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Amino Acid Substitutions That Increase the Thermal Stability of the λ Cro Protein

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ABSTRACT **A mutant Cro protein, which bears the Ile-30→Leu substitution, is thermally unstable and degraded more rapidly than wild-type Cro in vivo. Using an antibody screen, we have isolated five different second site suppressor substitutions that reduce the proteolytic hypersensitivity of this mutant Cro protein. Two of the suppressor substitutions increase the thermal stability of Cro by 12°C to 14°C. These amino acid substitutions affect residues 16 and 26, which are substantially exposed to solvent in the crystal structure of wild-type Cro.**

Key words: enhanced stability, λ Cro, genetic suppression, intracellular proteolysis, antibody screen

INTRODUCTION

The forces that contribute to protein stability have been understood in a general way for some time. Hydrophobic interactions, electrostatic interactions, van der Waals interactions, and hydrogen bonds all play important roles in stabilizing proteins, but it is still extremely difficult to assess the quantitative importance of specific interactions within the context of a folded protein. As a result, it has usually not been possible to predict accurately, from inspection of a protein structure, the effect of any particular amino acid substitution. This inability clearly presents a major obstacle to achieving the goals of rational protein modification and design. To examine the effects of residue substitutions on protein structure and stability, several groups have used genetic analysis to study proteins with known structures. One approach has been to use random mutagenesis to create a large pool of mutant genes, and a genetic screen or selection to identify individual amino acid changes that cause loss of activity in vivo. Among the mutants identified in this way are those that reduce protein stability. This class of substitutions may identify residues that are important for determining and/or stabilizing protein structure.[1–6] With such mutations in hand, reversion analysis can then be used to identify intragenic mutations that restore activity. These may provide information about the nature of the mutant defect or identify alternative means by which protein stability can be improved.[3,7–9] The advantage of using random mutagenesis for this type of analysis is that large numbers of amino acid changes can be surveyed in a rapid and relatively unbiased way, and interesting, useful, or surprising substitutions may be chosen for further analysis.

We have been studying the bacteriophage λ Cro protein, a small, sequence-specific DNA binding protein that acts as a dimer.[10,11] Its crystal structure has been determined[12] and the structure of its complex with operator DNA has been predicted based on model building studies.[13] In previous studies, we isolated a large collection of randomly generated Cro-defective mutations and observed that substitutions of structurally important residues, such as those in the hydrophobic interior of Cro, generally resulted in proteins that were rapidly degraded in vivo.[5] This observation led us to suggest that reduced stability of the folded structure of Cro was the cause of the increased proteolytic susceptibility of this class of mutants. Thus, we suspected that the rate of intracellular turnover could serve as a convenient indicator of thermal stability, and that substitutions that increase thermal stability could be isolated as second site suppressors of mutations that cause proteolytic sensitivity.

In this paper, we describe the isolation of intragenic mutations that suppress the rapid degradation of an unstable mutant Cro protein. Among these second-site mutations are two substitutions that dramatically increase the thermal stability of the revertant proteins. The extent to which these substitutions stabilize Cro is somewhat surprising since they affect surface residues, which, as a class, are usually thought to play relatively minor roles in the maintenance of protein structure.

MATERIALS AND METHODS

Plasmids and Bacteria

Escherichia coli strain X90[15] bears an F′ episome carrying the Lac repressor-overproducing allele *lacI*^{Q}. Strain X9T was derived from X90 by selection for resistance to a bacteriophage (probably T1) that

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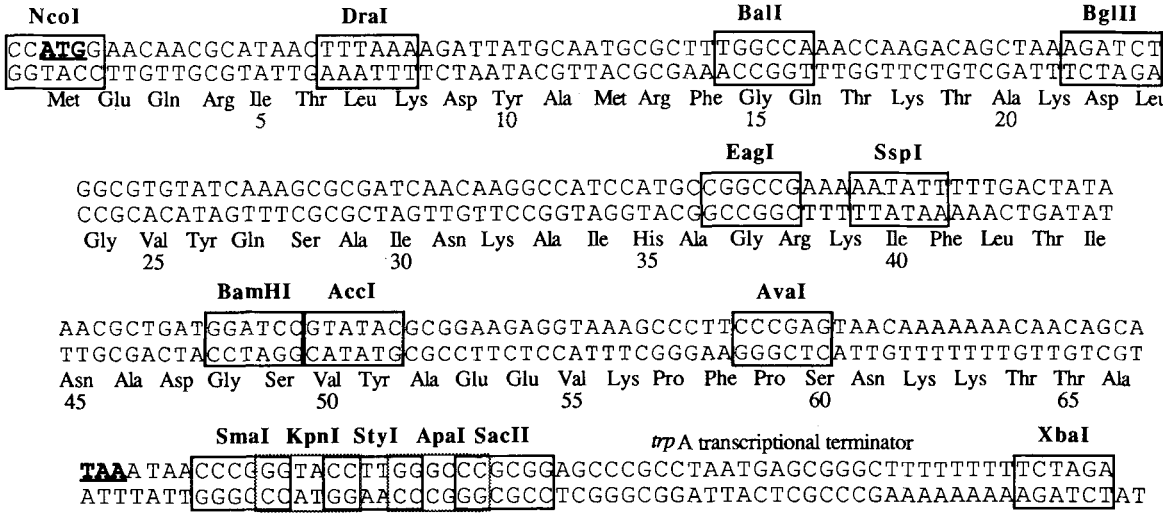

