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Role of an N_{cap} residue in determining the stability and operator-binding affinity of Arc repressor

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Abstract

The Arc repressor of bacteriophage P22 is a member of the ribbon–helix–helix family of transcription factors. Ser32 is a solvent-exposed position that serves a structural role as the N_{cap} residue of α-helix B of Arc, but also serves a functional role because its side chain is packed close to the sugar–phosphate DNA backbone in the repressor–operator complex. The tolerance of this N_{cap} position to amino-acid substitutions was probed by determining the repressor activity in vivo, the thermal stability and the operator-binding activity in vitro of a set of 13 mutant proteins. The stability of position-32 Arc variants, except for Cys32, correlated well with the frequencies observed for the corresponding residues at N_{cap} positions in α-helices of other proteins. Cysteine was quite stabilizing at the helix-B N_{cap} position in Arc, but surprisingly was the least frequent N_{cap} residue in the protein database. This latter finding may reflect a hyper-reactivity of N_{cap} cysteines, which makes them prone to chemical modification. In general, only Arc variants with small, uncharged residues at position 32 were active in vivo or showed strong operator binding in vitro. Based upon the results presented here, revised sequence alignments of the MetJ and NikR subfamilies with Arc and other ribbon–helix–helix proteins are proposed.

Keywords: Arc repressor; Transcription factor; Protein stability

1. Introduction

Most solvent-exposed side chains in proteins play minor roles in determining the stability of the molecule to denaturation. In P22 Arc repressor, for instance, individual alanine substitutions caused less than a 5 °C change in thermal stability at more than 90% of all surface positions [1], and a mutant Arc dimer bearing 30 alanine substitutions at surface positions was actually more stable than the wild-type protein [2]. Helical N_{cap} residues, however, provide a striking exception to the generalization that surface positions are unimportant in determining protein stability [1,3–15].

By definition, the N_{cap} or first residue of an α-helix has non-helical Φ,Ψ dihedral angles, but participates, through its main-chain –C=O group, in an intra-helical hydrogen bond with the main-chain –NH group of the N3 residue (residues

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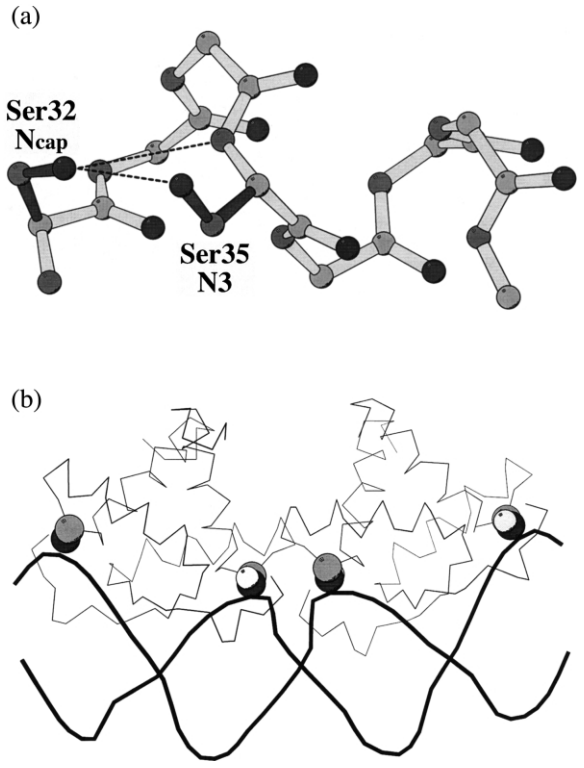

Fig. 1. (a) N_{cap} interactions in helix B of Arc repressor. The side chains of Ser32 and Ser35 are shown with main-chain atoms for residues 32–38. The dashed lines represent hydrogen bonds between the Ser32 side chain and the main chain and side chain of Ser35. (b) When two Arc dimers bind to operator DNA, the four Ser32 side chains (shown in CPK representation) are close to the DNA backbone. Both panels were prepared using MOLSCRIPT [40] and the coordinates of the Arc-operator cocrystal structure [18,42].

following the N_{cap} in the helix are numbered N1, N2, N3, etc.; residues preceding the N_{cap} are designated N′, N″, N‴, etc.) [16,17]. In addition, the side chain of the N_{cap} residue frequently accepts a hydrogen bond from the main-chain –NH of the N3 residue [16,17]. In Arc repressor, Ser32 is the N_{cap} residue for α-helix B, and the side-chain –OH of Ser32 forms hydrogen bonds with both the main-chain –NH group and the side-chain –OH group of the N3 residue, Ser35 [18] (Fig. 1a). Although the Ser32 side chain is 72% solvent-exposed, an alanine substitution at this position was found to be very destabilizing [1].

