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An Essential Proline in λ Repressor Is Required for Resistance to Intracellular Proteolysis†

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ABSTRACT: Pro^{78} is a solvent-exposed residue at the N-terminal end of α-helix 5 in the DNA binding domain of λ repressor. Random mutagenesis experiments have suggested that Pro^{78} is essential [Reidhaar-Olson, J. F., & Sauer, R. T. (1990) *Proteins: Struct., Funct., Genet.* (in press)]. To investigate the requirement for proline at this position, we constructed and studied the properties of a set of ten position 78 mutant proteins. All of these mutants have decreased intracellular activities and are expressed at significantly lower levels than wild type. Pulse–chase experiments show that the mutant proteins are rapidly degraded in the cell; the mutants examined had half-lives of 11–35 min, whereas the wild-type protein has a half-life of greater than 10 h. The rapid degradation of position 78 mutants is not suppressed by mutations that affect known *Escherichia coli* proteases. The $Pro^{78} \rightarrow$ Ala mutant could be overexpressed in a *dnaJ*⁻ strain and was purified. This mutant has full DNA binding activity in vitro, suggesting that its folded structure and ability to form active dimers are similar to those of wild type. The PA^{78} mutant (T_m = 48 °C) is less thermally stable than wild type (T_m = 55 °C). Double-mutant studies show that this instability contributes to but is not the main cause of its rapid intracellular degradation and also suggest that proteolysis proceeds from the denatured forms of proteins containing the PA^{78} substitution. The PA^{78} mutation does not appear to introduce a new cleavage site for cellular proteases, nor does the mutation enhance susceptibility to proteases such as thermolysin and trypsin in vitro. The mutation does decrease the *m* value in GuHCl denaturation experiments and may alter the properties of the denatured polypeptide, allowing it to be specifically recognized by an *E. coli* protease or auxiliary degradation factor.

In studies of protein structure and function, it is important to know which side chains play the most significant roles. It is often possible to address this question by examining a family of genetically or phylogenetically related protein sequences (Hampsey et al., 1986; Bashford et al., 1987; Bowie et al., 1990). Residues that are highly conserved are likely to be crucial to some aspect of the structure or function of the protein. Residues that accept a variety of substitutions are clearly less important.

We have been studying the mutability of residues in the N-terminal domain of λ repressor (Reidhaar-Olson & Sauer, 1988, 1990). This domain consists of residues 1–92 and mediates the DNA binding activity of λ repressor (Sauer et al., 1979; Pabo et al., 1979). The structure of the N-terminal domain, both alone (Pabo & Lewis, 1982) and complexed with operator DNA (Jordan & Pabo, 1988), is known. In random mutagenesis experiments, we have found that most buried positions in the N-terminal domain are invariant or tolerate only conservative substitutions. In contrast, most surface positions tolerate a wide range of substitutions, although there are exceptions to this rule. One of the most notable exceptions occurs at the first residue of α-helix 5, Pro^{78}, which is more than 80% exposed to solvent in the crystal structure (Figure 1). Following random mutagenesis of this position, proline was the only residue recovered in a set of 28 functional sequences, suggesting that other substitutions result in nonfunctional proteins. However, examination of the crystal structure does not reveal any obvious role for the Pro^{78} side chain; it is neither near the operator DNA nor involved in any obvious interactions that might stabilize the protein.

Here we report experiments aimed at understanding why proline is required at position 78. When other residues are substituted at this position, the resulting mutant proteins are subject to rapid degradation in vivo. Biochemical analysis of the $Pro^{78} \rightarrow$ Ala mutant shows that the purified protein has slightly decreased thermal stability, but the folded protein is as active as wild type in binding operator DNA. The thermal instability of the mutant contributes to but is not the sole cause of its rapid degradation. In fact, a multiply mutant protein that contains the $Pro^{78} \rightarrow$ Ala substitution but has wild-type thermal stability is still degraded rapidly and exhibits a defective phenotype in the cell. Overall, these results indicate that Pro^{78} is an essential residue because it protects the wild-type protein from intracellular degradation.

Materials and Methods

Strains and Plasmids. The following *Escherichia coli* K-12 strains were used in this work: strain X90 (Amann et al., 1983) is *ara* Δ(*lac pro*) *nalA argE*am *rif thi-1*/F′ *lac*⁺ *pro*⁺

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lacI^{Q}; strain AP401 (Pakula, 1988) is X90 *lon*::ΔTn10; strain MC4100 (Casadaban, 1976) is *araD*139 Δ(*argF*–*lac*)U169 *relA*1 *rbsR flbB*5301 *ptsF*25 *rpsL*150 *deoC*1; strain SG21118 (a gift from S. Gottesman) is MC4100 *clpA*::ΔKan (in the studies described here, MC4100 and SG21118 both carry an F′ *lac*$^{+}$ *pro*$^{+}$ *lacI*^{Q} episome); strain DP9270 is *thi leu lacY tonA supE*44 *lacZ*X90 F′128 (*lacI*^{Q} *lacZ*::Tn5); strain DP748 is DP9270 *dnaJ*259. Strain DP748 was derived from *Escherichia coli* strain CAG748 (Straus et al., 1988) (a gift from C. Gross), which has a Tn10 transposon in the *thr* gene linked to the *dnaJ*259 allele. During the course of mating F′128 into strain CAG748, this transposon was excised, restoring a *thr*$^{+}$ phenotype. The *dnaJ*$^{-}$ phenotype was unaffected by the Tn10 excision in that DP748 remained resistant to phage λ and deficient in proteolysis of denatured proteins. Each of the following strains contains a plasmid derived from pACYC184 that bears the *lacI*^{Q} allele: strain MH1 (a gift from H. Echols) is Δ*lacU galK str*^{R} *hsdR*; strain X9368 (Banuett et al., 1986) is MH1 *hflA*::Tn5; strain KS303 (Strauch & Beckwith, 1988) is Δ*lac*X74 *galE galK rpsL* (*str*^{R}) Δ*phoA lpp*5508; strain KS476 (Strauch & Beckwith, 1988) is KS303 *deg*P::ΔKan; strain UT2300 (Earhart et al., 1979) is *leu proC trpE thiA rpsL* Δ(*fepA*); strain UT5600 (Earhart et al., 1979) is UT2300 Δ(*fepA*–*ompT*).

Plasmid pRB104 is an ampicillin-resistant derivative of pBR322 that contains a gene encoding the N-terminal 102 residues of λ repressor (1–102), transcribed from an inducible p_{tac} promoter (Breyer, 1988). The 1–102 gene contains several unique restriction sites to allow cassette mutagenesis. Plasmid pRB104 also contains an M13 origin of replication to facilitate sequencing. Plasmid pDP160 is a pRB104 derivative in which the *Nhe*I–*Eco*RI fragment of the 1–102 gene is replaced by an unrelated "stuffer" fragment. Plasmid pLC57 is a pRB104-derived plasmid containing a Leu → Cys substitution at residue position 57 of the N-terminal domain (Parsell & Sauer, 1989). Plasmid pMH236 is a pBR322-based plasmid that contains the gene for intact λ repressor (residues 1–236) expressed from a P_{tac} promoter (Hecht et al., 1986). Plasmid pKB252 is a pMB9-derived plasmid that contains the λ O_R operator region (Backman et al., 1976).

