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Bacteriophage λ *cro* mutations: Effects on activity and intracellular degradation

(proteolysis/protein stability/repressor/DNA binding)

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ABSTRACT **Following random mutagenesis of the bacteriophage λ *cro* gene, we have isolated missense mutations that affect approximately half of the 66 residue positions of Cro. About two-thirds of the mutations change residues involved in the maintenance of Cro structure and stability. The corresponding mutant proteins are severely degraded in the cell but often have specific activities near that of wild-type Cro. The remaining mutations affect residues involved in DNA binding. These mutant proteins are present at moderately reduced intracellular levels, but their specific activities are much lower than that of wild type.**

The study of missense mutants provides a means of testing and adding to our understanding of the determinants of protein structure, stability, and function. In this paper, we describe mutations that affect the intracellular level and activity of bacteriophage λ Cro, a small repressor with a known three-dimensional structure (1–3). We find that a Cro^- phenotype results from replacing any 1 of at least half of the 66 residues of Cro; some of the mutations change residues on the DNA binding surface, some change solvent-exposed residues distant from the proposed binding surface, and some affect residues in the hydrophobic core. Mutations that would be expected to destabilize the folded structure of Cro generally result in extreme sensitivity to intracellular proteolysis. In many cases, the resulting reduction in concentration is severe enough to account fully for the observed mutant phenotype.

MATERIALS AND METHODS

Plasmids, Phage, and Bacteria. Three related Cro-producing plasmids—pAP100, pAP101, and pAP104—were constructed for this work. Each plasmid is a pBR322 derivative, and each bears the same fusion of the *lac* UV5 promoter to the phage λ *cro* gene. This fusion was derived from plasmid pTR214 (4). Plasmid pAP100 was constructed by inserting the 353-base-pair *Eco*RI/*Nae* I fragment from pTR214 between the *Eco*RI and filled-in *Cla* I sites of pBR322 (5). Plasmid pAP101 was constructed by replacing the 391-base-pair *Cla* I/*Bam*HI fragment of pAP100 with the 375-base-pair *Eco*RI/*Bam*HI fragment from pBR322. pAP101 has an intact *tet* promoter and, thus, confers a significantly higher level of tetracycline resistance than does pAP100. pAP104 is similar to pAP101 but contains the phage M13 origin of replication. pAP104 was constructed by replacing the small *Eco*RI/*Bam*HI fragment of pZ150 (6) with that from plasmid pAP101. Strains containing pAP100, pAP101, or pAP104 express the same level of phage λ Cro, as judged by the resistance of the strain to phage infection and by competitive radioimmunoassay. Thus, Cro^- mutations isolated in any of the three plasmid backgrounds can be directly compared.

λ200 is an immunity 21 bacteriophage that carries the *lacZ* gene under the control of the phage λ P_R promoter (7). Phage M13 appl was constructed by deletion *in vitro* of the *lac* promoter and operator sequences of M13 mp8 (8). DNA between the unique *Ava* II and *Eco*RI sites was deleted, the DNA was ligated, and candidates were screened for those with a regenerated *Eco*RI site. The DNA of one such phage, M13 appl, was sequenced and shown to contain a deletion of M13 mp8 bases 5013–6232.

Escherichia coli strains used for this study were grown at 37°C and include MM294 (9), JM103 (10), GW5100 (JM103 cured of P1 lysogen; L. Marsh and G. Walker, personal communication), KL731 (11), US3 (Thi^-, His^-, $lacZ^-$, sup^0, $recA^-$), LE30 (*mutD5, rpsL, azi, galU95.*; L. Enquist via D. Botstein), and DB6438 (ΔXIII, rif^r, *argEam, metB, mutT198*; J. Miller via D. Botstein).

Mutagenesis and Mutant Isolation. Plasmids were mutagenized *in vivo* with *N*-methyl-*N'*-nitrosoguanidine (MNNG) or 2-aminopurine (12) or by passage through mutator strains LE30 (*mutD*) or DB6438 (*mutT*). Purified pAP101 DNA was mutagenized *in vitro* with hydroxylamine (13).