Arc repressor is a member of the ribbon–helix–helix family of transcription factors [19,20]. The ribbon–helix–helix domains of these proteins fold as homodimers, in which each subunit contributes to a single hydrophobic core. Arc functions to repress phage P22 gene expression by binding, as a dimer of dimers, to a 21-bp operator DNA site [18,21]. In the Arc-operator complex, the four Ser32 side-chain –OH groups are all within 3.5–4.5 Å of the sugar–phosphate DNA backbone [18] (Fig. 1b). As a result, the side chain of residue 32 in Arc is positioned to play a role in determining the affinity of the repressor–operator interaction, as well as being an important determinant of protein stability.

In this paper, we have investigated the tolerance of the helix-B N_{cap} position in Arc to a set of amino-acid substitutions. With one exception, the thermal stability of these N_{cap} mutants in Arc correlated well with the frequencies observed for the corresponding residues at N_{cap} positions in α-helices of other proteins. Somewhat surprisingly, however, cysteine was one of the most stabilizing residues at position 32 in Arc, but was the least frequent N_{cap} residue in the protein database. Only a small subset of the position-32 mutants showed significant biological activity in vivo or strong operator DNA binding in vitro, with small, uncharged side chains generally resulting in the most active Arc proteins. The wild-type protein was neither the most stable nor the most active Arc variant, but represented the best compromise between stable folding and tight DNA binding. Based upon the N_{cap} substitutions studied here for helix B of Arc, we propose revised sequence alignments of the MetJ and NikR subfamilies with Arc and other ribbon–helix–helix proteins.

2. Materials and methods

A library designed to contain all possible position-32 codons was constructed by cassette mutagenesis in the *arc-st11* gene of pSA700 [22] and was transformed into *Escherichia coli* strain UA2F [23,24]. In UA2F/pSA700 cells, Arc variants with at least 5–10% wild-type activity repress transcription of the *cat* gene and the streptomycin sensitivity gene, rendering the host sensitive to

chloramphenicol and resistant to streptomycin [23–25]. Active position-32 variants were selected by streptomycin resistance, and inactive variants were chosen by screening for streptomycin sensitivity. The resulting plasmid-borne *arc* genes were sequenced to determine the identity of the position-32 side chain. UA2F/pSA700 colonies were also screened for chloramphenicol resistance. Active mutants were defined as those pSA700 plasmids that conferred streptomycin resistance and chloramphenicol sensitivity; partially active variants were streptomycin-resistant and chloramphenicol-resistant; and inactive mutants were streptomycin-sensitive and chloramphenicol-resistant.

The *arc-st11* gene encodes the C-terminal sequence H_6KNQHE, which allows affinity purification and improves protein expression by reducing intracellular degradation [22]. Mutant proteins were overexpressed in *E. coli* strain X90 and were purified by affinity chromatography using nickel–NTA resin (Qiagen) and cation exchange chromatography using SP-Sephadex (Pharmacia) [22]. For thermal denaturation studies, circular-dichroism ellipticity at 222 nm was monitored from 0 to 100 °C, with a step size of 0.5 °C, an averaging time of 45 s and an equilibration time of 1 min. The Arc concentration was 50 μM in monomer equivalents and the buffer was 50 mM Tris (pH 7.5), 250 mM KCl, and 0.2 mM EDTA. To obtain reversible denaturation of the Cys32 variant, thermal unfolding was performed in the presence of 3 mM β-mercaptoethanol. Thermal denaturation curves were fitted by non-linear least-squares methods to a reaction model in which Arc dimers denature in a concerted reaction to two unfolded monomers [24], using equations previously described [1,22].