Fig. 1. DNA and amino acid sequences of the synthetic *cro* gene. The translational initiation and stop codons are underlined and shown in bold characters. Restriction endonuclease recognition sequences are boxed. The transcriptional terminator from *trp*A consists of the base pairs between the *Sac*II and *Xba*I restriction sites.

contaminated the laboratory fermenters.[14] *E. coli* strain DB4729 is *endoI*$^-$, r_K^-, m_K^+, *thi*$^-$, *su2*$^+$, Δ(*srl-recA*) (obtained from D. Botstein). Plasmid pAP104[5] is a pBR322 derived plasmid that carries the λ *cro* gene under *lac* promoter control, confers resistance to ampicillin and tetracycline, and contains both an M13 and colE1 origin of replication. The presence of the M13 origin causes single-stranded plasmid DNA to be made and packaged upon infection with an M13 helper phage, such as RV1. Plasmid pAP5M is a derivative of pAP104, in which the *lac* promoter has been replaced by the *tac* promoter as described.[15] Plasmid pAP114 is a derivative of pAP5M in which the sequences between the *Eco*RI and *Nde*I sites of pBR322 were deleted and the *cro* sequences from the *Bgl*II site to the end of the gene have been replaced by the sequences from the synthetic gene shown in Figure 1. Plasmid pAP119 was derived from pAP114 by introducing the *Nco*I site shown in Figure 1. This was accomplished using a mismatch primer and the oligonucleotide directed mutagenesis kit from Amersham. The synthetic *cro* gene was constructed using automated DNA synthesis and standard cloning procedures. This gene encodes the wild-type amino acid sequence and incorporates a number of restriction enzyme recognition sites that simplify recombination in vitro and cassette mutagenesis.

Affinity Purification of Antibody

A λ Cro-Sepharose column was prepared using CNBr-activated Sepharose 4B essentially as described by the supplier (Pharmacia). Crude rabbit serum containing antibodies raised against wild-type Cro was loaded onto the column, which was washed with PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4) to remove unbound serum proteins. Bound antibodies were eluted with 3 M KSCN and, following dialysis, were stored in PBS with 3 mM NaN_3.

Mutagenesis and Mutant Screen

To isolate intragenic mutations that suppress the rapid degradation of an unstable Cro mutant, we used antibodies to identify colonies in which the Cro antigen was present at higher steady-state concentrations than in the original mutant colonies. Strain DB4729 carrying plasmid pAP104 bearing the IL30 *cro* gene[5] shows little or no antibody reactivity because the mutant IL30 protein is rapidly degraded and thus present at a low concentration. The *cro* gene of this plasmid was mutagenized in vitro using a modification of the precedure of Myers and Maniatis.[16] Single-stranded pAP104 DNA was prepared from transducing particles purified after infection of strain X90/pAP104(IL30) with the phage M13RV1.[17] The resulting DNA includes approximately equal amounts of single-stranded plasmid DNA and helper phage DNA, but the latter does not adversely affect the mutagenesis procedure. Single-stranded DNA was next subjected to one of three mutagenic regimens: (1) Incubation in 1 M $NaNO_2$, 0.2 M NaOAc (pH 4.3) for 5 hours at room temperature; (2) incubation in 0.13 mM $KMNO_4$ for 1 hour at room temperature; and (3) incubation in 25 mM sodium citrate/citric acid buffer (pH 4.8), 100 mM NaCl at 70°C for 3 hours. Following mutagenesis, DNA was recovered by EtOH precipitation and washed twice with 70% EtOH. An oligonucleotide primer that is complementary to a region 3′ to the

cro gene was then annealed to the single-stranded DNA and used to prime second strand synthesis by reverse transcriptase (Bethesda Research Laboratories). Partially double-stranded DNA was recovered by EtOH precipitation and was digested with restriction enzymes *Eco*RI and *Hin*dIII, each of which cuts once in pAP104, to yield a small double stranded fragment that includes the entire *cro* gene and *lac* promoter region. This fragment was isolated by agarose gel electrophoresis and ligated to the unmutagenized backbone fragment of pAP104.