After mutagenesis *in vivo*, pAP100 or pAP101 DNA was isolated by the small-scale rapid boiling method (14). pAP104 contains an M13 origin of replication, and packaged single-stranded DNA transducing particles were obtained from supernatants of cultures of GW5100 (pAP104) infected with M13 RV1 helper phage (6, 15). The supernatants were sterilized by incubation at 65–70°C for 30 min.

To identify plasmids bearing mutant *cro* genes, mutagenized plasmid DNA was introduced into strain US3/λ200 or its F^+ derivative. Cells transformed by Cro^- plasmids form red (Lac^+) colonies after growth on MacConkey–lactose–tetracycline (5 μg/ml) plates. Mutagenized pAP100 or pAP101 DNA was introduced into US3/λ200 by transformation. To introduce pAP104 into US3/λ200 F^+, a freshly saturated culture was incubated with transducing particles (multiplicity of infection = 0.1) for 30 min at 37°C and then plated.

DNA Sequencing. For each Cro^- mutant candidate, the entire *cro* gene and *lac* promoter region (P_{lac}) were sequenced by the dideoxy method (16). For mutations isolated in pAP100 or pAP101, the *Eco*RI/*Hin*dIII fragment that contains the P_{lac}-*cro* fusion was subcloned into M13 mp8 (8) or M13 appl. M13 appl is a useful cloning vector for restriction fragments containing the *lac* operator because these phages form blue plaques on F^+Lac^+ strains, such as KL731, when plated on 5-bromo-4-chloro-3-indolyl β-D-galactoside indicator plates. This phenotype is presumed to result from titration of Lac repressor from the chromosomal *lac* operator. Cro^- mutations in pAP104 backgrounds were sequenced with the single-stranded plasmid DNA template obtained from trans-

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Abbreviation: CRM, crossreacting material.

ducing particles present in supernatants of M13 RV1-infected cultures of GW5100(pAP104) (6).

Assays of Cro Activity and Level. β-Galactosidase assays were performed as described by Miller (12) on logarithmic-phase cultures of US3/λ200 or its F^+ derivative bearing wild-type or Cro^- plasmids. Competitive RIAs were performed on lysates of the same strains essentially as described by Johnson (17). For immunoblot analysis, lysates were electrophoresed in 20% polyacrylamide gels containing $NaDodSO_4$ and urea (18) and were transferred to nitrocellulose electrophoretically (19). The nitrocellulose-bound Cro was detected by incubation with rabbit anti-Cro antiserum and subsequently with ^{125}I-labeled *Staphylococcus* protein A (Amersham).

Pulse–Chase Experiments. Logarithmic-phase cultures of US3/λ200 carrying wild-type or Cro^- plasmids were pulse-labeled with L-[^{35}S]methionine (Amersham). Aliquots were removed 0.5, 10, and 60 min after addition of excess unlabeled L-methionine, and lysates were prepared. Cro protein was immunoprecipitated, electrophoresed on acrylamide gels (18), and detected by autoradiography.

RESULTS

After random mutagenesis of a plasmid containing the phage λ *cro* gene, Cro^- mutations were isolated by screening for Lac^+ transformants of an indicator strain containing a λ P_R-*lacZ* fusion. Cro represses transcription from the P_R promoter by binding at the O_R1 or O_R2 operator DNA sites (3); thus, β-galactosidase levels are inversely related to Cro activity in the indicator strain. For Cro^- mutants, high β-galactosidase levels indicate severe mutant defects.

