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Role of Arginine 67 in the Stabilization of Chymotrypsin Inhibitor 2: Examination of Amide Proton Exchange Rates and Denaturation Thermodynamics of an Engineered Protein†

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ABSTRACT: We have examined the contribution to protein stability of an interaction involving a charged hydrogen bond from an arginyl side chain (Arg67) in the serine proteinase inhibitor chymotrypsin inhibitor 2 (CI-2), by replacing this side chain with an alanyl residue by protein engineering. Using nuclear magnetic resonance spectroscopy (NMR), we have examined the effect of this mutation on the hydrogen–deuterium exchange rates of several backbone amide protons in the native and engineered proteins at 50 °C. These exchange rates provide a localized probe at multiple discrete sites throughout the protein and from comparison of native and mutant exchange rates allow calculation of the difference in free energy of exchange ($\Delta\Delta G_{ex}$) resulting from the mutation. The results show that for the majority of amides observed this mutation results in $\Delta\Delta G_{ex}$ of ca. 1.7 kcal mol^{-1} over the whole CI-2 molecule. However, for two relatively exposed amide protons the exchange rates are found to be far less perturbed, implying that local unfolding mechanisms predominate for these protons. Direct measurement of the stability of both proteins to denaturation by guanidinum hydrochloride shows that the interaction contributes 1.4 kcal mol^{-1} to the stability of the molecule. This value is comparable to those obtained from the NMR exchange measurements and indicates that the exchange processes reflect the differences in stability between the native and mutant proteins. Our results show (i) at the temperature of these experiments, NH exchange is due to unfolding processes; (ii) the whole CI-2 molecule (over the region for which data are available) forms a single cooperative folding unit; and (iii) the mutation studied results in a global destabilization of the protein by ca. 1.5 kcal mol^{-1}, a value consistent with the loss of the hydrogen-bonded interaction.

The conformation and stability of a protein depend upon a myriad of weak noncovalent interactions between the various constituent amino acid residues in the molecule. Collectively, these interactions produce a molecule that has a defined three-dimensional structure. Two relevant questions concerning the way these forces are used in the structural organization of proteins are (i) what is the precise energetic contribution of various individual interactions to the stability of the overall structure and (ii) how is the contribution of a single interaction distributed over the whole protein? Is the stabilization that is conferred localized to the immediate area of the protein around the particular residue involved or is this stabilization delocalized over the whole molecule?

Mutations within a protein are well-known to cause alterations in the stability of the molecule. Many "temperature-

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sensitive" mutants, usually involving a single amino acid substitution, have been produced by classical genetic techniques, the effects of the mutations on stability have been characterized, and recently protein engineering has been used to address specific questions concerning the strengths of various types of interactions involved in protein stability [reviewed by Alber (1989)]. Studies to date have quantitated the effect of various changes on the overall thermodynamic stability but have not provided direct evidence as to whether the destabilizing effects are confined to the region of mutation, are distributed globally over the protein, or involve a mixture of these effects.

Proteins with small numbers of amino acid substitutions, either natural mutations or obtained from related species, have been useful in the past to assist with NMR assignments (Jardetzky & Roberts, 1981; Williams et al., 1985). More recent advances make it now possible to assign most hydrogen atom resonances for small proteins by two-dimensional NMR spectroscopy (Wüthrich, 1986), and the advent of protein engineering allows us to create specific alterations to study protein function (Leatherbarrow & Fersht, 1986). Recently, assignments have been obtained for engineered proteins (Pielak et al., 1988; Folkers et al., 1989).

By use of two-dimensional NMR, the resonances from the amide protons of the polypeptide backbone are particularly easily identified and a large number of these can be individually resolved in one-dimensional spectra. In D_2O solution these NH resonances exchange to give ND with the concomitant decay of the NH signal. The rate of decay of the NH signal is dependent on the solvent accessibility of this group and also on the stability of the protein as unfolding exposes all the amides to the solvent. The mechanism of hydrogen–deuterium exchange is (Richarz et al., 1979; Wagner & Wüthrich, 1979)

$$C(H) \underset{k_2}{\overset{k_1}{\rightleftharpoons}} O(H) \xrightarrow[D_2O]{k_3} O(D) \underset{k_1}{\overset{k_2}{\rightleftharpoons}} C(D) \qquad (1)$$