Mutagenesis. A *Nhe*I–*Xho*I oligonucleotide cassette was designed to randomize residue position 78 of the N-terminal domain, by use of the inosine pairing method described previously (Reidhaar-Olson & Sauer, 1988). The cassette was ligated (Maniatis et al., 1982) to the large *Nhe*I–*Xho*I fragment of pDP160. The DNA was transformed (Hanahan, 1983) into strain X90, and transformants were selected for ampicillin resistance. Single-stranded plasmid DNA from 15 candidates was sequenced by the dideoxy method (Sanger et al., 1977), and 10 different amino acid substitutions at position 78 were recovered. In the case of the PA^{78} mutant, the entire 1–102 gene was sequenced, and no additional mutations were observed.

The 1–102 genes containing the GA^{46} and GA^{48} mutations were constructed by ligating the large *Nsi*I–*Sca*I fragment of pRB104 bearing either the Pro^{78} or Ala^{78} codon to the small *Nsi*I–*Sca*I fragment of pMH236 bearing the GA^{46}GA^{48} double mutation (Hecht et al., 1986). The gene containing the LC^{57}PA^{78} double mutation was constructed by ligating the large *Eco*RI–*Pst*I fragment of pRB104 bearing the PA^{78} mutation to the small *Pst*I–*Eco*RI fragment of pLC57.

To test whether the Pro^{78} → Ala substitution creates a specific proteolytic cleavage site, we constructed a gene in which either the wild-type or mutant sequence for residues 73–83 was introduced into a region encoding a C-terminal extension fused to the wild-type 1–102 gene (Bowie & Sauer, 1989). Oligonucleotide cassettes encoding the wild-type and mutant 73–83 sequences were inserted into the *Sfi*I–*Bss*HII backbone of plasmid pl–102LT (Bowie & Sauer, 1989). This plasmid is a pRB104 derivative that encodes the 1–102 protein fused to a 26 amino acid C-terminal extension. The construction replaces residues 9 and 10 of the C-terminal extension with residues 73–83 of the N-terminal domain. The sequence of the resulting 35-residue C-terminal extension (using the one-letter abbreviations for the amino acids) is R-K-V-E-A-P-T-A-V-E-E-F-S-X-S-I-A-R-E-V-R-A-S-V-V-S-K-S-L-E-K-N-Q-H-E, where X is either proline or alanine.

Activity Screens. Repressor activity in strains bearing mutant N-terminal domain genes was determined by spotting cell lawns with 2×10^5 phage from the following set of clear and virulent λ phages: KH54, *c*Ic17, vir, 3v, 4v, 5v, and 6v (Hecht & Sauer, 1985). Spot tests were developed at 37 °C on LB plates containing 100 μg/mL ampicillin. To measure activities at higher protein levels, expression of the N-terminal domain from the P_{tac} promoter was induced by including 100 μM isopropyl thio-β-D-galactoside (IPTG; Bachem) in the plates.

Determination of Steady-State Protein Levels. E. coli cells bearing plasmids encoding wild-type 1–102 or position 78 variants were grown to an OD_{600} of 1.0 at 37 °C in LB broth supplemented with 150 μg/mL ampicillin. IPTG was then added to a final concentration of 1 mM to induce P_{tac}-mediated transcription of the variants, and cells were grown for an additional 3 h. After being harvested, cells were lysed by boiling in Laemmli sample buffer, and lysates were electrophoresed on 15% SDS–polyacrylamide gels (Laemmli, 1970). When steady-state levels were measured in the *dnaJ*$^{-}$ strain DP748, the procedure above was followed except that cells were grown at 30 °C in M9 minimal medium supplemented as described for the pulse–chase experiments.

Protein Purification. A proteolytically deficient strain, DP748, bearing the appropriate plasmids was used as a source of wild-type 1–102 and mutant proteins PA^{78}, GA^{46}GA^{48}, and GA^{46}GA^{48}PA^{78}. Cells were grown at 30 °C to an OD_{600} of approximately 0.4 in M9 minimal medium (Miller, 1972) supplemented with 0.2% casamino acids, 1.7 μg/mL thiamin and 25 μg/mL ampicillin. Expression of the wild-type and mutant 1–102 proteins was induced by addition of 0.1 mg/mL IPTG, and cells were grown for an additional 4 h at 30 °C. Wild-type and mutant 1–102 proteins were purified by poly(ethylenimine) and ammonium sulfate precipitations, followed by chromatography on Affi-Gel Blue (Bio-Rad), Bio-Rex 70 (Bio-Rad), and Sephadex G-75 (Pharmacia) as described (Sauer et al., 1986). Each of the purified proteins was at least 98% pure as judged by electrophoresis on SDS gels (Figure 2B, lanes 9–12).

Thermal and GuHCl Denaturation Monitored by Circular Dichroism. Circular dichroism (CD) measurements were made on an AVIV Model 60DS spectropolarimeter. Thermal denaturation experiments were performed at protein concentrations of 50 μg/mL in 50 mM potassium phosphate (pH 7.0)–100 mM KCl. Denaturation was monitored at 222 nm, and the temperature was varied from 5 to 80 °C in 1 °C steps. Samples were equilibrated for 1 min at each temperature, and the signal was recorded for 1 min. When the samples were cooled to 20 °C following the melt, the ellipticity returned to 60–75% of the starting value, indicating only partial reversibility of the thermal denaturation.