The antibody screening method is a modification of the cDNA library screening procedure described by Helfman et al.[18] Mutagenized DNA was introduced into strain DB4729 by transformation and cells were plated on nitrocellulose filters overlaid on LB agar plates containing 100 μg/ml ampicillin. Colonies were then replicated onto a second filter, and regrown for several hours. One filter of each pair was treated with 0.1 N NaOH to lyse the bacteria. After neutralization with 2 M Tris–HCl (pH 7.0), filters were incubated for several hours in TBS buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl] containing 5% nonfat dry milk, 0.1 M $MgCl_2$, 1 μg/ml DNase I, 40 μg/ml hen egg lysozyme, and 3 mM NaN_3. Filters were washed, wiped clean of visible cell debris, and incubated for 1 hour in TBS containing 5% nonfat dry milk, 0.2% Tween 20, and affinity purified rabbit anti-Cro serum at a 1:500 dilution. Filters were then washed extensively in TBS with 0.2% Tween-20, incubated for 1 hour in TBS containing 5% nonfat dry milk, 0.2% Tween-20, and ^{125}I-labeled protein A (1:500 dilution; Amersham), and washed again. Bound radioactive protein A was visualized by autoradiography. Antibody reactive colonies were located on the unprocessed duplicate filters by comparison with autoradiograms, and were purified twice by restreaking for single colonies and retesting as in the initial screen.

DNA Sequencing

For each antibody-reactive isolate, the sequence of the entire *cro* gene and *lac* promoter region was determined using the dideoxy method.[19] Single-stranded template DNA was obtained from transducing particles in supernatants of M13RV1 infected cultures of X90 or X9T carrying the plasmid of interest. In some cases, the Sequenase dideoxy sequencing kit (US Biochemicals) was used.

Placing Genes Under *Tac* Promoter Control and Construction of Single Mutants

The suppressor mutations were isolated in plasmid pAP104, in which transcription of the *cro* gene is directed by the *lac* promoter. To express large quantities of the mutant or revertant proteins for purification, we placed the variant *cro* genes under the control of the stronger *tac* promoter.[15] For those suppressor mutations located after the naturally occurring *Bgl*II site in the *cro* gene, we isolated the small *Bgl*II–*Hin*dIII fragment from pAP104 and cloned this in place of the corresponding fragment of pAP5M. This places the IL30 mutation and the suppressor mutation in a *cro* gene under *tac* promoter control. To construct *tac*-promoted genes carrying the suppressor mutations alone, we synthesized the appropriate double-stranded cassettes and inserted them into pAP114. For the QL16 suppressor mutation, a cassette was prepared and inserted between the *Nco*I and *Bgl*II sites of pAP119.

Pulse–Chase Experiments

Logarithmic phase cultures of DB4729 carrying plasmids with wild-type or mutant *cro* genes were pulse labeled with [^{35}S]methionine (100 μCi) for 1 minute. Unlabeled methionine was then added to a concentration of 1.4 mg/ml to quench incorporation of the radioactive amino acid. Thereafter, portions were periodically removed, placed on ice, and *N*-ethylmaleimide was immediately added at a concentration of 2 mM to prevent further proteolysis. Cells were pelleted by centrifugation and lysed in 1% SDS. The lysates were resuspended in immunoprecipitation dilution buffer [1.25% Triton X-100, 190 mM NaCl, 60 mM Tris–HCl (pH 7.5), 6 mM EDTA], insoluble material was removed by centrifugation, and affinity purified rabbit anti-Cro antibody was added in molar excess over Cro. After a 30-minute incubation, a suspension of fixed Staph A cells (Cal-Biochem) was added, the mixture was incubated for an additional 30 minutes, and the cells and bound antibody and Cro were collected by centrifugation. The pellet was resuspended in wash buffer [50 mM Tris–HCl (pH 7.5), 5 mM EDTA, 150 mM NaCl] and centrifuged again. Labeled proteins were stripped from the Staph A cells by heating at 90°C for 3 minutes in electrophoresis loading buffer [0.125 M Tris–HCl (pH 6.8), 4% SDS, 20% glycerol, 0.14 M 2-mercaptoethanol]. Immunoprecipitated proteins were separated by electrophoresis in a 20% polyacrylamide gel containing SDS and urea.[20] Following treatment with Autofluor autoradiographic intensifier (National Diagnostics), the gel was dried and the amount of ^{35}S-labeled Cro was quantified by autoradiography and densitometry.

Protein Purification

Cultures of strains X90 and X9T carrying plasmids pAP5M, pAP114, or pAP119 were grown at 37°C in 2 liters of LB broth with 100 μg/ml ampicillin to an optical density of 1.5 at 600 nm. Expression of the wild-type or mutant Cro protein from the *tac* promoter was induced by the addition of 0.01% IPTG (w/v). The induced cultures were grown for an additional 3 hours, and cells were harvested by centrifugation and lysed by sonication. Cell lysis and all subsequent manipulations were performed at 4°C. Polyethylenimine was added to a final concentration

of 0.6% (v/v) and the precipitate was collected by centrifugation. Proteins were precipitated from the supernatant with 90% saturated $(NH_4)_2SO_4$, dialyzed into SB50 buffer [50 mM Tris–HCl (pH 7.0), 0.1 mM EDTA, 5% glycerol, 1.4 mM 2-mercaptoethanol, 50 mM KCl], and loaded onto a 2.5 × 10 cm DEAE Sephacel column (Pharmacia) equilibrated in SB50. The flow-through from this column was loaded directly onto a 1.5 × 8 cm Cellex P column (Bio-Rad Laboratories) equilibrated in SB50. This column was washed thoroughly and proteins were eluted with a gradient from SB50 to SB plus 1 M KCl. Fractions containing Cro were identified by SDS gel electrophoresis. The peak fractions were pooled, concentrated by precipitation with 90% saturated $(NH_4)_2SO_4$, and chromatographed on a 2.5 × 48 cm column of Sephadex-G50 F (Pharmacia).