where O represents the "open", solvent-exposed state and C the "closed" or folded solvent-inaccessible form of the protein. The rate of exchange, k_{ex}, is given in eq 2. At low pH it is

$$k_{ex} = k_1k_3/(k_2 + k_3) \qquad (2)$$

found that NH exchange in proteins generally follows EX_2 kinetics (Roder et al., 1985a), where the exchange rate, k_{ex}, is proportional to the equilibrium constant, K ($K = k_1/k_2$), between the solvent-exposed and unexposed states (as $k_2 \gg k_3$), giving

$$k_{ex} = Kk_3 \qquad (3)$$

The rate constant k_3 is the intrinsic chemical exchange rate. Although different residues have different k_3 values (Roder et al., 1985b), the major factor contributing to the different exchange rates of various residues is the solvent exposure, and hence the value of K, for these residues. A further factor influencing the exchange is whether the NH protons encounter solvent via generalized unfolding (global opening) or via localized unfolding. At low temperatures local structure fluctuations allowing solvent access are important, but at higher temperatures global unfolding mechanisms predominate (Wagner et al., 1984; Delepierre et al., 1988).

The system used for these studies is the serine proteinase inhibitor CI-2 from barley. This is one of the few proteins for which a high-resolution X-ray structure (McPhalen & James, 1987), complete NMR assignments (Kjaer et al., 1987), and a solution NMR structure (Clore et al., 1987) are available. In addition, the gene encoding CI-2 has been cloned

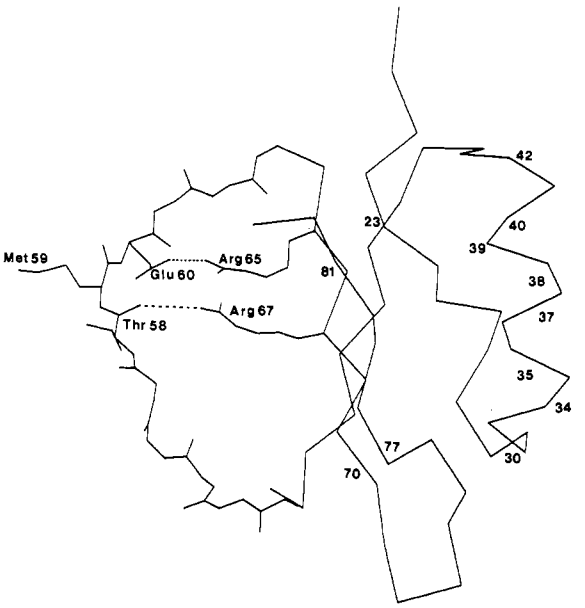

FIGURE 1: Structure of CI-2 as determined by X-ray crystallography (McPhalen & James, 1987). The main-chain atoms are drawn for the loop region (residues 52–62); over the rest of the molecule the α-carbon atoms alone are drawn. The interactions between the side chains of Arg65 and Glu60 and Arg67 and the main-chain carbonyl of Thr58 are indicated by dotted lines. The approximate locations of the amide protons whose exchange rates are monitored in this paper are indicated by numbers.

(Williamson et al., 1987) and expressed (Campbell, 1988; C. Longstaff, A. Campbell, and A. R. Fersht, manuscript in preparation), allowing the construction of mutants by protein engineering. CI-2 is a member of the potato proteinase inhibitor family (Laskowski & Kato, 1980), which is characterized by the absence of disulfides (which are thought to stabilize many other proteinase inhibitors to proteolysis by proteinase). In CI-2 it has been suggested that hydrogen-bonding interactions between arginine side chains from the main body of the protein (Arg65 and Arg67) and the residues on either side of the scissile Met59–Glu60 bond (Glu60 and Thr58) (Figure 1) play a similar role to the disulfide bridges of other inhibitors and stabilize the molecule (McPhalen & James, 1987). This paper determines the contribution made by the side chain of Arg67 by replacement of this residue by alanine.