Guanidine hydrochloride (GuHCl; Pierce Chemical Co.) denaturation experiments were performed at protein concen-

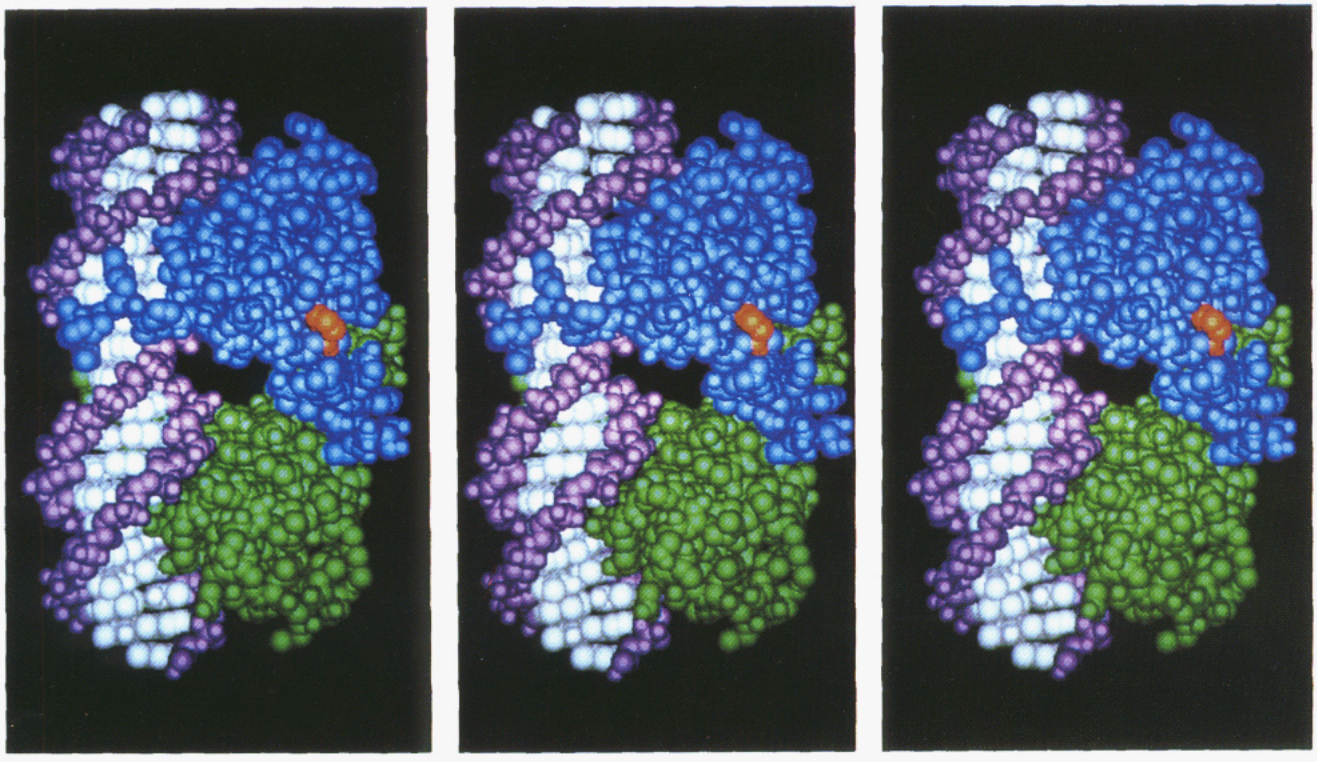

FIGURE 1: Stereoview of a dimer of the N-terminal domain of λ repressor bound to operator DNA. The monomers are blue and green, and Pro^{78} is red. These graphics were produced from coordinates provided by Jordan and Pabo (1988).

trations of 50 μg/mL in 50 mM potassium phosphate–100 mM KCl. The GuHCl concentration was varied from 0 to 4 M, and the pH was 6.6–6.8 throughout the denaturation transition region for each protein. Samples were incubated at each GuHCl concentration for at least 1 h at room temperature prior to transfer to the CD instrument and were then incubated at 20 °C until the ellipticity was constant (≤3 min). The signal was then recorded for 2 min. The CD spectra for proteins at the intermediate GuHCl concentration of 2 M were identical whether the protein sample began in the native (no GuHCl) or denatured (4 M GuHCl) state, indicating that the denaturation reaction is fully reversible.

Operator DNA Binding. DNA binding experiments were performed with the λ O_R operator region contained on a 368 base pair *Eco*RI–*Nsi*I restriction fragment from pKB252. The fragment was end-labeled with the Klenow fragment of DNA polymerase I (Boehringer-Mannheim) and [α-^{32}P]dATP (Amersham) and then purified by electrophoresis on a 5% polyacrylamide gel. DNase I protection experiments were performed at room temperature in 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1.5 mM $CaCl_2$, 0.1 mM EDTA, 50 mM KCl, 1 mM dithiothreitol, 25 μg/mL sonicated salmon sperm DNA, and 100 μg/mL bovine serum albumin (Sigma). Wild-type 1–102 and mutant PA^{78} proteins were incubated with labeled operator DNA (<0.3 nM) for 1 h at room temperature. DNase I (Boehringer-Mannheim) was then added to a final concentration of 2.4 ng/mL, and the samples were incubated at room temperature for 15 min. The DNA was ethanol precipitated and run on 6% polyacrylamide/urea gels. Gels were autoradiographed with Kodak X-OMAT film, and band intensities in the O_R1 operator site were measured on an LKB 2202 UltroScan laser densitometer. Band intensities within the protected region were normalized to the intensity of an unprotected band outside the operator.

Pulse–Chase Experiments. Cultures of *E. coli* strain X90 bearing plasmids encoding wild-type and mutant variants of the N-terminal domain were grown at 37 °C to an OD_{600} of 0.2 in M9 minimal medium supplemented with ampicillin (150 μg/mL), thiamin (1.7 μg/mL), and each of the 20 amino acids except methionine and cysteine. P_{tac}-mediated transcription of the N-terminal domain was induced with 1 mM IPTG. Twenty minutes after induction, cultures were pulse-labeled with 37.5 μCi of L-[^{35}S]methionine (Amersham; sp act. > 800 Ci/mmol) per milliliter of cells. After 2 min, cultures were chased with excess unlabeled methionine (1.4 mg/mL). At various times after this, 0.5-mL portions of cells were removed and added to 35 μL of a mixture of ice-cold protease inhibitors (60 mM phenylmethanesulfonyl fluoride, 30 mM *N*-ethylmaleimide, and 80 mM sodium azide). A 100-μL aliquot of a saturated culture of unlabeled cells was added to each sample to facilitate recovery during centrifugation. Cells were lysed by boiling in 200 μL of SDS sample buffer, and lysates were electrophoresed on 15% SDS–polyacrylamide gels (Laemmli, 1970). Gels were autoradiographed with Kodak X-OMAT film, and band intensities were measured on an LKB 2202 UltroScan laser densitometer.

Protease Digestions in Vitro. Wild-type or mutant 1–102 proteins were dissolved in 50 mM potassium phosphate (pH 7.0)–100 mM KCl at concentrations of 50, 75, and 100 μg/mL. Samples were equilibrated at 37 °C in the CD instrument until the ellipticity remained constant. Thermolysin (Sigma) was then added to a final concentration of 3.3 μg/mL. The ellipticity at 222 nm was monitored as a function of time, and rates of digestion were calculated from the initial linear portions of the reaction curves. In some cases, degradation was also assayed by monitoring cleavage of 1–102 on SDS gels. Similar rates were measured by the CD and gel experiments. Susceptibilities to proteolysis by trypsin (Millipore), chymotrypsin (Worthington), proteinase K (Sigma), and clostripain (Boehringer-Mannheim) were determined by CD experiments as described for thermolysin.

