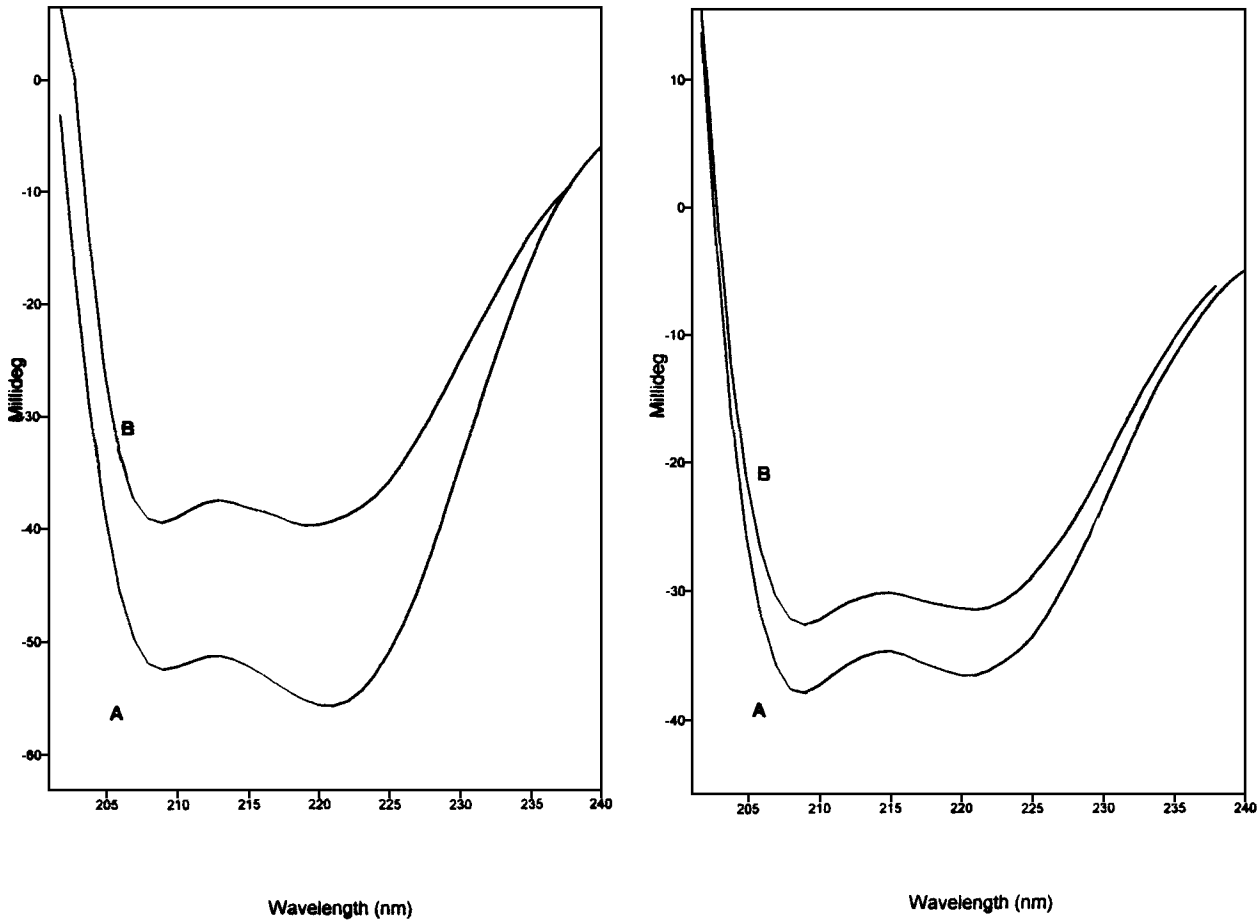

Fig. 4. Circular dichroism spectra of HtrX (left panel) and HtrXI (right panel) in the presence of 3.6 M NaCl (A) and 3.6 M KCl (B). Sample conditions are as described in Fig. 1.

range of ionic strength (roughly from 0.2 M NaCl to over 2 M NaCl), the intensity of the signal increases significantly (see Fig. 2). This suggests that processes other than the secondary structure formation occurs in this ionic strength range (e.g., tertiary structure formation/conformational transitions). Similar changes in CD intensities have been observed in the folding of TATA-box-binding protein of *H. salinarum* (Soppa and Link, 1997).