Materials and Methods

Protein Purification. CI-2 protein (native or Ala67 CI-2) was expressed in *E. coli* TG2 (Gibson, 1984) containing the plasmid pAC4 (Campbell, 1988) by overnight fermentation (MBR Bioreactor, 12L) at 37 °C in 2-TY medium supplemented with glucose (18 g L^{-1}), ampicillin (50 mg L^{-1}), and isopropyl β-D-thiogalactopyranoside (50 mg L^{-1}). After they were harvested, the cells were disrupted by sonication (Heat Systems-ultrasonicators, Model W-385). The pH of the resultant supernatant was then adjusted to 4.4 with glacial acetic acid and any precipitate was discarded. Ammonium sulfate was added to 40% w/v, and the precipitated proteins were removed. After raising the ammonium sulfate concentration to 65% w/v, the pellet containing CI-2 was recovered by centrifugation. The pellet was then redissolved in ammonium carbonate buffer (10 mM, pH 9.0) and size fractionated on a gel filtration column (2.7 cm × 101 cm, Sephadex G-75, Pharmacia). Fractions containing CI-2 were identified by their ability to inhibit hydrolysis of *N*-succinyl-ala-ala-pro-phe-*p*-nitroanilide (Sigma) by subtilisin Carlsberg (Sigma) and were pooled. These fractions were finally purified by passage

through a DEAE–Trisacryl column equilibrated in ammonium carbonate (10 mM, pH 9.0). The remaining protein impurities bound to this column, leaving pure CI-2 in the eluate. This was concentrated by lyophilization and then desalted on a column of Sephadex G-25 (2 cm × 30 cm, Pharmacia). The pH was adjusted to 4.2 in preparation for the NMR experiments, and the sample was lyophilized. The dry protein was used directly for the NMR and denaturation studies.

Mutagenesis. The mutant Ala67 CI-2 was prepared by the double-priming technique (Norris et al., 1983), using the mutagenic oligonucleotide 5′-CTGGCGCAG*CG*GGAGA-AAC-3′ with transformation into repair-deficient *E. coli* in order to increase mutation efficiencies (Kramer et al., 1984; Carter et al., 1985). The sequence of the mutant was confirmed by DNA sequencing of the entire coding region of the gene. Oligonucleotides were synthesized on an Applied Biosystems 380B synthesizer.

Two-Dimensional NMR Spectroscopy. NMR spectra were recorded on a Bruker AM 500 NMR spectrometer equipped with an Aspect 3000 data system. COSY (Aue et al., 1976; Marion & Wüthrich, 1983; Neuhaus et al., 1985) spectra of wild-type and Ala67 CI-2 were recorded by using 5 mM solutions of protein at 25 °C, pH 4.2, in D_2O. Typically, 512 increments of 2K data points were collected and zero filled to 2K points in the F1 dimension and 4K points in F2.

NMR Exchange. For proton exchange measurements, 3 mM protein solutions were prepared by dissolving 14 mg of lyophilized CI-2 in 0.5 mL of D_2O (99.96 atom % D, Sigma). Measurements were performed by following a simple quench procedure (Richarz et al., 1979). A zero time point spectrum was recorded immediately after sample preparation. Subsequent spectra were recorded at various time intervals. Between NMR measurements the sample tube was placed in a thermostated water bath at 50 °C. After an incubation period, the sample was rapidly cooled to 25 °C. Subsequently, the ^1H NMR spectra were recorded at a probe temperature of 25 °C ± 0.5. This procedure ensured that no significant exchange occurred during the time taken for data acquisition. Typically 64 transients of 32K data points were recorded per spectrum. The spectra were resolution enhanced by Gaussian multiplication. Assignments of the amide resonances in the one-dimensional (1D) spectra were confirmed by recording COSY spectra (not shown) for a duplicate sample, both at the start of the exchange period and when approximately half of the resonances had exchanged.

The exchange rates were determined by measuring the time-dependent decrease in resonance intensities as hydrogen atoms were replaced with deuterium. The exchange followed first-order kinetics within experimental error for all of the resolved NH resonances. Rate constants were determined by fitting the measured peak heights to a single exponential decay curve by nonlinear regression by using the program GraFit (Leatherbarrow, 1989).