Binding of Arc variants to a ^{32}P-end-labeled *arc* operator fragment (O1) was assayed by gel-mobility shifts using minor variations of established methods [21,26]. To avoid oxidation, purified Arc variants were stored in lyophilized form and resuspended on the day of the assay. Proteins were diluted in 10 mM Tris (pH 7.5), 3 mM $MgCl_2$, 250 mM KCl, 0.1 mM EDTA, 0.1 μg/ml BSA, and 0.02% Nonidet NP40 and incubated with 10 pM operator DNA for 3–4 h at 25 °C. For the Cys32 variant, binding assays also contained 3 mM β-mercaptoethanol. Samples were electrophoresed on 7% polyacrylamide gels, and band intensities on the dried gel were determined using a Molecular Dynamics phosphor-imager and ImageQuant software. Binding data were fitted to a model, in which unfolded Arc monomers are in equilibrium with folded dimers and DNA-bound tetramers [21,26] using a non-linear least-squares subroutine implemented in the program KALEIDAGRAPH.

3. Results

3.1. Effects of position-32 replacement on Arc activity in vivo

Using cassette mutagenesis, we constructed a plasmid library in which all possible amino-acid substitutions were permitted at position 32 of Arc repressor. Active variants were selected by streptomycin resistance, as Arc binding to its operator results in resistance to this antibiotic in strain UA2F/pSA700 [23,24]. Among *arc* genes encoding active variants, Pro32 was recovered seven times, Ser32 (wild type) and Ala32 were recovered five times each, and Cys32 and Thr32 were recovered four times each. The *arc* genes from randomly chosen clones from the initial library were also sequenced, resulting in identification of nine additional position-32 mutants. Representatives of all 14 different substitutions at position 32 were then screened for resistance/sensitivity to streptomycin and to chloramphenicol (Table 1). In strain UA2F/pSA700, Arc-operator binding also represses the *cat* gene and results in sensitivity to chloramphenicol [23–25]. Only two mutants (Ala32 and Cys32) had intracellular activity similar to wild-type Arc (Ser32) according to the criteria that host cells were resistant to streptomycin and sensitive to chloramphenicol. Two additional mutants (Pro32 and Thr32) had partial activity according to the criterion that the UA2F/pSA700 host showed resistance to both streptomycin and to chloramphenicol. Mutants containing Asp32, Asn32, Glu32, Gln32, Gly32, His32, Met32, Val32 and Leu32 showed no detectable activity in the cell; their host cells were killed by streptomycin and were resistant to chloramphenicol.

Table 1
Activity in vivo of Arc position-32 mutants

Residue 32	Activity
Ser	+
Cys	+
Ala	+
Thr	+/−
Pro	+/−
Asp	−
Asn	−
Glu	−
Gln	−
Gly	−
His	−
Met	−
Val	−
Leu	−

(+) *E. coli* UA2F cells expressing an active position-32 mutant were streptomycin-resistant and chloramphenicol-sensitive. (+/−) cells expressing a partially active mutant were streptomycin-resistant and chloramphenicol-resistant. (−) cells expressing an inactive mutant were chloramphenicol-resistant and streptomycin-sensitive.

Arc repressor activity in the cell depends on the expression level of the protein, the fraction of the protein that forms native Arc dimers, and the intrinsic operator-binding activity of the dimer. Mutants in which the stability of the Arc dimer is reduced are degraded more rapidly in the cell, and thus have reduced expression levels [22]. Mutations that decrease Arc stability also increase the fraction of denatured monomers relative to native dimers, which also decreases repressor activity. Thus, the inactivity observed in vivo of the Asp32, Asn32, Glu32, Gln32, Gly32, His32, Met32, Val32 and Leu32 variants could result from alterations in either Arc stability or DNA affinity.

3.2. Thermal stability of purified variants

Individual mutant variants were purified to greater than 95% homogeneity by Ni^{2+} affinity and ion exchange chromatography. Each of the mutants, like wild-type Arc, had a far-UV circular dichroism (CD) spectrum at 15 °C expected for a protein with approximately 60% α-helical structure (spectra not shown). Moreover, each mutant showed a cooperative thermal-unfolding transition, as monitored by CD ellipticity (Fig. 2 and data not shown). Only one mutant, Asp32, was more thermally stable than wild-type Arc, with an increase in T_m of 7 °C (Fig. 2; Table 2). The remaining mutants had T_m values reduced by 4–29 °C relative to wild-type Arc (Fig. 2; Table 2). Clearly, the chemical identity of residue 32 plays