Thermal Denaturation

Wild-type and mutant Cro proteins at a concentration of 120 μg/ml [in 10 mM KPi (pH 7.0), 0.1 M KCl] were heated at a rate of 0.8°C per minute. Protein unfolding was monitored by recording the changes in ellipticity at 222 nm with a AVIV model 60S circular dichroism (CD) spectropolarimeter. The ellipticity was found to remain constant for long periods when the temperature was held near the T_m, and the melts were reversible. The value of T_m was determined, after baseline correction, as the temperature at which the measured ellipticity is midway between the native and denatured baseline values. The transition from the Cro dimer (N_2) to two unfolded monomers (2U) is a concerted reaction in which the folded monomer appears to be an unpopulated intermediate. As a consequence, the T_m varies with Cro concentration. All unfolding reactions reported here were performed at the same concentration of wild-type or mutant Cro (120 μg/ml). The equilibrium constant for unfolding is defined as $K_u = [U]^2/[N_2]$. $\Delta\Delta G_u$ values are calculated as $\Delta\Delta G_u = -RT \ln[K_u^{mutant}/K_u^{wt}]$ or $-RT \ln[K_u^{double\ mutant}/K_u^{single\ mutant}]$ using values of K_u measured at 45°C.

Fractional solvent accessibilities were calculated using the method of Lee and Richards[21] from the coordinates of the wild-type Cro protein.[12,13]

DNA Binding

The gel shift assay was performed essentially as described[22,23] using a radiolabeled fragment from pKB252[24] generated by digestion with *Bgl*II and *Nsi*I. This fragment contains the λ O_R1, O_R2, and O_R3 operators. The O_R3 site is the strongest binding site for Cro.[11] Binding assays were performed at room temperature in a buffer consisting of 10 mM Tris–HCl (pH 7.0), 200 mM potassium glutamate, 1 mM EDTA, 100 μg/ml bovine serum albumin, and 100 μg/ml sonicated salmon sperm DNA.

RESULTS

Isolation of Suppressor Mutations

Our aim, in this study, was to identify amino acid changes that increased the thermal stability of the λ Cro protein. One might expect that such changes would arise in a genetic screen for second site suppressor mutations that restore activity to a mutant protein with reduced stability. However, the revertant mutations that we isolated in this way appeared to restore activity by improving DNA binding without increasing thermal stability (unpublished results). Enhanced activity mutations of this type had previously been isolated and characterized in λ repressor.[25] As a result, we decided to attempt to isolate stabilizing substitutions based on the anticipation that such substitutions would suppress the intracellular proteolytic sensitivity of an unstable mutant Cro protein. To isolate such mutations, a mutant *cro* gene was subjected to random mutagenesis, and colonies containing increased intracellular Cro levels were identified using an antibody screen (see Methods). A variant Cro protein bearing the IL30 (Ile-30→Leu) substitution was used for these studies. The destabilizing effect of this amino acid change is presumably due to disrupted packing interactions, since the Ile-30 side chain forms part of the hydrophobic core of Cro.[12] The IL30 Cro protein is present in the cell at about 10% of the wild-type level,[5] and is both less thermally stable than wild-type Cro in vitro and degraded more rapidly than wild-type Cro in vivo (see below).

Locations of Amino Acid Substitutions

The *cro* genes and *lac* promoter regions of 14 independent revertant candidates were analyzed by DNA sequencing. Each of these isolates retained the IL30 mutation and had acquired additional sequence changes as shown in Table I. In all, we found four revertants with changes in upstream sequences, nine genes encoding single second-site mutations in the *cro* gene, and two genes encoding second and third site changes in the *cro* gene. We presumed that base changes in the regions upstream from the *cro* gene increase expression of the IL30 Cro protein and did not pursue their study. Similarly, we did not study the isolates containing second and third site revertant mutations further. In the nine genes containing single second-site changes, there were five different reverting substitutions.