Table 1. Phage λ Cro mutations

Allele	Amino acid change	Mutagens*	Base change (position)	β-Gal activ.†	CRM, % wt
wt	—	—	—	38	100
z1	—	A^1	C→G(−100)	106	7
z2	—	H^2M^2	C→T(−95)	797	3
z3	—	A^1	T→C(−75)	86	13
z4	—	T^1	T→G(−75)	1292	1
z5	—	T^1	T→G(−70)	2162	<1
z6	—	A^1	G→T(−8)	1085	2
z7	—	T^1	A→C(−7)	132	6
z8	—	A^1	T→C(−1)	45	11
z9	—	T^1	T→G(2)	2252	<1
Qoc3	Gln-3→TAA	H^1	C→T(7)	2278	<1
f1	Frameshift	T^1	+T(9)	ND	ND
TI6	Thr-6→Ile	H^1N^4	C→T(17)	1563	2
LR7	Leu-7→Arg	T^1	T→G(20)	2408	<2
DY9	Asp-9→Tyr	S^1	G→T(25)	1238	3
YD10	Tyr-10→Asp	T^2	T→G(28)	2378	<1
YS10	Tyr-10→Ser	T^1	A→C(29)	2385	<1
FL14	Phe-14→Leu	T^1	T→G(42)	207	2
GR15	Gly-15→Arg	D^1H^1	G→A(43)	1490	1
GE15	Gly-15→Glu	N^3	G→A(44)	2169	<1
Qoc16	Gln-16→TAA	H^1	C→T(46)	2091	<1
QP16	Gln-16→Pro	T^1	A→C(47)	1774	41
QH16	Gln-16→His	T^2	A→C(48)	2022	28
TP19	Thr-19→Pro	T^1	A→C(55)	2127	<1
AT20	Ala-20→Thr	$D^1H^1N^2$	G→A(58)	2529	3
AV20	Ala-20→Val	$A^1H^3N^3$	C→T(59)	2245	<1
LF23	Leu-23→Phe	N^5	C→T(67)	509	2
LP23	Leu-23→Pro	A^1	T→C(68)	2366	<1
LR23	Leu-23→Arg	T^1	T→G(68)	2353	<1
LH23	Leu-23→His	D^1	T→A(68)	2125	3
VA25	Val-25→Ala	M^1	T→C(74)	937	9
YD26	Tyr-26→Asp	T^1	T→G(76)	2018	54
Qoc27	Gln-27→TAA	H^1	C→T(79)	2090	<1
QH27	Gln-27→His	T^2	A→C(81)	1934	44
SR28	Ser-28→Arg	T^2	A→C(82)	2246	13
SN28	Ser-28→Asn	N^1	G→A(83)	1860	25
IL30	Ile-30→Leu	T^1	A→C(88)	1203	9
IT30	Ile-30→Thr	A^1	T→C(89)	2384	<1
IS30	Ile-30→Ser	T^2	T→G(89)	2371	<2
KQ32	Lys-32→Gln	T^1	A→C(94)	1194	42
KT32	Lys-32→Thr	T^3	A→C(95)	2158	22
KN32	Lys-32→Asn	S^2	G→T(96)	1089	12
AT33	Ala-33→Thr	H^2N^5	G→A(97)	2231	<4
AV33	Ala-33→Val	A^1N^4	C→T(98)	2252	<1
AG33	Ala-33→Gly	D^1T^1	C→G(98)	2039	6
IT34	Ile-34→Thr	A^1	T→C(101)	1972	<2
Rop38	Arg-38→TGA	D^1N^2	C→T(112)	2017	<1
RL38	Arg-38→Leu	S^1	G→T(113)	2212	2
RQ38	Arg-38→Gln	N^1	G→A(113)	2298	18
IF40	Ile-40→Phe	D^3	A→T(118)	2247	<4
IL40	Ile-40→Leu	T^3	A→C(118)	2282	<4
f2	Frameshift	D^2T^1	−T(119)	2157	<1
f3	Frameshift	D^2	+T(119)	2018	<1
IS40	Ile-40→Ser	T^1	T→G(119)	2503	<5
IM40	Ile-40→Met	T^2	T→G(120)	2376	2
Lop42	Leu-42→TGA	T^1	T→G(125)	2403	<1
LF42	Leu-42→Phe	T^7	A→C(126)	1542	<2
LF42'	Leu-42→Phe	T^1	A→T(126)	ND	ND
TA43	Thr-43→Ala	A^1	A→G(127)	145	2
DY47	Asp-47→Tyr	S^2	G→T(139)	2037	<1
DH47	Asp-47→His	M^1	G→C(139)	1584	5
DG47	Asp-47→Gly	N^3	A→G(140)	1239	1
Gop48	Gly-48→TGA	S^1	G→T(142)	2229	<1
GR48	Gly-48→Arg	$D^1M^3N^1$	G→A(142)	1072	<1
GE48	Gly-48→Glu	N^8	G→A(143)	531	1
GA48	Gly-48→Ala	M^2	G→C(143)	1126	5
SN49	Ser-49→Asn	M^2	G→A(146)	1450	<7
VG50	Val-50→Gly	T^1	T→G(149)	2384	<2
AT52	Ala-52→Thr	H^1N^1	G→A(154)	2177	<4
AS52	Ala-52→Ser	M^1	G→T(154)	1798	2
AV52	Ala-52→Val	D^1N^1	C→T(155)	2107	12
EK53	Glu-53→Lys	M^1	G→A(157)	398	5
EK54	Glu-54→Lys	$D^1H^1M^1N^1$	G→A(160)	2216	2
EA54	Glu-54→Ala	T^1	A→C(161)	2329	2
ED54	Glu-54→Asp	S^1	G→C(162)	2220	<2
KQ56	Lys-56→Gln	T^2	A→C(166)	2269	15
KT56	Lys-56→Thr	T^5	A→C(167)	2188	41
KN56	Lys-56→Asn	D^1	G→C(168)	2117	29
KN56'	Lys-56→Asn	T^1	G→T(168)	ND	ND
FV58	Phe-58→Val	T^3	T→G(172)	2389	10
FC58	Phe-58→Cys	T^3	T→G(173)	2250	8
PS59	Pro-59→Ser	N^2	C→T(175)	2230	6
PL59	Pro-59→Leu	D^1	C→T(176)	2076	6