Chemical Denaturation Experiments Using Fluorescence Spectroscopy. Guanidinium hydrochloride (ARISTAR grade) was obtained from BDH Limited. Guanidinium hydrochloride solutions were prepared by serial dilutions from an 8 M stock solution. For each data point, 300 μL of CI-2 solution in sodium acetate buffer (pH 4.2, 50 mM) was diluted into 2700 μL of the appropriate denaturant solution. The final CI-2 concentration was 6.7 μg/mL. The protein/denaturant solutions were preequilibrated at 25 °C for 1 h. Fluorescence spectroscopy measurements were recorded after equilibration to 50 °C in a thermostated fluorimeter (Perkin-Elmer MPF-44A). The excitation wavelength was 290 nm and emission spectra were recorded at 356 nm. Data were analyzed by nonlinear regression by using the program GraFit (Leatherbarrow, 1989).

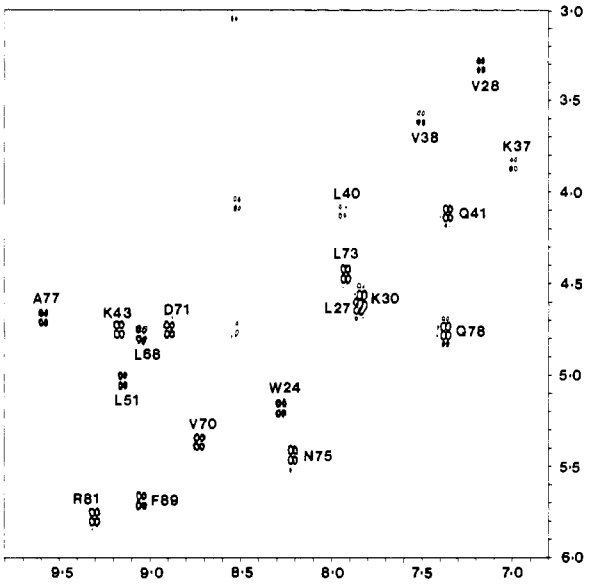

FIGURE 2: NH–C$^\alpha$H "fingerprint" region from COSY spectra of Ala67 CI-2. Spectra were recorded in D_2O solution, pH 4.2, 25 °C, and show cross-peaks for the more slowly exchanging amide protons. The positions and identity of these slowly exchanging cross-peaks are essentially the same as for wild-type CI-2 under the same conditions (Kjaer et al., 1987; Kjaer & Poulsen, 1987), allowing their assignment.

RESULTS

Assignment of NH Protons. The slowly exchanging NH protons in Ala67 CI-2 were identified from COSY and NOESY spectra of samples freshly dissolved in D_2O. Assignment of the amide protons in the mutant protein was by reference to the complete assignment data available for wild-type CI-2 (Kjaer et al., 1987), as most proton resonances were unaffected by the mutation. The NH–C$^\alpha$H fingerprint regions of the COSY spectra recorded for Ala67 CI-2 are shown in Figure 2. CI-2 contains several slowly exchanging amide protons, principally located in the β-sheet and α-helix regions of the molecule (Kjaer & Poulsen, 1987). The equivalent NH protons are found to be in slow exchange for the wild-type and mutant proteins, confirming that the basic secondary structural organization of the protein remains unaltered. All NH protons whose exchange rates were monitored in this study had chemical shift values that differed from those of the wild-type protein by <0.1 ppm in spectra recorded under identical conditions.

NMR Amide Proton Exchange. Exchange experiments were performed by observing the decay of the amide proton signals in the 1D NMR spectrum when a fresh protonated sample of protein was dissolved in D_2O (Figure 3). Sufficient resolution is obtainable in the 1D spectra to obtain the exchange rates for most of the amide protons without the need to use 2D techniques. This gives a considerable advantage in terms of speed (2D spectra take longer to acquire), which was important for the Ala67 CI-2 due to the much faster rates of amide exchange. Representative decay curves are shown in Figure 4. The rates of NH exchange of various assignable amide protons in native CI-2 and Ala67 CI-2 were calculated from these data and are given in Table I. It was found that all the measured rate constants for hydrogen–deuterium exchange were significantly increased in the mutant protein.

The thermodynamic effects of the mutation on the amide proton exchange rates can be calculated on the basis of eq 3.