The physiological concentration of KCl in *H. salinarum* is roughly 5 M and, under these conditions, both HtrX and HtrXI exhibit predominately α-helical character. In addition, previous studies have demonstrated that soluble proteins either isolated from this organism or expressed from *Escherichia coli* are unstable at low ionic strengths due to unfolding of the protein (Soppa and Link, 1997; Reupp *et al.*, 1995; Blecher *et al.*, 1993). The low-ionic-strength data for HtrXI are consistent with these observations. It is somewhat surprising, however, that HtrX exhibits considerable α-helical character at low ionic strengths despite the fact that HtrX and HtrXI share considerable C-terminal sequence homology. In addition, both HtrX and HtrXI share similar molecular weights (53 kDa for HtrX and 49 kDa for HtrXI), isoelectric points (3.78 for HtrX and 3.95 for HtrXI), and percentage of charged amino acids (41% charged, 27% acidic, 10% basic, 22% polar, and 33% hydrophobic for HtrX and 42% charged, 28% acidic, 10% basic, 22% polar, and 29% hydrophobic for HtrXI). Thus, differences in folding patterns between HtrX and HtrxI suggest significant differences in tertiary structure contributing to protein stability.

3.2. Solvent Accessibility of Trp35 in HtrX and both Trp47 and Trp74 in HtrXI

The quenching of tryptophan fluorescence by exogenous quenchers allows for estimation of the solvent

accessibility of the given tryptophan residue. Residues which are at or near the protein surface would be expected to be highly quenched by KI or acrylamide, while tryptophan molecules imbedded within the protein matrix would experience significantly less quenching. Both HtrX and HtrXI contain intrinsic tryptophan fluorophores that can be used to probe for solvent accessibility of the analogous peptide regions. HtrX contains a single tryptophan (Trp35), while HtrXI contains two residues (Trp47) and (Trp74). Quenching of HtrX fluorescence by KI under both high- and low-ionic-strength conditions is shown in Fig. 5. In the case of HtrX, the degree of quenching, as judged by the Stern–Volmer constant, is distinct for both high and low ionic strengths ($K_{sv} = 3.3 \pm 0.1$ M^{-1} for low-ionic-strength and 2.1 ± 0.1 M^{-1} for high-ionic-strength conditions). In addition, the Stern–Volmer constants are similar to that obtained for the quenching of free tryptophan in aqueous detergent solution in which the tryptophan is partially exposed to the solvent ($K_{sv} = 28$ M^{-1} for free indole in aqueous solution and $K_{sv} = 2$ M^{-1} for indole in an aqueous solution containing 5 mg/ml laurylmaltoside) (Hill *et al.*, 1986). These results suggest that the single tryptophan (Trp35) is partially exposed to the solvent in the folded form of the protein.

The corresponding KI quenching of wild-type HtrXI displays nonlinear Stern–Volmer plots at both high and low ionic strengths. This observation is consistent with the two tryptophan residues (Trp74 and Trp47) being in different protein environments in both the partially unfolded and folded forms of the protein. The correspond-

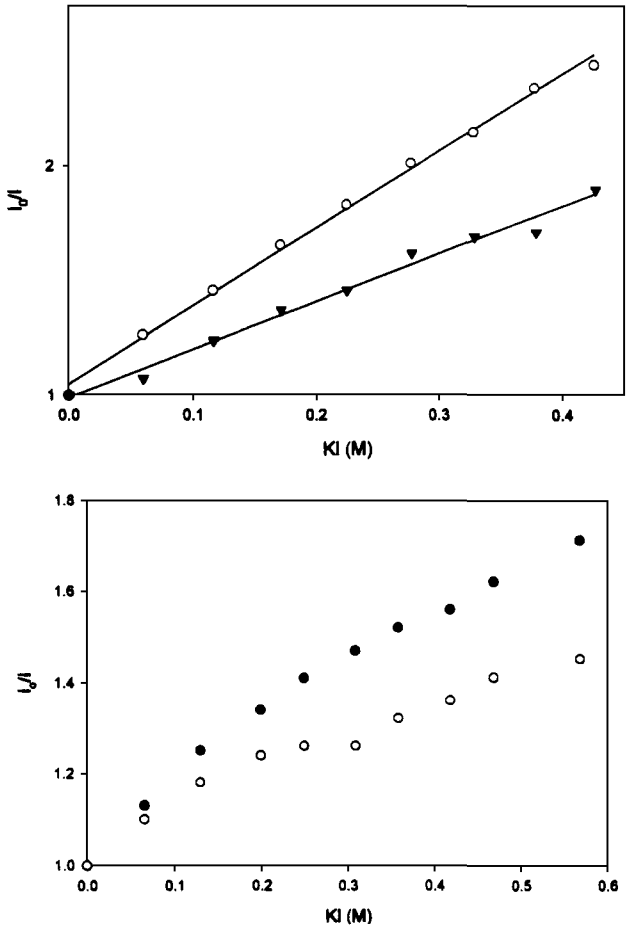

Fig. 5. Stern–Volmer plots for KI quenching of tryptophan fluorescence of HtrX (top panel) and wild-type HtrXI (lower panel) at 0.2 M NaCl (open circles) and 3.6 M NaCl (closed triangles). Protein concentrations used were roughly 10 μM in 0.5 ml of 10 mM potassium phosphate buffer, pH 6.5. The protein solution was titrated with KI from a 4 M stock solution. Intensities were corrected for dilution.