RESULTS

Position 78 Mutants. To investigate the apparent requirement for proline at position 78, we randomly mutagenized

Table I: Intracellular Activities of N-Terminal Domain Variants[a]

residue at position 78	activity level: uninduced	activity level: induced
In Wild-Type Background		
Pro	++	+++++
Glu	–	++++
Thr	–	+++
Ala	–	++
Asn	–	++
Gly	–	++
Leu	–	++
Phe	–	++
Tyr	–	++
Arg	–	+
Cys	–	–
In $GA^{46}GA^{48}$ Background		
Pro	++	+++++
Ala	–	++++

[a] Immunity levels are as follows: (+) resistance to phage λ KH54; (++) resistance to λ *c*Ic17; (+++) resistance to λ vir; (++++) resistance to λ 4v; (+++++) resistance to λ 6v; (–) sensitivity to KH54.

this codon in the gene encoding repressor residues 1–102 and picked 15 unselected candidates for DNA sequence analysis. Table I shows the ten different amino acid substitutions that were recovered. The intracellular activities of these position 78 mutants were assayed in *E. coli* strain X90 under conditions of low and high transcription from the P_{tac} promoter. Under repressed conditions, where transcription levels are low, none of the mutants displayed any repressor activity (Table I). Under derepressed conditions, where transcription levels are high, all but one of the mutants exhibited some activity; however, all were less active than wild type (Table I). These results confirm the conjecture that Pro^{78} plays an important role of some kind in mediating the intracellular activity of the N-terminal domain of λ repressor.

SDS gel electrophoresis was used to assay the intracellular levels of the mutant proteins in lysates of X90 cells grown under derepressed conditions. As shown in lane 2 of Figure 2A, the wild-type 1–102 protein is present at approximately 2% of the total cellular protein. Each of the position 78 mutant proteins is present at levels that are at least 10-fold lower; lanes 3–6 of Figure 2A show these data for the Ala, Cys, Leu, and Arg variants. The reduced steady-state levels must contribute to the lower activity observed for the mutant proteins in the cell.

To investigate the biochemical properties of a protein bearing a position 78 mutation, we chose the mutant containing the Pro → Ala substitution, PA^{78}, for further characterization. The intracellular level of the PA^{78} variant in strain X90, grown in rich medium at 37 °C, was too low to allow purification (Figure 2A, lane 3). We found, however, that higher levels of the mutant protein could be obtained in the *dnaJ*⁻ strain DP748, grown in minimal medium at 30 °C (Figure 2A, lane 7). Using these cells as a source of protein, we were able to purify the PA^{78} protein to homogeneity (Figure 2B, lane 10). As a control, wild-type 1–102 protein was also purified (Figure 2B, lane 9) from strain DP748 grown under the same conditions. The stability properties of the wild-type 1–102 protein (discussed below) are the same whether it is purified as described here or from strain X90 grown at 37 °C in rich medium (Parsell & Sauer, 1989).

DNA Binding Activity and Thermal Stability of the PA^{78} Mutant. The binding of the wild-type and PA^{78} proteins to the λ O_R1 operator site was assayed by DNase I protection experiments. As shown in Figure 3, the two proteins show virtually identical operator binding with half-maximal binding

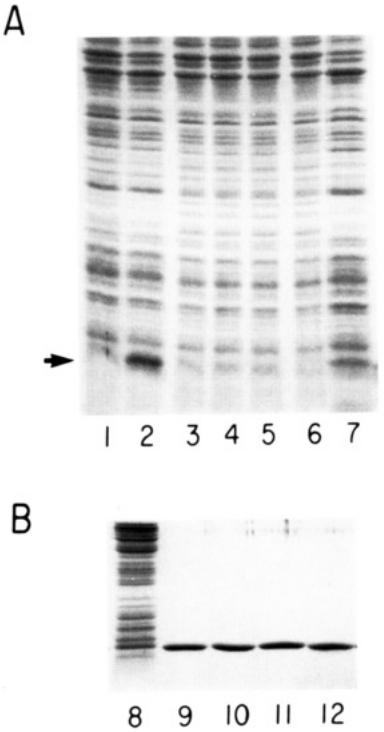

FIGURE 2: (A) Coomassie-stained Laemmli gel showing steady-state levels of position 78 variants. The arrow indicates the position of the 1–102 protein. Lanes 1–6 show the steady-state levels of 1–102 variants in *E. coli* strain X90 grown in LB broth at 37 °C. In lanes 2–6, expression of 1–102 protein was induced by addition of 1 mM IPTG. Lane 1, uninduced; lane 2, wild type; lane 3, PA^{78}; lane 4, PC^{78}; land 5, PL^{78}; lane 6, PR^{78}. Lane 7 shows the induced steady-state level of the PA^{78} protein in *E. coli* strain DP748 grown in minimal medium at 30 °C. These latter conditions were used for protein purification as described under Materials and Methods. (B) Coomassie-stained gel showing purified N-terminal domain variants. Lane 8, lysate showing the steady-state level of PA^{78} protein prior to purification; lane 9, 1 μg of wild-type 1–102 protein; lane 10, 1 μg of PA^{78} protein; lane 11, 1 μg of $GA^{46}GA^{48}PA^{78}$ protein; lane 12, 1 μg of $GA^{46}GA^{48}$ protein.

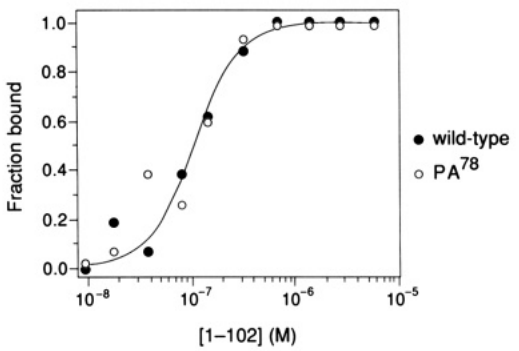

FIGURE 3: DNA binding curves for the wild-type and PA^{78} 1–102 proteins. The data were obtained from DNase I protection experiments by monitoring changes in the intensity of bands in the O_R1 region. Each point is the average of three determinations. The theoretical curve for the second-order reaction $2R + O \rightleftarrows R_2O$ was calculated from $\theta = 1/(1 + [R_{1/2}]^2/[R]^2)$, where θ = fraction bound, [R] concentration of 1–102, and $[R_{1/2}] = 1.1 \times 10^{-7}$ M, the protein concentration at which half-maximal binding is observed.

occurring at a free protein concentration of 1.1×10^{-7} M. Binding of the wild-type and mutant proteins to the O_R2 operator site was also indistinguishable (data not shown). The binding curves in Figure 3 show a second-order dependence on protein concentration. This is expected because the 1–102 protein is predominantly monomeric at these concentrations (Weiss et al., 1987) but binds to single operator sites as a dimer