The identity of the five second-site amino acid substitutions and their locations in the three-dimensional structure of the Cro monomer are shown in Figure 2. Each of the affected residue positions is distant from the dimer interface region, which is located primarily near residues 52 through 58. The AV11 suppressor mutation (Ala-11→Val) changes a residue that is buried in the wild-type crystal struc-

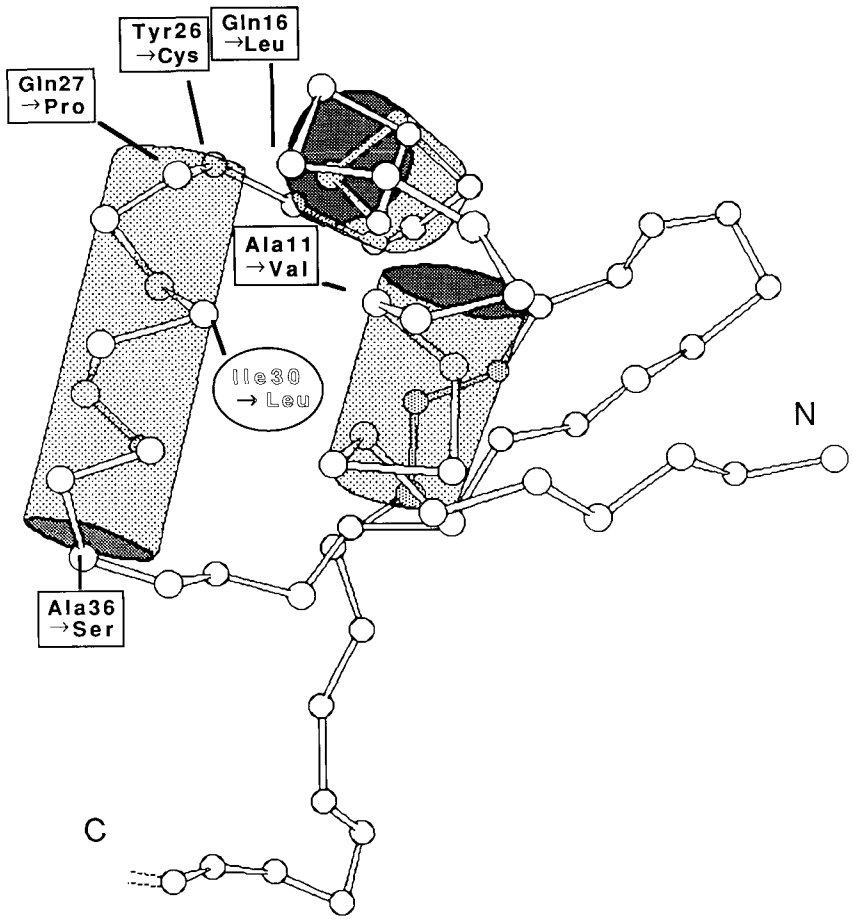

Fig. 2. The location of each suppressor substitution and the destabilizing Ile-30→Leu substitution are shown in a diagramatic representation of a Cro monomer. The four most C-terminal amino acids are not shown, as they are disordered in the crystal structure. Figure adapted from Anderson et al.[12]

TABLE I. DNA and Inferred Amino Acid Sequence Changes in the *cro* Genes of Revertant Candidates*

Revertant	Base and amino acid change(s)		Isolates
AV11	Ala-11→Val GCA→GTA		4
QL16	Gln-16→Leu CAA→CTA		1
YC26	Tyr-26→Cys TAT→TGT		1
QP27	Gln-27→Pro CAA→CCA		1
AS36	Ala-36→Ser GCC→TCC		1
AT29/T165	Ala-29→Thr GCG→ACG	Thr-65→Ile ACA→ATA	1
AS36/AS29	Ala-36→Ser GCC→TCC	Ala-29→Ser GCG→TCG	1

*Four candidates were found to have acquired changes in the region upstream from the *cro* gene.

ture; the remaining suppressors change side chains located on the surface of Cro. Inspection of the Cro structure suggests that the side chain of a valine at position 11 could contact the mutant leucine side chain at position 30.[12] Hence, the AV11 substitution might correct a packing defect caused by the IL30 substitution. In this case, the effect of the AV11 change on stability would be expected to differ, depending on the identity of the residue present at position 30; in genetic terms, the suppression would be allele specific.

Each of the remaining four suppressor mutations affects a surface residue near the end of a α-helix. The QL16 change (Gln-16→Leu) is located at the beginning of α-helix 2; the YC26 and QP27 changes (Tyr-26→Cys and Gln-27→Pro, respectively) are located at the N-terminal end of α-helix 3; and the AS36 change (Ala-36→Ser) is located near the C-terminal end of α-helix 3. Moreover, three of these substitutions replace residues that are believed to make DNA-binding contacts.[13] The Gln-16 side

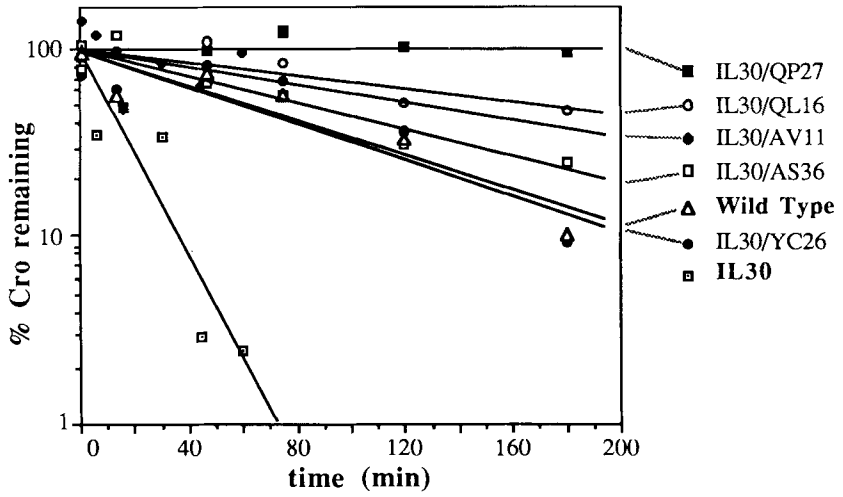

Fig. 3. Pulse–chase experiment. The intracellular half-life of the IL30 Cro protein is about 11 minutes, and that of wild type is over 60 minutes. Each revertant protein has a half-life that is at least 1 hour.