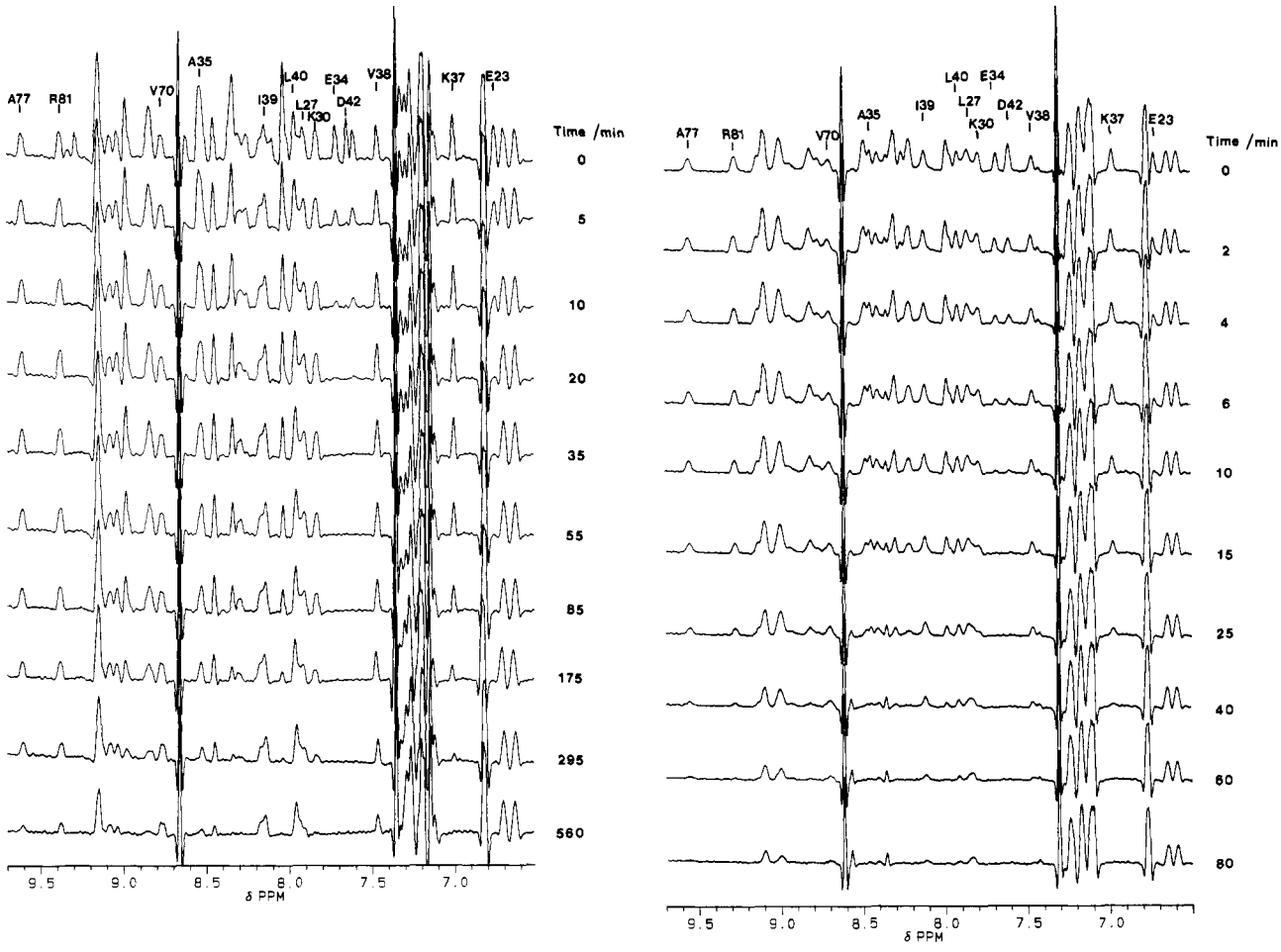

FIGURE 3: Time course of amide proton exchange. Hydrogen–deuterium exchange was allowed to occur at 50 °C for the periods indicated; the ^{1}H NMR spectra shown were then recorded at a probe temperature of 25 °C: (top) wild-type CI-2; (bottom) Ala67 CI-2. The resonance positions of the amide protons monitored in the present study are indicated.