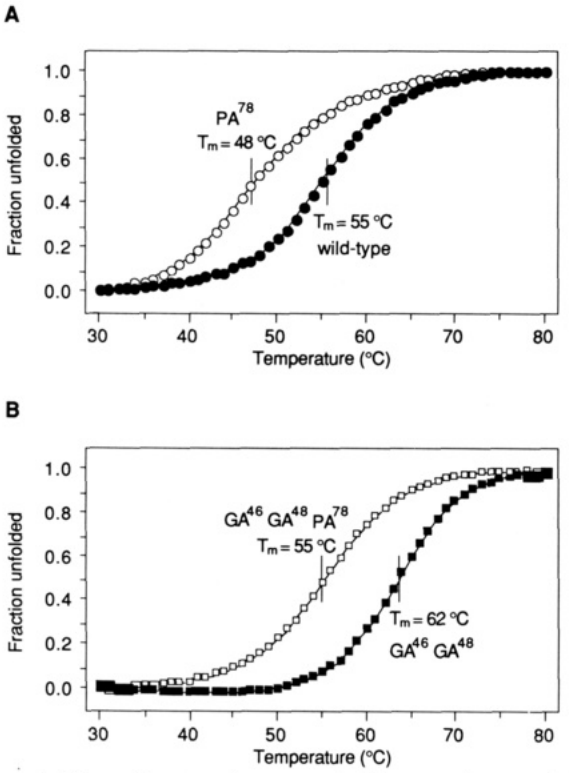

FIGURE 4: Thermal denaturation curves of wild-type and mutant 1–102 proteins. Measurements were made with 50 μg/mL protein in 50 mM potassium phosphate (pH 7.0)–100 mM KCl. Unfolding was monitored by changes in circular dichroism at 222 nm. (A) Wild-type and PA^{78} proteins. (B) $GA^{46}GA^{48}$ and $GA^{46}GA^{48}PA^{78}$ proteins.

(Jordan & Pabo, 1988). The full DNA binding activity of the PA^{78} variant in vitro suggests that both the folded structure and the ability of the protein to form dimers are similar to those of wild type. Therefore, the Pro^{78} side chain is not essential for maintaining an active DNA binding conformation.

The thermal stabilities of the wild-type and PA^{78} proteins were determined with circular dichroism as a probe of the α-helical structure of the N-terminal domain. As shown in Figure 4A, the PA^{78} protein is somewhat less thermally stable than wild type. The mutant has a T_m of 48 °C, whereas the wild-type protein has a T_m of 55 °C. However, both proteins are greater than 90% folded at 37 °C. This result makes it unlikely that unfolding of the PA^{78} protein is the direct cause of its severely reduced intracellular activity.

Susceptibility to Intracellular Proteolysis. The reduced intracellular levels of the position 78 mutants suggest that these variants are rapidly degraded. To test this possibility, pulse–chase experiments were performed to measure the intracellular stability of the PA^{78} mutant. As shown in Figure 5, the PA^{78} protein is degraded rapidly, whereas the wild-type protein shows no degradation after 2 h. The half-life of the PA^{78} protein is approximately 11 min; the half-lives of three other position 78 mutants (PR^{78}, PC^{78}, and PL^{78}) were measured and found to range from 15 to 35 min (data not shown). In previous experiments, the half-life of the wild-type protein was found to be greater than 10 h (Parsell et al., 1990). For technical reasons, the pulse–chase experiments are performed in minimal medium, and thus the degradation rates measured in these experiments cannot be assumed to accurately reflect precise rates of protein turnover in cells growing in rich medium. Nevertheless, the relative proteolytic susceptibilities of N-terminal domain variants measured in pulse–chase experiments are well correlated with their steady-state levels in cells growing in rich medium. Thus, intracellular degradation

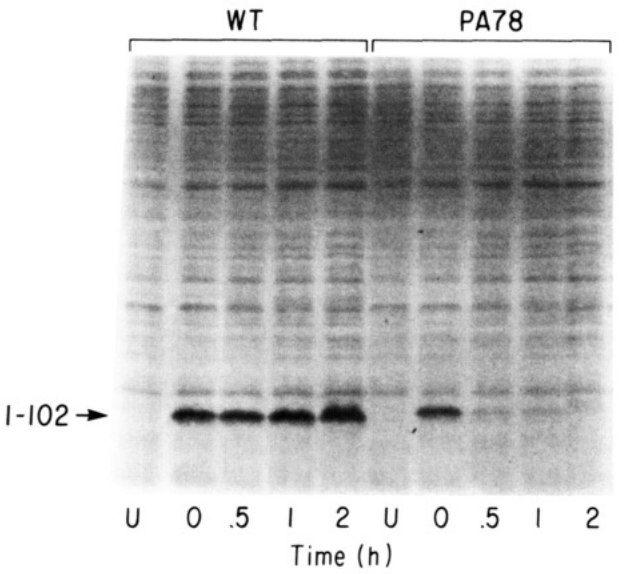

FIGURE 5: Pulse–chase analyses of the degradation of wild-type and PA^{78} 1–102 proteins. The arrow indicates the position of the 1–102 proteins on the autoradiogram. The uninduced lane (U) is a control in which expression of 1–102 protein was not induced by addition of IPTG.

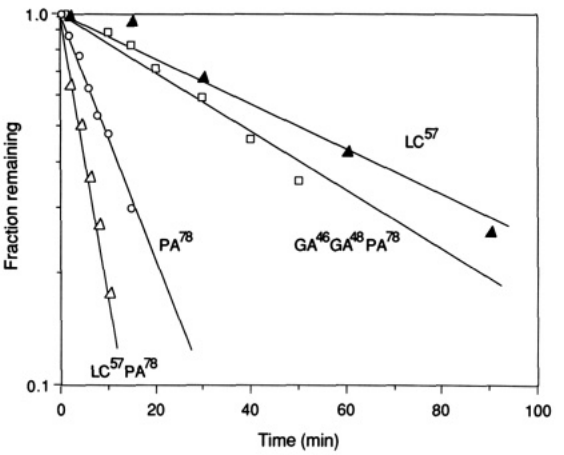

FIGURE 6: Intracellular degradation of the LC^{57}, $GA^{46}GA^{48}PA^{78}$, PA^{78}, and $LC^{57}PA^{78}$ 1–102 proteins. These data were obtained from pulse–chase experiments similar to those shown in Figure 5. Half-life values are 50 min (LC^{57}), 40 min ($GA^{46}GA^{48}PA^{78}$), 11 min (PA^{78}), and 4 min ($LC^{57}PA^{78}$).

appears to be a major cause of the phenotypic defects observed for the position 78 mutants.