CRM levels for mutant Cro proteins are expressed as a percentage of the wild-type (wt) levels of Cro. ND, not determined; TAA, ochre termination codon; TGA, opal termination codon; β-Gal activ., β-galactosidase activity.

*A, 2-aminopurine; H, hydroxylamine; N, MNNG; M, MNNG-mutagenized packaged single-stranded plasmid introduced into tester strain via transduction; T, MutT; D. MutD; S, spontaneous mutagenesis arising during growth of the M13 origin plasmid pAP104 as single-stranded DNA. The number of isolates of a particular mutation obtained with each mutagen is superscripted.

†Units of β-galactosidase determined as described (12).

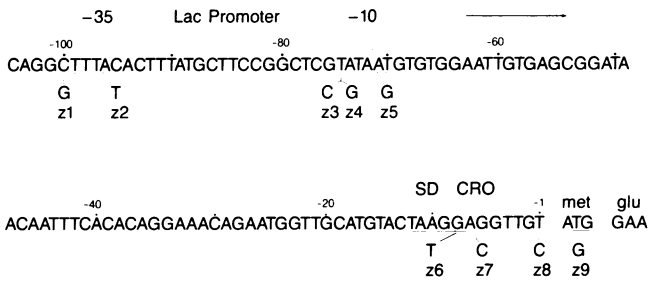

FIG. 1. DNA sequence of the promoter region and 5′ noncoding sequences. Mutations and their allele names are shown below the wild-type sequence. The numbering is relative to the start point of translation. The presumptive start point of transcription is denoted by an arrow. SD Cro is the ribosome binding site of the phage λ *cro* gene.

Sequence Changes and Activities of Cro⁻ Mutants. The mutation in each Cro⁻ plasmid was identified by DNA sequencing and was found to be a substitution, deletion, or insertion of a single base pair. Table 1 lists the DNA sequence changes identified for 175 independent Cro⁻ mutant isolates and, where appropriate, indicates the inferred amino acid changes. A total of 79 different Cro⁻ mutations were isolated. Sixty-two are missense mutations; six are nonsense mutations; four affect translational start signals; five are *lac* promoter mutations; and two are frameshift mutations. Table 1 also lists the β-galactosidase levels in the phage λ P_R-*lacZ* fusion strain for each mutant.

The specificities of the mutations induced by the chemical mutagens and mutator strains are similar to those reported by other workers (20–22). Mutations that arose spontaneously during growth of the M13 origin plasmid, pAP104, as single-stranded DNA were either G→T or G→C transversions. These mutations arose at a moderate frequency (10^{-5} to 10^{-4}) and may result from replication past apurinic sites in the single-stranded DNA (23).

Promoter and Translational Start Mutations. Nine of the Cro⁻ mutations change bases in the *lac* UV5 promoter, in the Shine–Dalgarno ribosome binding sequence, or in the ATG translation initiation codon, as shown in Fig. 1. The steady-state level of Cro, as measured by RIA, was reduced by all of these mutations (Table 1). Combining the steady-state level data for these mutants with the corresponding β-galactosidase activity measurements establishes a reference curve for wild-type Cro. By comparison of the corresponding data for a mutant Cro protein, the relative specific activity of the mutant protein *in vivo* may be determined.