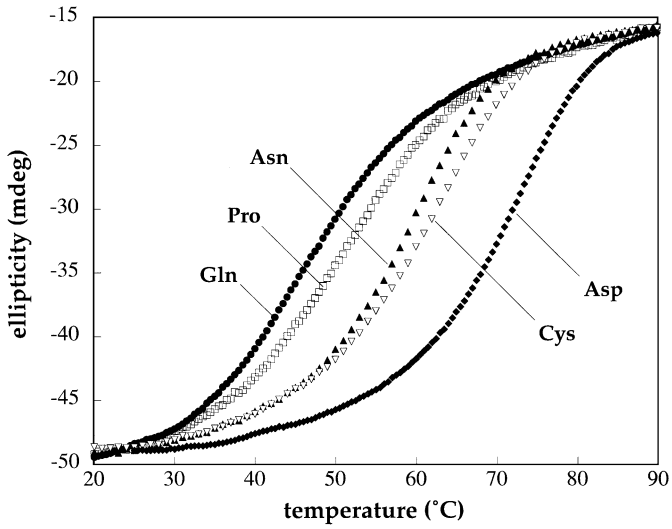

Fig. 2. Melting curves for five position-32 variants at 50 μM protein concentration in 50 mM Tris (pH 7.5), 250 mM KCl, 0.2 mM EDTA. The buffer for the Cys32 melt also contained 3 mM β-mercaptoethanol.

Table 2
Melting temperatures of Arc position-32 mutants

Residue 32	T_m (°C)	ΔT_m (°C)
Asp	71	+7
Ser	64	–
Cys	60	−4
Thr	58	−6
Asn	56	−8
Glu	51	−13
Pro	49	−15
Gly	48	−16
His	46	−18
Ala	45	−19
Gln	43	−21
Met	40	−24
Val	36	−28
Leu	35	−29

ΔT_m is the change in thermal stability relative to wild-type Arc (Ser32).

Table 3
Protein concentrations in monomer equivalents required for half-maximal binding of variants to the O1 *arc* operator.

Residue 32	Half-maximal concentration (nM)
Pro	0.56
Ser (wt)	0.57
Cys	4.5
Asn	5.5
Ala	5.5
Gly	48
Thr	73
Val	79
His	140
Glu	850
Gln	1300
Met	2000
Leu	22 000
Asp	33 000

a large role in determining the intrinsic stability of the Arc dimer.

3.3. DNA binding affinity of purified mutants

Gel electrophoretic mobility shifts were used to assay the operator–DNA binding of each of the purified position-32 variants. A representative experiment for the Cys32 mutant and binding curves for wild-type Arc and the Asn32, Cys32 and Thr32 mutants are shown in Fig. 3. Table 3 lists the protein concentrations required to observe half-maximal binding for each mutant. These values range from approximately 0.6 nM for wild-type Arc (Ser32) and the Pro32 mutant to 33 μM for the Asp32 variant. In addition to the Pro32 variant, the Cys32, Asn32 and Ala32 mutants were the most active, with half-maximal DNA-binding activity within 10-fold of wild-type Arc.

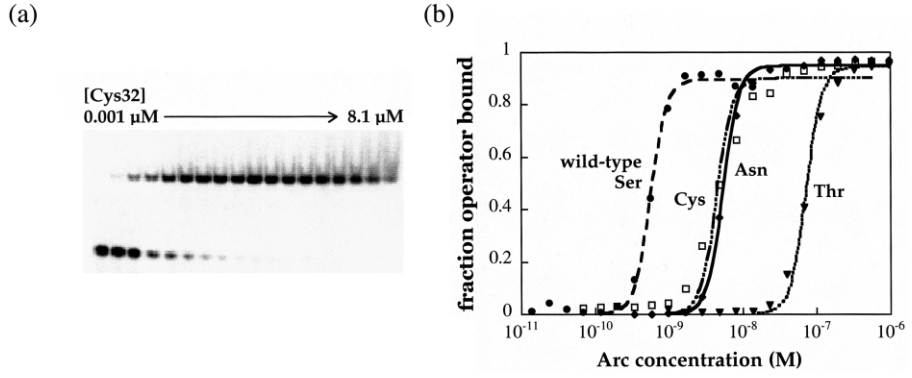

Fig. 3. DNA mobility shift assays. (a) Binding of the Cys32 variant to a ^{32}P-labeled oligonucleotide corresponding to the *arc* O1 operator [26]. The protein concentration was increased in 1.7-fold increments from 1 nM to 8.1 μM. (b) Binding curves for wild-type Arc and three position-32 variants. In all experiments, protein and DNA were incubated at 25 °C in a buffer containing 10 mM Tris (pH 7.5), 3 mM $MgCl_2$, 250 mM KCl, 0.1 mM EDTA, 0.1 mg/ml BSA, and 0.02% Nonidet NP-40. Buffer for the Cys32 experiment also contained 3 mM β-mercaptoethanol.