Relation of Thermal Instability to Rapid Degradation. On the basis of previous studies of thermally unstable variants of the N-terminal domain (Parsell & Sauer, 1989), it seemed likely that the decreased thermal stability of the PA^{78} protein is at least partially responsible for its increased susceptibility to intracellular proteolysis. By this model, increasing the thermal stability of the PA^{78} protein should reduce its rate of degradation. To test this, we introduced into the mutant two Gly → Ala substitutions, at positions 46 and 48, which are known to increase the T_m of the N-terminal domain (Hecht et al., 1986; Stearman et al., 1988). The triple-mutant protein $GA^{46}GA^{48}PA^{78}$ was purified (Figure 2B, lane 11) and found to be more thermally stable than the PA^{78} protein (cf. Figure 4). In fact, the melting curves measured for the wild-type and $GA^{46}GA^{48}PA^{78}$ proteins are indistinguishable. Thus, the two Gly → Ala mutations compensate almost exactly for the reduced thermal stability caused by the Pro^{78} → Ala substitution.

Pulse–chase experiments were performed to determine the intracellular half-life of the $GA^{46}GA^{48}PA^{78}$ protein. As shown

Table II: Relative Half-Lives of N-Terminal Domain Variants in Proteolytically Deficient Strains[a]

strains	relevant genotypes	relative half-lives PA[78]	relative half-lives GA[46]GA[48]PA[78]
AP401/X90	*lon*$^-$/*lon*$^+$	1.8	1.0
SG21118/MC4100	*clpA*$^-$/*clpA*$^+$	1.7	1.1
X9368/MH1	*hflA*$^-$/*hflA*$^+$	1.0	0.8
KS476/KS303	*degP*$^-$/*degP*$^+$	1.3	1.4
UT5600/UT2300	*ompT*$^-$/*ompT*$^+$	0.5	2.1
DP748/DP9270	*dnaJ*$^-$/*dnaJ*$^+$	2.9	1.1

[a] Each value is the ratio of the half-lives of the N-terminal domain variants in the two strains indicated. The *lon*, *clpA*, *hflA*, *degP*, and *ompT* alleles encode proteases, whereas the *dnaJ* allele encodes a heat-shock protein that is thought to play an auxiliary role in proteolysis. Many of the pulse–chase experiments were performed only once, and thus, the reproducibility of these ratios is not certain.

in Figure 6, this triple mutant has a half-life of about 40 min. Although this is about 4 times longer than the half-life of the PA[78] protein, the GA[46]GA[48]PA[78] protein is still degraded much more rapidly than wild type and still has a defective phenotype in the cell (Table I). These results suggest that while the decreased thermal stability of the PA[78] protein contributes to its degradation, this global thermal instability cannot be the sole cause of its increased sensitivity to proteolysis. We note that the proteolytic instability of the triple mutant is not likely to be directly influenced by the GA[46]GA[48] substitutions, as the GA[46]GA[48] protein has an intracellular half-life at least as long as wild type (data not shown).

Because the GA[46]GA[48]PA[78] protein is more thermally stable than the PA[78] mutant, a smaller fraction of the GA[46]GA[48]PA[78] protein will be unfolded at 37 °C. The 4-fold reduction in the degradation rate of the GA[46]GA[48]PA[78] variant relative to that of the PA[78] protein is consistent with the idea that the denatured form of these proteins is the target for proteolysis. If this is the case, then it should be possible to increase the proteolytic susceptibility of the PA[78] protein by introducing additional mutations that decrease thermal stability. A 1–102 variant containing a Leu → Cys substitution at position 57 has a T_m of 35 °C, which is 20 °C lower than that of the wild-type protein (Parsell & Sauer, 1989). The double mutant LC[57]PA[78] was constructed, and its half-life was determined by a pulse–chase experiment. As shown in Figure 6, the LC[57]PA[78] protein is degraded more rapidly than the PA[78] protein, as expected if degradation of these variants proceeds from the denatured form.

Effects of Cellular Mutations That Affect Proteolysis. To assess the possible roles of cellular genes in the turnover of the PA[78] and GA[46]GA[48]PA[78] variants, the half-lives of these proteins were measured in isogenic protease-competent and protease-deficient strains (Table II). In some of the strains, a small (2–3-fold) effect is observed, but in no case is a significant stabilization of both variants observed. These results indicate that none of the tested alleles plays a major role in degradation of the N-terminal domain proteins containing the PA[78] substitution. Moreover, because of possible pleiotropic effects, stabilization per se cannot be taken as evidence for a direct role. For example, an approximate 2-fold stabilization of the GA[46]GA[48]PA[78] variant is observed in the *ompT*$^-$ strain, but since OmpT is an outer membrane protease, it is unlikely to be directly involved in turnover of cytoplasmic proteins.

Susceptibility to Protease Digestion in Vitro. The results presented so far suggest that degradation of mutant proteins containing the Pro[78] → Ala substitution proceeds from the denatured form. However, the increased susceptibility to intracellular proteolysis does not appear to be caused solely by an increase in the concentration of denatured protein. How

Table III: Thermodynamic Stability and Proteolytic Susceptibility of N-Terminal Domain Variants

protein	T_m (°C)	$t_{1/2}$ in vivo (min)	relative k_{cat}/K_m for thermolysin[a]	$\Delta G°_u$ (kcal/mol)	m [kcal/(mol·M)]
PA[78]	48	11	9.0	3.6	2.1
wild type	55	>120	1.0	5.2	2.3
GA[46]GA[48]PA[78]	55	40	1.3	5.4	2.2
GA[46]GA[48]	62	>120	0.2	6.9	2.4

[a] The value of k_{cat}/K_m for wild type is 2.1×10^4 M^{-1} s^{-1}.

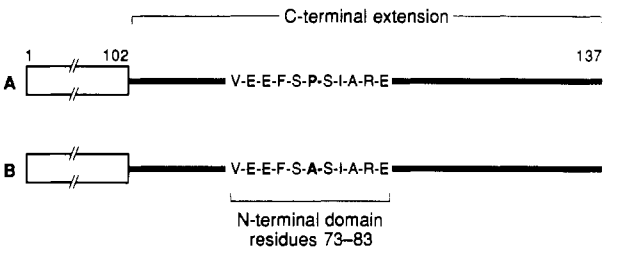

FIGURE 7: N-Terminal domain variants containing C-terminal extensions. N-Terminal domain variants in which the local sequence around Pro[78] (A) or Ala[78] (B) is inserted into a C-terminal extension fused to the 1–102 protein.

then does the mutation increase susceptibility to degradation? One possibility is that the Pro[78] → Ala substitution alters the denatured polypeptide chain in a way that makes it generally more accessible to proteases. To test this idea, we measured the rates at which the purified wild-type and mutant proteins were degraded by proteases such as thermolysin, which preferentially digests the denatured form of the N-terminal domain (Hecht et al., 1983). The PA[78] protein, which is less thermally stable than wild type, is digested significantly faster, whereas the GA[46]GA[48] variant, which is more stable, is digested significantly more slowly (Table III). However, the key result is that wild type and the triple mutant GA[46]GA[48]PA[78], which have comparable thermal stabilities, are digested at similar rates. These results suggest that the denatured forms of the wild-type and mutant proteins are roughly equivalent in terms of their suitability as substrates for thermolysin. The wild-type and GA[46]GA[48]PA[78] proteins were also digested at similar rates by chymotrypsin, proteinase K, and clostripain (data not shown). These results indicate that the Pro[78] → Ala substitution does not make the denatured protein a better substrate for proteases in general.