Missense Mutations. Fig. 2 shows the wild-type amino acid sequence of Cro and the missense mutations which, as a group, affect 32 of the 66 residue positions. The mutant sites are distributed throughout the protein sequence. We have not saturated all of the possible sites of missense mutation. Caruthers *et al.* (26) have constructed site-directed changes at three residue positions not represented in our collection and report that these changes confer a Cro-defective phenotype. Since many of the mutants in our collection were isolated only once and certain mutagenic specificities are underrepresented, it is likely that there are a number of other sites in Cro where amino acid substitutions will cause a defective phenotype.

The levels of crossreacting material (CRM) for each mutant were measured by competitive RIA. These data are listed in Table 1 and are represented schematically in Fig. 3. All of the mutant proteins appear to be present in reduced amounts relative to wild type. Of the 62 missense mutants, 24 have CRM levels from 5% to 60% of the wild-type level, and the remaining 38 are present at levels below 5% of wild type. When mutant levels were estimated by immunoblot analysis with high antisera concentrations, results comparable to the RIA data were obtained (data not shown).

The severely reduced levels of the mutant Cro proteins probably result from increased rates of cellular proteolysis. Fig. 4 shows pulse–chase experiments for wild-type Cro and three of the mutant proteins. Wild-type Cro turned over with a half-life of 30–60 min, whereas the Asp-9→Tyr, Asp-47→Gly, and Arg-38→Leu mutant proteins turned over with half-lives of 1–3 min. Higher levels of transcription did not prevent degradation of these mutant proteins. When mutant *cro* genes encoding the Asp-47→Gly and Arg-38→Leu substitutions were cloned under control of the highly efficient *tac*-promoter (27), the levels of the mutant proteins relative to wild type were not increased.

DISCUSSION

In principle, there are three general factors that could contribute to the inability of any given mutant Cro protein to bind operator DNA. The folded form of the mutant protein

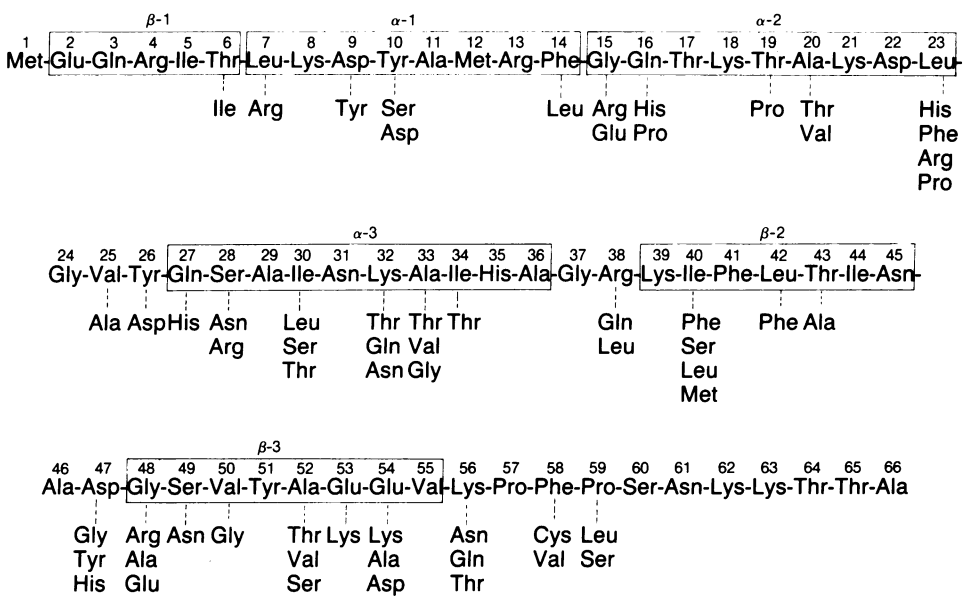

FIG. 2. Protein sequence of wild-type Cro (24, 25) and positions of mutant amino acid substitutions. α-Helix and β-sheet regions in wild-type Cro (1) are boxed.