4. Discussion

The N-cap interactions in α-helix B of Arc are similar, but not identical to those found in N-capping boxes [17,27]. Canonical N-capping boxes contain one hydrogen bond between the N_{cap} side chain and the N3 main-chain amide (present in Arc), another hydrogen bond between the N_{cap} main-chain amide and the N3 side chain (absent in Arc), and hydrophobic interactions between the side chains of the N′ and N4 residues (replaced by a salt bridge in wild-type Arc, but present in the MYL Arc mutant [28,29]). The results presented here show that Ser32, the N_{cap} residue of helix B in Arc repressor, plays an important role in determining the stability of this protein to thermal denaturation. Approximately 70% of the surface of the wild-type Ser32 side chain is exposed to solvent, and mutant side chains at this position would also be highly solvent-accessible. Although surface residues do not usually play a major role in determining protein stability, the T_m values of our set of 14 position-32 Arc variants showed a 36 °C range (Table 2), with the SL32 substitution causing a 29 °C reduction compared with wild-type Arc. By contrast, amino-acid substitutions at Ser35, another solvent-exposed residue for which the side chain makes hydrogen-bond interactions in helix B, caused only small changes in T_m, and the SL35 mutant was 2 °C more stable than the wild type [30]. These differences among surface positions, which otherwise seem quite similar, highlight the special role that N_{cap} residues seem to play in stabilizing the native structure of proteins [3–15].

The great majority of the most stabilizing residues at position 32 in Arc are also commonly found at the N_{cap} positions in α-helices of other proteins. In fact, the T_m values of the position-32 variants in Arc correlate reasonably well with the frequencies of N_{cap} occurrence of the corresponding side chains in a library of 2101 α-helices in high-resolution protein structures (Fig. 4) [31]. In Arc, Asp32, Ser32, Thr32 and Asn32 represent four of the five most stable variants. The side chains of each of these residues could hydrogen bond with the main-chain nitrogen of the N3 residue (Ser35), a defining feature of the N-capping box motif. Among these four residues, Asp32 was the most stabilizing in Arc, probably because its negative charge interacts favorably with the partial positive charge at the N-terminus of the α-helix [3,32].

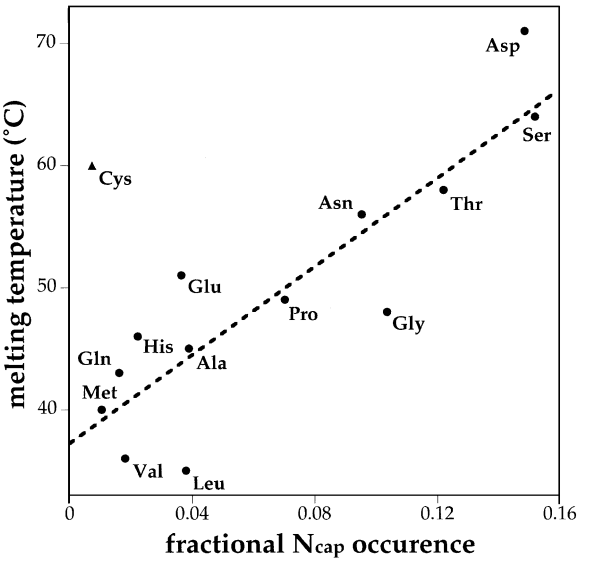

Fig. 4. Correlation between the T_m values of position-32 variants and the fractional occurrence of the variant residue at N_{cap} positions in a library of 2101 α-helices [31]. The line is a linear fit to all of the points except cysteine and has a correlation coefficient of 0.88. When cysteine is included, the correlation coefficient is 0.72.