Table I: Rates of Amide Proton Exchange of Native and Ala 67 CI-2[a]

residue	wild-type CI-2 k_{ex} (10^{-6} s^{-1})	Ala 67 CI-2 k_{ex} (10^{-6} s^{-1})	$\Delta\Delta G_{ex}$ (kcal mol^{-1})
Glu23	2120.0	3170	0.3
Leu27	43.8	698	1.8
Lys30	79.3	898	1.6
Glu34	2300.0	3630	0.3
Ala35	57.8	653	1.6
Lys37	83.8	708	1.4
Val38	26.8	432	1.8
Ile39	17.8	328	1.9
Leu40	22.2	422	1.9
Asp42	1360.0	13100	1.5
Val70	17.8	300	1.8
Ala77	39.8	533	1.7
Arg81	45.3	643	1.7

[a] The standard errors of the $\Delta\Delta G_{ex}$ values are $\sim\pm0.2$ kcal mol^{-1} in each case.

If we assume that the chemical exchange rate of an individual amide proton, k_3, is not grossly affected by the mutation (this is likely to be reasonable if the mutation is distant from the amide proton observed), we can calculate the thermodynamic effect on the exchange rates from eq 4. This free energy term,

$$\Delta\Delta G_{ex} = -RT \ln (K_{[\text{wild type}]}/K_{[\text{mutant}]}) = -RT \ln (k_{ex[\text{wild type}]}/k_{ex[\text{mutant}]}) \quad (4)$$

$\Delta\Delta G_{ex}$, reflects the effect of the mutation on the exchange process at the location of the particular amide observed. Values of $\Delta\Delta G_{ex}$ for the amide protons measured are given in Table I.

Solvent-Induced Denaturation. The stabilities of native CI-2 and Ala67 CI-2 were compared by measuring their unfolding in solutions of guanidinium hydrochloride (Kellis et al., 1989; S. Jackson and A.R. Fersht, personal communication). Unfolding was monitored by observation of intrinsic protein fluorescence, which in CI-2 arises from the sole tryptophan side chain, Trp24. It is found that denaturation of the protein is accompanied by a 4.5-fold increase in fluorescence. The denaturation curves for both proteins are shown in Figure 5.

Denaturation thermodynamics were calculated on the basis of the experimental observation that the free energy of protein unfolding in the presence of urea or guanidinium chloride is linearly related to the concentration of denaturant (Pace, 1986), eq 5. ΔG_{H2O} is the apparent energy of unfolding in the absence of denaturant; ΔG_U is the free energy of unfolding in the presence of the denaturant. The equilibrium constant for unfolding in the presence of denaturant, K_U, is given by eq 6 (Kellis et al., 1989)

$$\Delta G_U = \Delta G_{H2O} - m[\text{denat}] \quad (5)$$

$$K_U = (F_N - F)/(F - F_U) \quad (6)$$

where F is the observed fluorescence and F_N and F_U are the fluorescence of the native and unfolded forms of the protein, assuming a two-state transition. Analysis of denaturation data is performed from eq 7, which is derived from eq 5 and 6. The

$$F = F_N - (F_N - F_U) \exp[(m[\text{denat}] - \Delta G_{H2O})/RT]/\{1 + \exp[(m[\text{denat}] - \Delta G_{H2O})/RT]\} \quad (7)$$