Does the Pro[78] Substitution Create a Cleavage Site? Dipeptide sequences containing proline are often resistant to cleavage by model proteases (Beynon & Bond, 1989). For this reason, we considered the possibility that changing Pro[78] to any other amino acid might create a cleavage site for some cellular protease. As a test of this model, we constructed the 137-residue N-terminal domain variants shown in Figure 7. In these variants, the sequence around position 78 was introduced into a C-terminal extension fused to the wild-type N-terminal domain gene (Bowie & Sauer, 1989; Parsell & Sauer, 1990). The C-terminal extension is unstructured, and thus sequences within this region should be accessible to proteases. If a specific cleavage occurred at or near the Ser-**Ala**-Ser sequence in the mutant, the protein would be cleaved to a protein of about 116 residues. To test for this, we performed pulse–chase experiments but observed no cleavage of either 137-residue fusion protein over the course of 2 h (data not shown). Thus, the Pro[78] → Ala substitution does not create a local site for proteolytic cleavage that is recognized within the context of this unstructured region of the polypeptide chain.

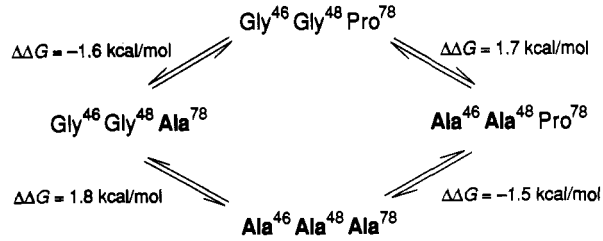

FIGURE 8: Thermodynamic cycle showing differences in the free energy of unfolding among wild-type 1–102 and mutants PA^{78}, $GA^{46}GA^{48}$, and $GA^{46}GA^{48}PA^{78}$. $\Delta\Delta G$ values were calculated by taking the differences between $\Delta G°_u$ values presented in Table III.

GuHCl Denaturation Studies. To quantitate the effects of the PA^{78}, $GA^{46}GA^{48}$, and $GA^{46}GA^{48}PA^{78}$ mutations on thermodynamic stability, GuHCl denaturation experiments were performed for the wild-type and mutant proteins. The free energy of unfolding, ΔG_u, was determined for each protein at 20 °C as a function of denaturant concentration. The data were least-squares fit to

$$\Delta G_u = \Delta G°_u - m[\text{GuHCl}]$$

where $\Delta G°_u$ is the free energy of unfolding extrapolated to 0 M GuHCl (Schellman, 1978; Pace, 1986). As expected, the wild-type and $GA^{46}GA^{48}PA^{78}$ proteins have comparable values of $\Delta G°_u$, whereas PA^{78} is less stable and $GA^{46}GA^{48}$ is more stable than wild type (Table III). Figure 8 shows a thermodynamic cycle relating the stabilities of the four proteins. The near equivalence of the cross terms in the cycle indicates that the effects of the destabilizing Pro → Ala mutation and the two stabilizing Gly → Ala substitutions are independent (Carter et al., 1984).

As shown in Table III, the PA^{78} substitution also decreases the value of m by 0.2 kcal/(mol·M) in both the wild-type and $GA^{46}GA^{48}$ backgrounds. Changes in the value of m have been proposed to reflect differences in the relative solvent accessibility of the native and denatured forms (Schellman, 1978; Shortle & Meeker, 1986). Interpreted in this way, proteins containing the Pro^{78} → Ala substitution would be expected to have a more compact denatured form than the corresponding proteins containing proline at position 78.

Discussion

Strict conservation of a specific residue in a family of protein sequences is often taken as evidence that the residue plays a crucial role, but the precise nature of this role cannot generally be deduced in the absence of biochemical experiments. Our previous studies suggested that Pro^{78} in the N-terminal domain of λ repressor is an essential residue (Reidhaar-Olson & Sauer, 1990). Here, we have confirmed that Pro^{78} is indeed important and have investigated the nature of the defect when mutations are introduced at this position. We find that a protein bearing the Pro^{78} → Ala substitution has wild-type activity but differs from the wild-type protein in two ways. First, the folded structure of the mutant protein is somewhat less stable than that of wild type. Second, and most importantly, the mutant protein is a much better substrate for intracellular proteolysis than wild type. Even when the thermal instability of the mutant is suppressed by additional mutations, the protein is still subject to rapid degradation in the cell. Hence, we conclude that Pro^{78} is essential primarily because it protects the protein from intracellular proteolysis. Similar mechanisms may operate in cases of conserved or invariant residues for other proteins as well.

The results seen here make it difficult to argue that a residue is *directly* required for protein folding, structure, or activity simply because it is conserved within a family of sequences. Another example is seen in the cytochrome *c* family, where phylogenetic comparison of over 90 sequences indicates that lysine is highly conserved at position 32. However, when this lysine is replaced by leucine or glutamine, the thermodynamic stability of the protein is unchanged (Hickey et al., 1988), and the variants are fully active in vitro (Das et al., 1988). Surprisingly, mutants bearing these substitutions also seem to function at very near wild-type levels in vivo (Hickey et al., 1988). This could indicate that natural selection can discriminate on the basis of very small activity differences or that the residue is needed only under some special set of growth or environmental conditions. For example, the residue might be required for resistance to proteolysis under certain stress conditions.

Why are position 78 variants of the N-terminal domain of λ repressor degraded so rapidly? Several factors are known to affect the degradation of the N-terminal domain in vivo. These include both thermal stability and the C-terminal sequence of the protein (Parsell & Sauer, 1989; Parsell et al., 1990). However, in the case of the $GA^{46}GA^{48}PA^{78}$ protein, the thermal stability and C-terminal sequence are identical with those of wild type. Consequently, some other aspect of the structure or sequence of position 78 variants must be responsible for their increased rates of degradation. On the basis of double-mutant studies, the degradation of the PA^{78} variants seems to proceed primarily from their denatured forms. It could be argued that a locally unfolded region of the otherwise native PA^{78} protein is recognized by the proteolytic machinery of the cell, with global unfolding of the protein being required in some subsequent, rate-limiting step of the degradation reaction. However, because Pro^{78} is the first residue of the dimerization helix (Pabo & Lewis, 1982), it seems improbable that this region could be locally unfolded without affecting dimerization and therefore decreasing DNA binding. Since the PA^{78} protein shows wild-type DNA binding, we consider this model unlikely.