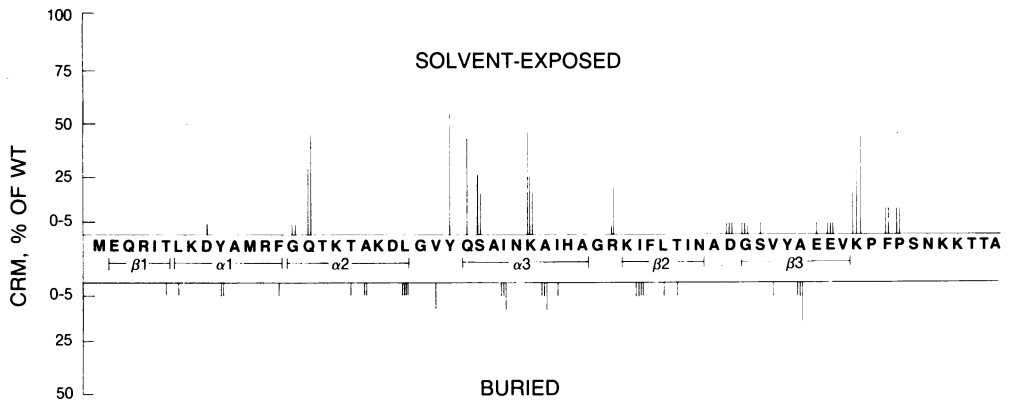

FIG. 3. CRM levels of missense mutant proteins. Wild-type residues are indicated by the single letter amino acid code. CRM levels for substitutions affecting solvent-exposed residues are shown above and those affecting buried residues are shown below the sequence.

could have decreased affinity for the operator; the mutant protein might be inactive because it is unfolded; or, the mutant protein might be rapidly degraded by cellular proteases. Our data do not address the first possibilities directly but do indicate that proteolysis plays an important role in the phenotypic defects displayed by most of the Cro$^-$ missense mutants. This raises several questions. Which cellular proteases degrade the mutant Cro proteins? What determines whether a given Cro mutant will be hypersensitive or relatively resistant to degradation? How do missense proteins in other systems avoid cellular proteolysis? In the discussion that follows, we consider possible answers to these questions.

It is widely recognized that nonsense fragments, missense mutants, and other abnormal proteins can be sensitive to proteolysis *in vivo* (28–33). Moreover, many of these proteins are stabilized in strains of *E. coli* bearing *lon*$^-$, *htpR*$^-$, or *hfl*$^-$ mutations (34–36). However, as judged by pulse–chase experiments, these cellular mutations do not prevent rapid turnover of the mutant Cro proteins (data not shown). These data suggest that the mutant Cro proteins are degraded by a proteolytic system, which has not yet been characterized genetically.

What properties of the mutant Cro proteins make them susceptible to proteolysis? In our view, protein stability is the most likely determinant of proteolytic sensitivity, since denatured proteins, but not their folded counterparts, are good substrates for most proteases. By this model, the mutant Cro proteins with the lowest thermodynamic stabilities should be the ones that are most sensitive to proteolysis. As we argue below, this appears to be the case.

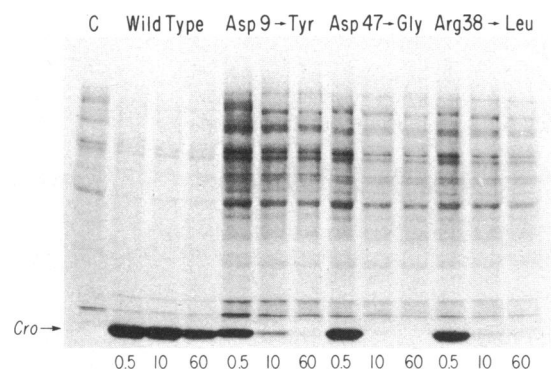

FIG. 4. Pulse–chase experiments. Cells were labeled with [^{35}S]methionine for 0.5 min. The time of the unlabeled methionine chase in minutes is indicated below each lane. Lane C shows a 0.5-min chase for a control strain carrying pBR322 instead of a Cro plasmid.