chain is thought to contact the sugar-phosphate backbone of the operator, while the side chains of Tyr-26 and Gln-27 are thought to make sequence specific major groove contacts.

Intracellular Half-Lives

The rates of intracellular degradation for wild-type Cro, IL30 Cro and each revertant protein were measured by pulse–chase experiments. These data are shown in Figure 3; the half-life of each protein is listed in Table II. Each revertant protein has a substantially longer half-life than the IL30 parent. Moreover, in each case, the half-life of the revertant protein is as long, or longer, than that of wild type Cro. For example, the QP27/IL30 protein shows essentially no turnover during the 3-hour chase period of this experiment, whereas the half-lives of the IL30 and wild-type proteins are approximately 11 and 63 minutes, respectively.

Protein Purification

To examine the effects of the suppressor mutations on the physical properties of Cro, we purified wild-type Cro, the IL30 protein, and several of the revertant proteins. We also sought to purify several proteins bearing suppressor mutations without the IL30 mutations. Genes containing the QL16, YC26, and AS36 alleles in otherwise wild-type backgrounds were constructed in a straightforward manner using cassette mutagenesis (see Methods). However, despite repeated attempts, we were unable to introduce the QP27 change into a wild-type *cro* gene; the QP27 allele was recovered, but always in combination with additional changes, predominantly frameshifts. We suggest that this substitution causes Cro to be deleterious to the cell.

In all, seven variant Cro proteins were purified. These included the IL30, IL30/YC26, IL30/QP27, IL30/AS36, QL16, YC26, and AS36 proteins. The IL30/QP27 double mutant protein tended to precipitate at high concentrations. Each of the remaining proteins, however, behaved like wild-type during all purification steps including gel filtration and ion-exchange column chromatography. Thus, the mutant Cro proteins do not appear to have oligomeric structures or surface charge properties that are significantly different from those of wild-type Cro. We were able to isolate 10 to 120 mg quantities of each protein at purity levels of 95% or better.

Thermal Stability

The stabilities of wild-type Cro and the purified variants to denaturation were determined by experiments in which thermal unfolding was monitored by the change in CD ellipticity at 222 nm. Figure 4 shows the resulting melting curves. Table II lists the melting temperature (T_m) for each protein and the change in T_m brought about by the suppressor mutation (ΔT_m). Wild-type Cro is half-denatured at 40°C, under the conditions used here. The IL30 protein is less thermally stable with a melting temperature 5°C below the wild-type value. Thus, these data are consistent with our original surmise that the cellular instability of the IL30 mutant is caused by thermal instability.

Two of the revertant substitutions—YC26 and QL16—cause large increases in the thermal stability of Cro. The effect of the YC26 substitution was measured in an otherwise wild-type Cro protein, and in Cro with the IL30 substitution. As shown in Table II, the increase in stability is not strongly dependent upon the identity of the residue at position 30. In the