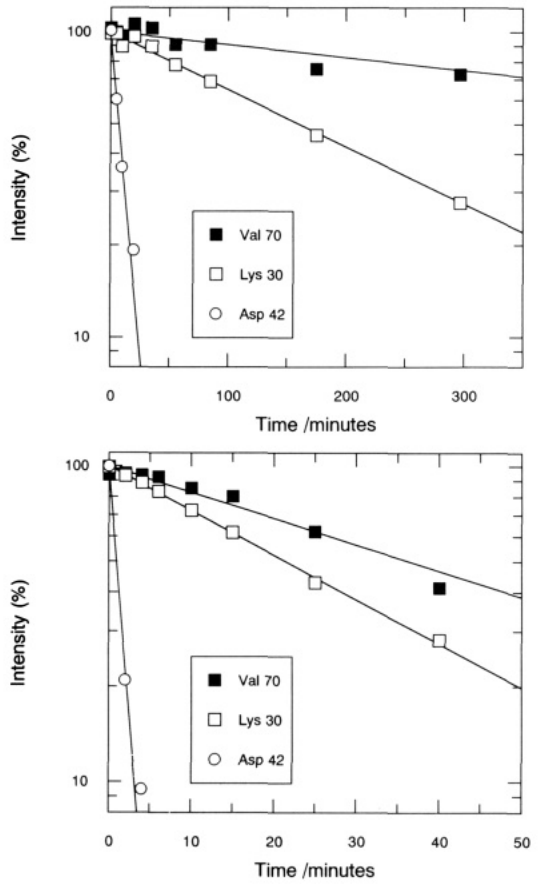

FIGURE 4: Amide proton exchange rates. The peak heights of the amide resonances indicated in Figure 2 were plotted against time to obtain the exchange rate constants. Illustrated are the exchange time courses for residues Asp42, Lys30, and Val70: (top) wild-type CI-2; (bottom) Ala67 CI-2. The relative exchange rates of these amide protons are approximately the same for these residues in the two proteins, but the absolute rates are very different (note the abscissa scale).

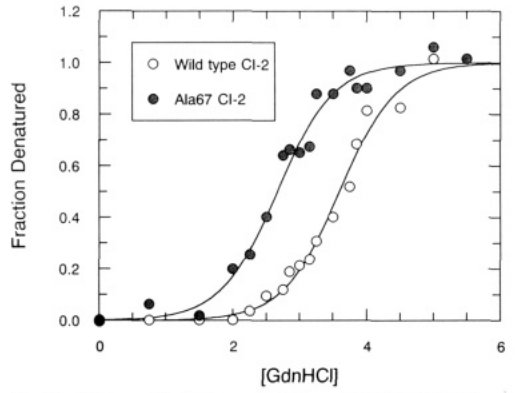

FIGURE 5: Unfolding of wild-type CI-2 and Ala67 CI-2 in solutions of guanidinium hydrochloride at 50 °C, pH 4.2. Unfolding was monitored by intrinsic tryptophan fluorescence, which shows a 4.5-fold increase on denaturation. The data have been normalized to facilitate comparison; the curves represent best fits to the data by nonlinear regression from eq 7.

difference in free energy of unfolding between wild-type and mutant enzymes, $\Delta\Delta G_U$, is given by $\Delta G_{H2O(\text{wild type})} - \Delta G_{H2O(\text{mutant})}$. As has been pointed out by Kellis et al. (1989), calculation of separate ΔG_{H2O} values for two sets of data involves long extrapolations of the data, reducing the accuracy of any derived difference. Increased accuracy in calculating the $\Delta\Delta G_U$ value may be obtained by assuming that the m value is the same for the two data sets. Kellis et al. (1989) used this to derive an equation to give the $\Delta\Delta G_U$ value directly (eq 8).

$$\Delta\Delta G_U = RT \ln \{([\text{folded}]/[\text{unfolded}])_{\text{wild type}}/([\text{folded}]/[\text{unfolded}]_{\text{mutant}})\} \quad (8)$$

However, for this equation to be successfully applied $\Delta\Delta G_U$ must be small. We used a different approach in which paired wild-type and mutant data sets were analyzed by multiple nonlinear regression by using the program GraFit. Each of the data sets was fitted simultaneously to eq 7 in which the value of m was forced to be the same for both sets of results but the ΔG_{H2O} values were independent. This has the same overall effect as eq 8, but is not affected by the magnitude of $\Delta\Delta G_U$ and requires no rearrangements of the data.

As is evident from Figure 5, the mutant protein unfolds at lower concentrations of denaturant and so is less stable than the native. The stabilities (ΔG_{H2O}) of native CI-2 and Ala67 CI-2 are determined from this experiment to be 5.5 kcal mol^{-1} and 4.1 kcal mol^{-1}, respectively, under the conditions used, showing that the mutation has resulted in destabilization of the protein by 1.4 kcal mol^{-1}.

Discussion

The crystal structure of CI-2 shows it to possess an unusual hydrogen-bonding network, which has been suggested to stabilize the reactive-site loop (McPhalen & James, 1987) (Figure 1). Hydrogen bonds between the side chains of Arg65 and Arg67 and the side chain and main chain of residues Glu60 and Thr58, respectively, which are adjacent to the scissile Met59–Glu60 bond, are thought to stabilize the loop structure. The mutation Arg67 to Ala67 was constructed in order to probe this stabilization. The NMR spectra of the mutant protein are, qualitatively, similar to those of the wild type (Figures 2 and 3). In addition, the same amide protons are found to be in slow exchange with solvent. This strongly suggests that the mutation has not resulted in any large conformational changes, although quantitative indications of the structural consequences of this mutation must await calculation of an NMR solution structure.