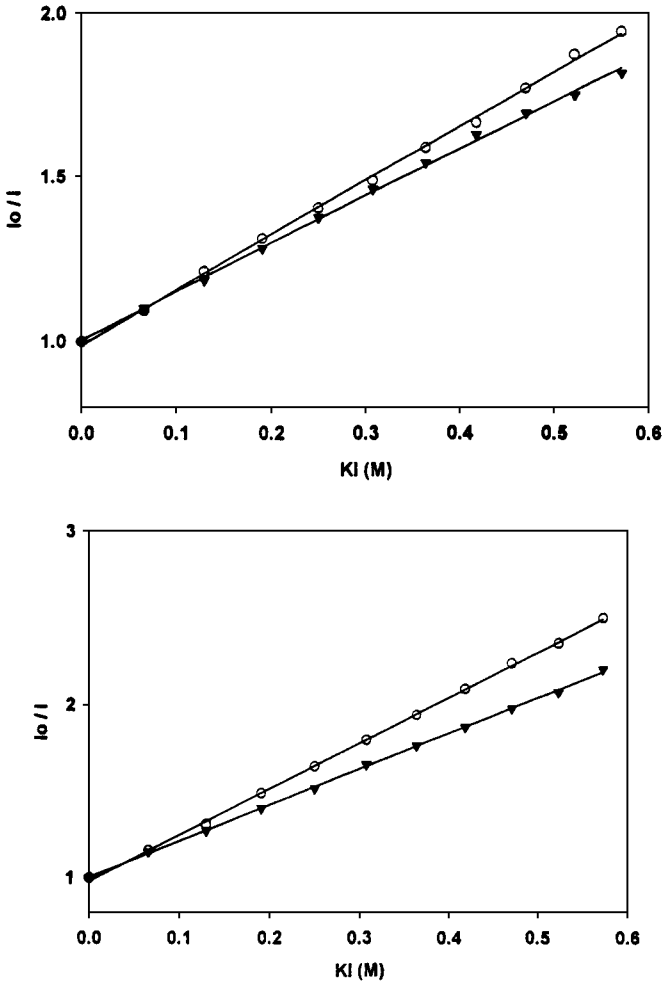

Fig. 6. Stern-Volmer plots for KI quenching of the single-tryptophan mutants of HtrXI. Top panel: Trp74Ala. Bottom panel: Trp47Ala. Sample conditions are as described in Fig. 5.

ing Stern–Volmer plots of the mutants Trp74Ala and Trp47Ala give Stern–Volmer constants of 1.66 ± 0.02 and 2.62 ± 0.02 M^{-1} under low-ionic-strength (respectively) and 1.44 ± 0.02 and 2.05 ± 0.02 M^{-1} (respectively) under high-ionic-strength conditions. The fact that the Stern–Volmer constants are similar in magnitude and resemble the value obtained for the Trp/detergent value under low-ionic-strength conditions indicates that both Trp47 and Trp74 are most likely located within the partially folded domain (i.e., corresponding to the 30% α-helical character observed in the CD spectrum of HtrXI under low-ionic-strength conditions). Upon complete folding (high ionic strengths) this region changes conformation, resulting in slightly less solvent exposure of both tryptophan residues. The data further reveal that Trp74 experiences greater solvent accessibility than Trp47 as judged by the magnitudes of the two Stern–Volmer constants (1.66 versus 2.05 M^{-1} for Trp47 and Trp 74, respectively).

The 154 N-terminal residues of HtrXI are not homologous to the conserved cytoplasmic signaling domain of the transducer superfamily. In general, the ligand-binding domains of different transducers vary in primary structure. It is striking, therefore, that the N-terminal region of HtrXI contains an arginine cluster similar to those found in the ligand-binding domains of the eubacterial chemoreceptors Tar and Tsr (Chen *et al.*, n.d.). These arginine residues are involved in Asp sensing in Tar (Wolff and Parkinson, 1988). We therefore hypothesize that the 154 N-terminal residues of HtrXI are involved in chemosensing. Examination of the amino acid sequence of HtrXI reveals that Trp 47 is two residues away from the last Arg in the cluster (Arg44) and seven residues away from the first Arg in the cluster (Arg39). The steady-state fluorescence data of the Trp74Ala clearly demonstrate that Trp47 is at least partially exposed to the solvent (i.e., at or near the protein surface) indicating that this region of the protein may also be exposed to the solvent.

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