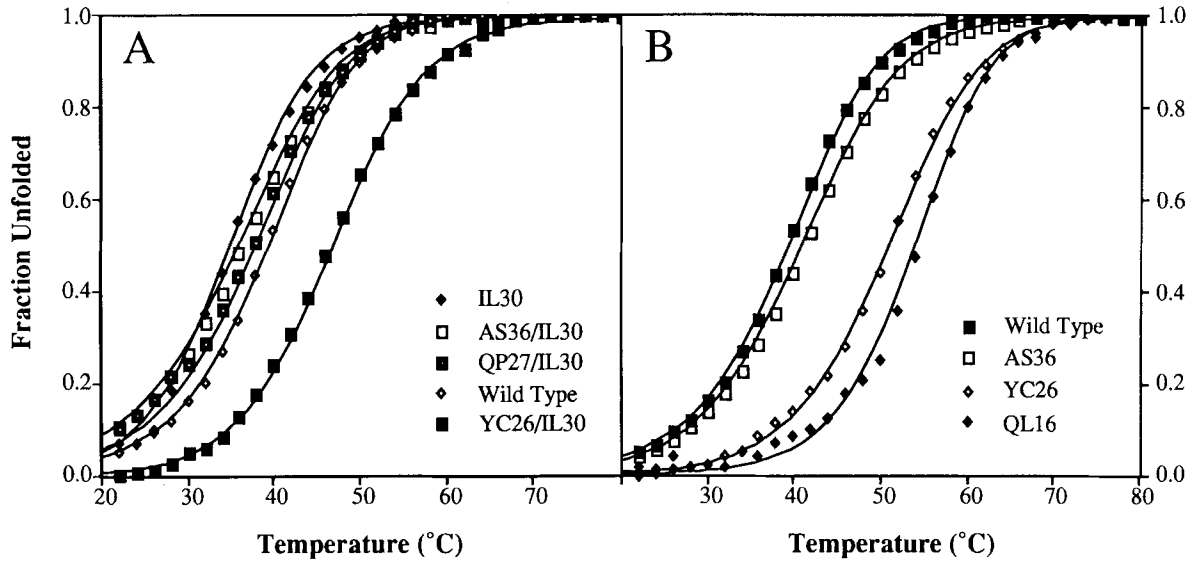

Fig. 4. **A:** Thermal denaturation profiles for wild-type Cro, IL30 Cro, and Cro proteins with the IL30 substitution and one of the three suppressor substitutions. **B:** Thermal denaturation profiles for wild-type Cro and Cro with the AS36, QL16, or YC26 substitutions. The solid lines are theoretical curves for a two-state transition (folded dimer to two unfolded monomers) calculated using the Gibbs–Helmholtz equation and best fit values for ΔH and ΔC_p.

IL30 background, the YC26 change causes a 12°C increase in melting temperature ($\Delta\Delta G_u$ = 1.8 kcal/mol); in the wild-type background, an 11°C increase is observed ($\Delta\Delta G_u$ = 2.2 kcal/mol). The QL16 substitution also causes a dramatic stabilization of Cro. The QL16 change increases the T_m of otherwise wild-type Cro by 14°C ($\Delta\Delta G_u$ = 2.8 kcal/mol); its effect in combination with IL30 was not determined. The QP27 and AS36 changes cause only 1–3°C increases in the T_m of Cro.

The increased thermal stability afforded by the YC26 and QL16 substitutions presents a plausible mechanism by which these amino acid changes prevent proteolytic degradation of the IL30 mutant. Specifically, the IL30 mutant is thought to be proteolytically susceptible in the cell because it is unfolded to a greater extent than wild-type. By increasing stability, the YC26 or QL16 changes will reduce the fraction of the IL30 protein that is unfolded and, as a consequence, reduce its susceptibility to proteolysis. In accord with this model, correlations between increasing thermal stability and decreasing proteolytic susceptibility in vivo have been reported for a number of proteins.[26,27]

It is important to note, however, that we do not observe a direct relationship between stability and proteolytic susceptibility for the mutant proteins. For example, the QP27/IL30 protein has a longer half-life than that of YC26/IL30, whose melting temperature is 9° higher. Similarly, the IL30/YC26 and wild-type proteins have the same half-lives but melting temperatures that differ by 7°. These observations presumably reflect the involvement of properties, other than intrinsic thermal stability, that affect proteolytic sensitivity. These may include binding to nonspecific DNA[27] and/or aggregation of the revertant protein. These mechanisms may play some role in suppression of degradation by QP27 and AS36, as these substitutions cause only small changes in melting temperature.

TABLE II. Stabilities of Wild-Type and Variant Cro Proteins*

Protein	T_m (°C)	ΔT_m (°C)	$\Delta\Delta G_u$ (kcal/mol)	$t_{1/2}$ (min)
IL30	35	0	0.0	11
IL30/YC26	47	+12	+1.8 ± 0.2	64
IL30/QP27	38	+3	+0.3 ± 0.2	>300
IL30/AS36	36	+1	+0.3 ± 0.2	84
IL30/QL16	ND	—	—	168
IL30/AV11	ND	—	—	126
Wild-type	40	0	0.0	63
QL16	54	+14	+2.8 ± 0.2	—
YC26	51	+11	+2.2 ± 0.2	—
AS36	41	+1	+0.4 ± 0.2	—

*ΔT_m and $\Delta\Delta G_u$ are the changes in melting temperature and free energy of unfolding, respectively, that result from the suppressor substitutions. These values are relative to IL30 Cro for the doubly substituted proteins and relative to wild-type Cro for the proteins with single mutations. $t_{1/2}$ is the intracellular half-life as measured by pulse–chase experiments. ND, not determined. $\Delta\Delta G_u$ values were calculated at 45°C.

DNA Binding

The antibody screen employed in this study identifies revertant Cro proteins solely by their increased intracellular level and does not specifically require or preclude any effect of the substitutions on DNA binding activity. In fact, three of the five sec-

ond site suppressor substitutions (QL16, YC26, and QP27) replace residues that have been proposed to contribute to the affinity and/or specificity of the Cro-operator DNA complex.[13] We examined the effects of these substitutions on sequence-specific DNA binding activity in vitro using the gel mobility shift assay.[22,23] Under the conditions used, half-maximal binding of wild-type Cro to O_R3 occurs at a concentration of 1×10^{-10} M. The QL16 and QP27 substitutions reduce the apparent affinity of Cro for operator DNA by 1,500- and 150-fold, respectively (data not shown). These severe effects support the idea that these residues play key roles in operator recognition. The YC26 substitution, on the other hand, has only a small (2- to 8-fold) effect, suggesting a less important role for the Tyr-26 side chain in binding to operator DNA.