Effects on Amide Proton Exchange Rates. Measurement of amide proton exchange rates allows determination of the thermodynamic effect of mutation at a range of different sites throughout the protein by application of eq 4. The meaning of the parameter calculated, $\Delta\Delta G_{ex}$, depends upon the mechanism whereby the exchange occurs. If, as is the most likely explanation at the temperature of this experiment, exchange occurs as a result of unfolding processes, $\Delta\Delta G_{ex}$ measures the destabilization resulting from the mutation at the position where the amide proton is located in the protein. As we can measure this parameter for many different amide protons, we can determine the specific consequences of mutation throughout the molecule. One can therefore test whether the mutation causes changes in stability that are confined to the region of mutation, globally distributed throughout the protein, or confined to a subdomain of the molecule. Interpretation of amide proton exchange rates from an individual proton within a protein is difficult, as several processes may contribute to the exchange mechanisms (Dempsey, 1986; Delepierre et al., 1988). However, by measuring *differences* in exchange rates between two closely related proteins, we can determine the effect of a mutation without requiring a detailed interpretation of the exchange mechanism itself.

The $\Delta\Delta G_{ex}$ values calculated from the NH exchange data are given in Table I. Although these residues are distributed throughout the protein (Figure 1), in the majority of cases the destabilization caused by the mutation is found to be identical

within error, even though the rates of exchange for these protons differ by up to 2 orders of magnitude. The median effect is 1.7 kcal mol^{-1}. However, there are two residues (Glu23 and Glu34) for which the NH exchange rates are not so markedly affected, resulting in much smaller $\Delta\Delta G_{ex}$ values. These residues are distant from the site of mutation and have relatively fast exchange rates. In addition, the X-ray structure shows that they are located near the surface of the protein and are not in any of the defined secondary structural elements of the molecule. It seems likely that the exchange processes that expose these protons to solvent are dominated by local opening, and so the mutation has relatively little effect on their exchange rates.

For the remaining 11 amide protons, the mutation affects their exchange rate constants in a global fashion. The average value of $\Delta\Delta G_{ex}$ for these residues is 1.7 kcal mol^{-1}. This value is close to that for destabilization of the whole protein, which was measured by unfolding in guanidinium hydrochloride solutions (1.4 kcal mol^{-1}). This suggests that both experiments measure the same destabilization effects. These results show (i), at the temperature used in these experiments (50 °C), NH exchange for the majority of the residues observed occurs due to global unfolding processes and (ii) the unfolding appears to be cooperative and involves all the secondary structural elements in the molecule, as residues in the β-sheet and α-helix parts of the molecule are affected identically.

The value of the $\Delta\Delta G$ obtained for this mutant, ca. 1.5 kcal mol^{-1}, is indicative of the effective stabilization by the side chain of Arg67, which primarily involves a charged hydrogen bond to the backbone carbonyl group of Thr58. Substitution of Arg by Ala will delete this charged hydrogen bond and additionally disrupt any hydrophobic interactions made by the aliphatic portion of this side chain. The effect of this mutation confirms the role of Arg67 in stabilizing the CI-2 molecule. The value may be compared with the 1.0–1.6 kcal mol^{-1} per methylene group for hydrophobic effects (Kellis et al., 1989), 0.5–2 kcal mol^{-1} for deletion of uncharged hydrogen bonds in enzyme–substrate (E·S) systems, and 3–6 kcal mol^{-1} for a range of charged hydrogen bonds in E·S systems (Fersht et al., 1985; Fersht, 1987). The mutation here, however, is different from the latter group where the large effect is primarily due to leaving an unpaired charged group; the Arg → Ala substitution does not result in an unpaired charge. As pointed out by Fersht et al. (1985), this is expected to produce an effect comparable to the 0.5–2 kcal mol^{-1} group for the removal of the hydrogen bond. Our results fit well into this range and suggest that hydrogen bond deletion is likely to be the predominant factor in the destabilization observed. This is consistent with the crystal structure data which show that the hydrophobic portion of the Arg67 side chain is exposed to solvent and makes few interactions with the rest of the molecule.

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