The Cys32 mutant had the third-highest thermal stability in the library of position-32 Arc variants. This does not seem surprising, given that the cysteine side chain is sterically most similar to the wild-type serine side chain, is capable of forming weak hydrogen bonds [33,34], and could also interact favorably with the positive end of the helical dipole when deprotonated. Remarkably, however, cysteine is the rarest N_{cap} residue in natural proteins, comprising less than 1% of all of these positions [31]. Relative to other protein helices, there is nothing obvious about the structure surrounding the N_{cap} position of Arc helix-B that would suggest that this is a unique or highly favorably environment for cysteine. If this is true, however, then cysteine should also be a good N_{cap} residue in other proteins. Why then is cysteine so rarely used in this capacity? One possibility is

that the interaction with the helical dipole in an N_{cap} context reduces the pK_a of the cysteine side chain, resulting in a higher population of the deprotonated species under physiological conditions. Because this species is reactive in nucleophilic displacements, the solvent-exposed cysteine side chain at N_{cap} positions might be highly reactive and potentially prone to oxidation and other types of chemical modification. The Cys32 Arc variant was active in our screen, but under conditions where the cells were dividing rapidly and new protein was constantly being synthesized. Indeed, the purified Cys32 Arc mutant was highly prone to oxidation in the absence of reducing agents. Interestingly, when Cys32 is removed from the comparison of T_m values in the Arc variants with N_{cap} frequencies, the correlation coefficient improves from 0.72 to 0.88 (Fig. 4).

The identity of the side chain at position 32 in Arc also plays a critical role in protein function by influencing operator binding affinity. In the wild-type protein–DNA complex [18], Ser32 is close to the DNA backbone, but the wild-type Ser32 side chain does not make any direct hydrogen bonds with the operator. Nevertheless, mutant side chains at position 32 could destabilize the complex via steric or electrostatic clashes with the DNA. For example, modeling Thr32 into the protein–DNA complex without allowing structural relaxation results in steric clashes (2.4 Å) between the γ-CH_3 of the mutant side chain and the phosphate backbone. Indeed, even though the Thr32 Arc variant is quite thermally stable, this mutant is only partially active in vivo and displays a significant reduction in operator binding in vitro. Not surprisingly, most large and/or negatively charged side chains at position 32 (His, Glu, Gln, Met, Leu, Asp) resulted in Arc proteins that were inactive in the cell and displayed dramatic reductions in operator binding in vitro. The Pro32, Cys32, Asn32 and Ala32 variants had activity within 10-fold of the wild-type Ser32 protein in vitro, and, except for Asn32, showed some activity in vivo. Thus, relatively small and uncharged side chains are tolerated in the protein–DNA complex better than larger ones. The inactivity of the Asn32 variant in vivo was puzzling, as this variant was almost as stable as the Thr32 variant and was significantly more active in operator binding in vitro. One possibility is that the Asn32 side chain makes favorable interactions with the DNA backbone in complexes with non-operator sites, thereby increasing affinity for non-specific DNA and reducing the concentration of free dimers available for operator binding in the cell.

The Pro32 mutant was as active as wild-type Arc in vitro, despite its 15 °C reduction in thermal stability. In fact, after correcting for the reduction in the concentration of the Pro32 dimer caused by its reduced stability, the Pro32 dimer would half-maximally bind operator DNA at a concentration approximately 14-fold lower than the wild-type dimer. Previous studies of the Ala32 mutant dimer also revealed that it bound more tightly to operator DNA than the wild-type protein [2]. Electrostatic calculations indicate that the wild-type Ser32 side chain makes small, favorable DNA contacts in the operator complex, but also show that desolvation of this side chain is more energetically costly, resulting in a net interaction that is slightly unfavorable (S. Spector, B. Tidor, personal communication). This may explain the strengthened operator binding by the Ala32 and Pro32 mutants. Alternatively, these mutations may result in small conformational changes that improve other contacts between Arc and operator DNA.

The choice of side chain for position 32 in Arc repressor clearly represents an evolutionary compromise between protein stability and DNA binding activity. The wild-type residue, Ser32, does not result in the most stable protein or in the highest intrinsic operator affinity but is ranked near the top of both categories. Variants with Asp32 would be more stable, but inactive, and variants with Pro32 or Ala32 would probably be too unstable to permit strong DNA binding in the cell.