Based on their positions in the wild-type structure (1, 2), about two-thirds of the missense substitutions would be expected to reduce Cro stability. Thirty-six of the 40 mutants in this "stability" class have intracellular levels between 0 and 5% of wild type, and three have levels between 5% and 10% of wild type. Most stability mutations (30 of 40) affect residues in the tightly packed protein interior. Here, some substitutions (e.g., Phe-14→Leu) would remove favorable packing and hydrophobic interactions, whereas others (e.g., Ala-33→Val) probably introduce unfavorable interactions. Some of the stability substitutions (5 of 40) disrupt charge-stabilized hydrogen bonds on the surface of Cro; for example, the Asp-47→Tyr and Ser-49→Asn mutations affect the hydrogen bond that links the wild-type side chains at these positions. For Gly-48→Ala and four similar substitutions, stability appears to be reduced because a glycine in a tight turn has been replaced with a residue that cannot readily assume the positive ϕ,ψ dihedral angles required for the turn (37).

Most of the stability mutants have levels that are too low to determine whether the mutant protein has significant activity. However, some proteins in the stability class seem to have specific activities that are near wild type. For example, strains containing the Asp-47→Gly protein have CRM levels of 1% and β-galactosidase levels of 1239 units, while strains containing wild-type Cro expressed from the *z4* mutant promoter have CRM levels of 1.5% and β-galactosidase levels of 1292 units. Other mutant proteins with specific activities near wild type are Asp-9→Tyr, Leu-23→Phe, Gly-48→Glu, and Glu-53→Lys.

About one-third (22 of 62) of the Cro$^-$ mutations affect DNA-binding residues in the α2 and α3 helices, and the C-terminal β-sheet region of Cro (1, 2). These mutations could decrease operator affinity by removing favorable interactions with the DNA or introducing unfavorable interactions. For example, mutations in or near the α3 recognition helix (e.g., Tyr-26→Asp, Gln-27→His, Ser-28→Asn, Lys-32→Thr, Arg-38→Gln) change residues that have been proposed to contact bases in the major groove of the operator site. Other mutations (e.g., Gln-16→His in α helix 2 and Lys-56→Gln in the C-terminal β-sheet region) probably disrupt hydrogen bonds or salt bridges with the phosphate oxygens of the DNA backbone.

We will refer to the mutations described immediately above as "DNA binding" mutations. The mutant proteins in this class appear to have reduced affinity for operator DNA because their specific activities *in vivo* are lower than that of wild-type Cro. The DNA binding mutant proteins are also present at reduced intracellular levels, but their levels are

generally higher than those of the stability mutants. Most (18 of 22) of the DNA binding mutants have levels between 5% and 60% of wild type, whereas only 4 of 40 of the stability mutants have levels this high. Why are the levels of the DNA binding mutant proteins reduced at all? There are several possibilities. (*i*) These proteins may be slightly less stable than wild type. If Cro were marginally stable, even small changes in stability could increase its turnover rate. (*ii*) If Cro were less susceptible to proteolysis when bound to DNA, then mutations that decreased DNA binding could lead to increased proteolysis. Nonoperator binding would be an essential component of such a mechanism because Cro bound to operator DNA represents an insignificant fraction of the total Cro in the cell. (*iii*) It is conceivable that wild-type Cro preferentially inhibits the expression of cellular genes whose products participate in protein degradation. In such a case, the mutant strains would have increased proteolytic levels or activities that could be responsible for the increased degradation of the mutant proteins.

Missense mutants of different proteins show radically different patterns of sensitivity to intracellular proteolysis. Almost all of the phage λ Cro mutants are degraded to some extent. However, the phage λ repressor mutants studied previously in this laboratory were not degraded (38–40). To account for these differences and the known properties of many of the mutant proteins, at least three classes of missense mutation must be postulated. The first class includes mutations that do not have significant effects on the thermodynamic stability of the protein. About half of the phage λ repressor mutants have this property. These missense proteins are protease resistant because they are stably folded. The second class includes mutations that destabilize the native structure and thereby increase the fraction of protein molecules in an unfolded, protease-sensitive state. This model seems reasonable for most of the phage λ Cro mutations since there is a good correlation between substitutions that would be expected to destabilize the protein and mutant proteins whose intracellular levels are severely reduced. The third class also includes mutant proteins with reduced thermodynamic stability, but in this case the unfolded chains escape proteolysis. Many of the phage λ repressor mutants fall in this category. Since the unfolded chains of these mutant repressors aggregate during denaturation *in vitro* (40), it is possible that a similar process of aggregation accounts for their protease resistance in the cell.

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