The PA^{78} substitution may alter some structural feature of the denatured protein, making it an intrinsically better substrate for proteolysis. In several proteins, a molten-globule state has been observed under denaturing conditions (Dolgikh et al., 1981; Ohgushi & Wada, 1983; Goto & Fink, 1989). This form is more compact than a true random coil and usually contains elements of secondary structure. It is possible that under physiological conditions the denatured state of the N-terminal domain actually contains a significant degree of structure. In the context of such a model, we considered the possibility that the denatured PA^{78} mutant might have a conformation different from that of wild type that makes it more proteolytically susceptible. The fact that the wild-type and $GA^{46}GA^{48}PA^{78}$ proteins are degraded at the same rate by thermolysin or any of several other proteolytic enzymes tested in vitro argues against a generally more extended structure in the denatured state of the mutant protein. In fact, the m values from GuHCl denaturation experiments (Table III) suggest that the denatured form of the PA^{78} protein is actually more compact than wild type, consistent with the possibility that it is somewhat more structured. By this model, structural elements within the denatured state of proteins containing the Pro^{78} → Ala substitution could be recognized by an *E. coli* protease or auxiliary degradation factor.

We also considered the possibility that substitutions at position 78 create a specific cleavage site for an intracellular protease. The results of experiments in which the local sequence around either Pro^{78} or Ala^{78} was introduced into an

unstructured region of the polypeptide chain suggest that this is not the case. However, it is possible that a specific cleavage site depends on the presence of a larger region of the local sequence than was tested in this experiment. A third possibility is that the position 78 variants fold slowly in the cell and as a consequence are rapidly degraded. We cannot rigorously exclude this kinetic model, but the wild-type and PA^{78} proteins refold completely in vitro within 10 s at 37 °C, following dilution from GuHCl (unpublished observations). Unless folding occurs much more slowly in vivo, one would need to argue that the mutant protein is committed to degradation on a time scale that is much faster than the observed degradation rate in the cell.

At 20 °C, the folded structure of the PA^{78} mutant is 1.6 kcal/mol less stable than that of the wild-type protein. Since the mutant protein is fully active in DNA binding, its folded structure is likely to be similar to that of wild type. The decreased stability of the mutant protein can be rationalized most simply if we assume that the folded proteins have similar free energies and that the stability changes arise from differences in the free energies of the denatured states. Since the proline side chain is covalently bonded to the backbone amide nitrogen, the available repertoire of ϕ angles is greatly reduced in comparison to that of other amino acids. As a result, replacing proline with alanine (or any other residue) should increase the conformational entropy of the denatured state, thereby decreasing the free energy difference between native and denatured protein (Matthews et al., 1987). In other words, the entropic cost of folding will be lower for the wild-type Pro^{78} protein than for the Ala^{78} mutant.

It is possible to make a rough calculation of the magnitude of the energetic effect expected when a proline residue is replaced by alanine. The proline ϕ angle is limited to 60° ± 15° (Balasubramanian et al., 1971). Therefore, the area of a ϕ–φ map available to proline is about one-eighth the area available to alanine. The entropic contribution to the free energy of unfolding is given by

$$\Delta G_{conf} = -T\Delta S_{conf} = -R \ln N$$

where N is the number of conformational states available to the denatured polypeptide chain. The difference in conformational free energy between the proline and alanine variants is then

$$\Delta\Delta G_{conf} = -R \ln (N^{Ala}/N^{Pro})$$

If the ratio of isoenergetic conformational states is 8, then $\Delta\Delta G_{conf} = -1.2$ kcal/mol at 20 °C. This value is quite close to the experimentally observed $\Delta\Delta G$ value of −1.6 kcal/mol. By this type of analysis, all amino acid substitutions except glycine at position 78 would be expected to destabilize the protein by roughly the same amount. The slightly increased activity of the PE^{78} mutant in vivo compared to that of the other position 78 variants (Table I) may be due to a stabilizing effect afforded by favorable interaction of the negatively charged glutamate side chain with the helix dipole (Shoemaker et al., 1987; Nicholson et al., 1988). Although it is possible that factors besides entropy are responsible for the decreased stability of the PA^{78} protein, it is interesting to note that the stability change of 1.6 kcal/mol caused by this mutation is similar in magnitude to the destabilizing changes observed in cytochrome *c* when Pro^{71} is replaced with valine, threonine, or isoleucine (1.0–1.7 kcal/mol) (Ramdas et al., 1986) and the stabilizing change in T4 lysozyme when Ala^{82} is replaced with proline (0.8 kcal/mol) (Matthews et al., 1987).

The thermal stability of the PA^{78} protein can be increased to the level of the wild-type protein by introducing the $GA^{46}GA^{48}$ double mutation. These latter mutations presumably increase stability by decreasing the conformational entropy of the denatured form of the protein (Hecht et al., 1986). This mechanism is exactly the opposite of that proposed to explain the destabilizing effect of the PA^{78} mutation. The opposing effects of the Pro → Ala mutation and the two Gly → Ala substitutions are independent (Figure 8), as expected if they exert their effects solely through changes in the conformational entropy of the denatured state.

Pro^{78} is the first residue of α-helix 5 in the N-terminal domain of λ repressor (Pabo & Lewis, 1982). Prolines frequently occur at the N-termini of α-helices, and it has been suggested that this may prevent extension of the helix toward the N-terminus (Richardson & Richardson, 1988). In T4 lysozyme, replacement of a proline at the start of an α-helix did in fact lead to extension of the helix, resulting in conformational changes that brought about minor changes in stability and somewhat larger changes in activity (Alber et al., 1988). In the absence of structural data, we cannot exclude the possibility that replacing Pro^{78} allows extension of helix 5. Nevertheless, if this does occur, then it must not affect either dimerization of the N-terminal domain, which is mediated by α-helix 5, or the conformation of the DNA binding surface of the protein. If extension of the helix does not occur, then other structural features must determine the precise extent and geometry of helix 5, even in the absence of proline at position 78.

Although the mechanism remains uncertain, the results presented here clearly indicate that the presence of proline at the first position of α-helix 5 protects the protein from intracellular proteolysis. A similar result has been observed in the λ Cro protein, where a Gln → Pro substitution at the first position of α-helix 3 has been shown to suppress the rapid intracellular degradation of a thermally unstable mutant (Pakula & Sauer, 1989). The proline substitution increased the T_m of the Cro protein by just 3 °C but increased the intracellular half-life of the protein from 11 min to over 5 h. Further experiments will be required to determine whether similar mechanisms operate in the Cro case and the example presented here. It may be that the insertion of prolines at positions where they are tolerated structurally can confer enhanced resistance to intracellular proteolysis to a variety of proteins.

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Registry No. Pro, 147-85-3.

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