Arc is a member of the ribbon–helix–helix family of transcription factors, and it is instructive to ask how other family members choose the N_{cap} residue for helix B. In all previously published alignments [19,35–38], Ala50 or His49 of *E. coli* MetJ repressor is aligned with Ser32 of Arc as the helix-B N_{cap}. Inspection of the MetJ crystal structure [39], however, clearly shows that Thr51 is the N_{cap} residue for helix B of this protein. The

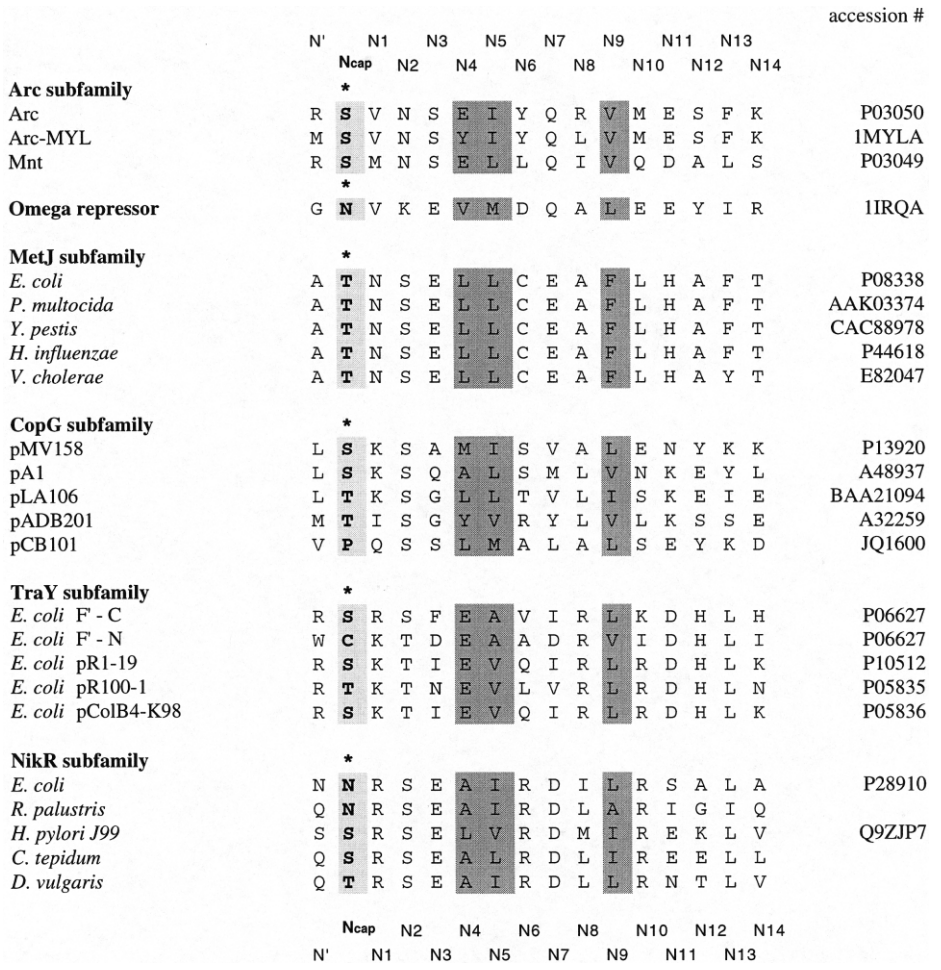

Fig. 5. Alignment of the helix-B region of ribbon–helix–helix proteins. The N_{cap} positions for Arc, Mnt, omega repressor, *E. coli* MetJ, and pMV158 CopG are known from crystal or NMR structures [18,37–39,41]. The N4, N5 and N9 positions are generally hydrophobic. When the N4 position is glutamic acid, the N′ position is arginine in all cases but one. The helix-B N′ and N4 positions form a salt bridge in Arc and Mnt. Sequences without accession numbers are from unfinished genomes.

position of the MetJ orthologs in the multiple alignment shown in Fig. 5 was offset by one residue at the beginning of helix B to reflect this fact. This same change was also made for NikR subfamily members. Although structural information is not available for NikR, our new alignment results in helix-B N_{cap} residues for NikR that are more consonant with those found in other family members and with the mutational studies presented here. The previous alignment, by contrast, had Gln, Glu and Met—which are unstable and inactive in Arc—as the N_{cap} residues in some NikR orthologs [36]. In our revised sequence alignment, most of the MetJ, CopG, TraY and NikR subfamily members use Ser, Thr, Asn or Pro as the helix-B N_{cap} residue. These results are consistent with those presented here, in that each of these side chains results in Arc molecules with biological activity in vivo, or reasonably strong operator binding in vitro. In this case, knowledge of the structural and functional effects of numerous substitutions at an important sequence position in one family mem-

ber—the helix-B N_{cap} residue in Arc—has suggested an improved alignment with more distantly related proteins.

Acknowledgments

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