One of the mechanisms by which susceptibility to intracellular proteolysis might be reduced is increased nonspecific binding affinity. The effects of the QL16, YC26, QP27, and AS36 substitutions on the formation of nonspecific DNA binding complexes were investigated using the gel shift assay, but no significant changes were observed.

DISCUSSION

We have used an antibody screen to identify randomly generated second site mutations that confer increased resistance to intracellular proteolysis upon an unstable Cro mutant. At least three classes of mechanisms could conceivably account for the increased resistance to proteolysis in vivo. (1) Decreasing the fraction of Cro that is present in the unfolded state would be expected to increase resistance to proteolysis of the revertant proteins because unfolded proteins are preferentially digested by proteases. This could be accomplished directly by raising the protein's intrinsic thermal stability or indirectly by increasing the binding of the folded protein to bulk cellular DNA. (2) Structural or chemical features of the folded or unfolded protein that serve as recognition signals for one or more proteases may be altered. (3) The formation of protein aggregates or complexes with other cellular components could physically prevent access by proteases.

Two of the revertant substitutions, YC26 and QL16 (Tyr-26→Cys and Gln-16→Leu, respectively), increase the thermal stability of Cro to a significant extent. The side chains of both residues are exposed on the surface of Cro.[12] The Tyr-26 side chain (fractional solvent accessibility = 1.37) is completely solvent exposed, while the Gln-16 side chain (accessibility = 0.38) is partially exposed. It will be important to understand the mechanisms by which the YC26 and QL16 substitutions stabilize Cro, as substitutions at many surface positions in proteins have been shown to have little or no effect on stability.[6,28] The increased stability of the YC26 and QL16 proteins does not appear to involve a change in oligomeric state, as both proteins behave like wild-type Cro and chromatograph as dimers in gel filtration experiments. Inspection of the structure of wild-type Cro shows that the mutant side chains could be positioned to contact nearby side chains, but it is not clear whether such interactions occur in the mutant proteins, or, if they do occur, whether they would be sufficient to account for the observed stabilization. The λ repressor and Cro proteins have a very similar tertiary structure in the DNA-binding regions where Gln-16 (α-helix 2) and Tyr-26 (α-helix 3) are located.[28] Interestingly, a mutation in λ repressor, Gln-33→Tyr, at the position analogous to Gln-16 of Cro, also increases thermal stability.[6] Perhaps a particular structural feature common to this homologous region of the two proteins is responsible for the ability of substitutions at position 16 in Cro and 33 in λ repressor to increase stability.

It seems likely that the changes in stability afforded by the YC26 and QL16 substitutions are responsible for the increased resistance to proteolysis in vivo as these substitutions do not appear to have other effects that would reduce protease sensitivity. For example, they do not appear to cause increased DNA binding, or aggregation in solution, even at high concentration. Moreover, these proteins are soluble in crude lysates and show no tendency to copurify with other cellular components. The AS36 and QP27 substitutions do not, however, increase thermal stability dramatically. These mutations may reduce proteolytic sensitivity by one of the other mechanisms mentioned above. The AS36 substitution partially restores the activity of the IL30 protein in vivo, suggesting that DNA binding is altered, although this is not apparent in our assay in vitro. Aggregation might be involved in the reduced proteolytic susceptibility of IL30/QP27, since this protein displays a tendency to precipitate at high concentration in vitro.

The screening method that we have used to isolate the suppressor mutations is unusual because it does not require restoration of activity in vivo. A similar approach could be used to isolate suppressor mutations in other unstable proteins when no other convenient phenotype exists. The ability to screen for a phenotype that does not depend on activity is also desirable when investigating issues of protein structure and stability. For example, *cro* mutations like QL16 that increase stability but adversely affect specific DNA binding, would not be identified in an activity-based screen or selection. This may be a particularly important consideration for small proteins that make extensive contacts with large molecules. For Cro, the fraction of surface area that is involved in DNA binding is relatively large, so that sites where stabilizing changes may be made, without affecting DNA binding, could be relatively rare. Another type of problem that may be approached

using a mutant screen that does not involve activity is the relationship between protein flexibility and activity. Among the class of mutations that increase a protein's stability, but reduce its activity, might be those that restrict functionally important motions or conformational shifts.

The use of the random genetic approach and the isolation of suppressor mutations in *cro* have provided a number of interesting and unexpected results for further study. The striking increases in thermal stability conferred by two amino acid substitutions on the surface of the Cro protein may provide new insight into the ways in which surface residues can influence protein stability. Since substitution of surface residues does not commonly increase or decrease thermal stability, these unusual positions would not likely have been chosen for study using directed mutagenesis. Although further investigation of these mutations will require the construction of specific amino acid substitutions at these sites, their identification as important positions demonstrates the utility of the genetic approach in the study of protein structure.

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