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Mutational studies of protein stability and folding of the hyperstable MYL Arc repressor variant

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Abstract

The Arc-MYL variant is hyperstable and has an earlier transition state than wild type as a consequence of replacing the wild-type salt-bridge triad formed by R31, E36 and R40 with hydrophobic interactions formed by M31, Y36 and L40. Amino acid substitution mutations were constructed at 16 positions in the Arc-MYL background and the equilibrium stabilities of the corresponding mutant proteins were determined. At three positions, mutations were found to be less destabilizing in MYL than in wild-type Arc, and, at one position, the opposite result was obtained. The kinetics of refolding and unfolding were determined for a subset of the Arc-MYL core mutants. Three mutations—VA18, LA19 and LA40—had their major energetic effects on the refolding rate. The interactions perturbed by these mutations appear to be substantially formed in the transition state. V18 and L19 are in the N-terminal turn of helix A and L40 is in the center of helix B. The remaining mutations—VA22, MA31, VA33, YA36, VA41, MA42 and FA45—had some effects on refolding but exerted their major effects on the unfolding rate. Approximately 30% of the energetic interactions mediated by these latter side chains seem to be present in the transition state of Arc-MYL.

Keywords: Arc-MYL variant; Fast-folding mutants; Transition state; Context dependence; Buried salt bridge

1. Introduction

Does the basic structural topology of a protein fold determine its mechanism of folding and tolerance to mutations or do the specific interactions that stabilize the fold determine these factors? The wild-type and MYL variants of the Arc repressor dimer afford an opportunity to study this question. In wild-type Arc, a salt-bridge triad between R31, E36 and R40 forms part of the protein core, whereas in Arc-MYL hydrophobic interactions between M31, Y36 and L40 replace the salt bridge [1,2]. The remaining portions of the wild-type dimer and the MYL dimer, however, are very similar with an average RMSD deviation of only 0.7 Å. Arc-MYL shows two-state behavior in equilibrium and kinetics studies of unfolding and refolding but is approximately 4 kcal/mol more stable than wild type [2]. Interestingly, MYL also folds approximately 20-fold faster than the wild type in a reaction that shows a reduced denaturant dependence and smaller m_f/m value [3]. The latter observation suggests that the MYL transition state

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is more solvent exposed and thus less structured than that of the wild type. Consistent with this model, recent studies suggest that fewer α-helical hydrogen bonds are formed in the transition state of Arc-MYL than in the transition state of wild-type Arc [4].

Here, we examine the effects of a set of 17 mutations, located largely in the hydrophobic core, on the equilibrium stability and folding kinetics of Arc-MYL. Five of these mutations were too destabilizing to study in wild-type Arc, and thus the MYL experiments provide the first information concerning the participation of these residues in the folding mechanism. At the remaining positions, mutational effects could be compared in the both sequence backgrounds. Although, most mutations caused approximately similar stability changes in both protein contexts, a few core mutations were significantly less destabilizing in Arc-MYL and one N-cap mutation was less destabilizing in wild-type Arc. Surprisingly, some side-chain interactions appeared to be more fully developed in the transition state of MYL than in wild-type Arc, despite the fact that the MYL transition state seems to be located earlier in the folding pathway.

2. Materials and methods

Site-directed mutations were constructed by cassette mutagenesis in the *arc-MYL-st11* gene of pSA700-MYL and the corresponding mutant proteins were expressed and purified as described [5]. Protein concentrations in monomer equivalents were calculated using an extinction coefficient at 280 nm of 7953 M^{-1} cm^{-1} for Arc-MYL variants with one tryptophan and two tyrosines and 6756 M^{-1} cm^{-1} for Arc-MYL-YA36-st11 and Arc-MYL-YA38-st11, which have one tryptophan and one tyrosine. Far-UV circular dichroism and fluorescence spectra were used to evaluate the low-resolution structures of mutant proteins. For NMR experiments, the LA19, VA33 and IV37 mutants were subcloned into a pET21 vector in Arc-st11 and Arc-MYL-st11 backgrounds, and ^{15}N-labeled proteins were purified following expression in *E. coli* strain BL21(λ*DE3*/*pLysS*) cells in M9T minimal medium containing $^{15}NH_4Cl$ (0.8 g/l) as the only nitrogen source. HSQC-NMR experiments were performed at 25 °C using 1 mM or higher concentrations of ^{15}N-labeled protein in 20 mM sodium phosphate (pH 5.1), 10% D_2O.

Equilibrium urea-denaturation reactions [25 °C, 10 mM Tris·Cl (pH 7.5), 250 mM KCl] were performed and analyzed as described [6,7]. Kinetic experiments at 25 °C were performed at protein concentrations of 10 to 60 μM and assayed by changes in fluorescence (excitation 280 nm; emission 320 nm) using an Applied Photophysics DX17.MV stopped-flow instrument. For unfolding experiments, protein in 10 mM Tris·Cl (pH 7.5), 250 mM KCl was diluted into buffer containing 2–8 M urea. For refolding experiments, protein in 8.2 M urea, 10 mM Tris·Cl (pH 7.5), 250 mM KCl was diluted into buffer containing 0.8–4 M urea. Kinetic trajectories were fit to a two-state relaxation model consisting of unimolecular unfolding and bimolecular refolding reactions [6]. Relaxation kinetics were constrained by using the experimental equilibrium constant for dimer dissociation and unfolding determined at the same final denaturant concentration.

3. Results

Sixteen alanine-substitution mutants in Arc-MYL were initially constructed, expressed and purified. The LA12 mutation modified a core residue in the β-sheet of Arc-MYL. The VA18, LA19, VA22, and VA25 mutations altered core residues in α-helix A. Three mutations—GA30, MA31 and SA32—changed residues in the turn between helix A and helix B. Finally, the VA33, YA36, IA37, YA38, LA40, VA41, MA42 and FA45 sequence changes affect core residues in α-helix B. The positions of these mutations in the MYL structure are shown schematically in Fig. 1. The IA37 mutant was found to be too unstable to study even in the MYL background. As a result, we also constructed and purified the IV37 mutant, which was sufficiently stable to characterize its folding properties.

Under native conditions, the LA12, VA18, LA19, VA22, VA25, GA30, MA31, SA32, VA33, IV37, LA40, VA41, MA42 and FA45 mutants had far-UV circular dichroism (CD) and fluorescence spectra that were very similar to the Arc-MYL

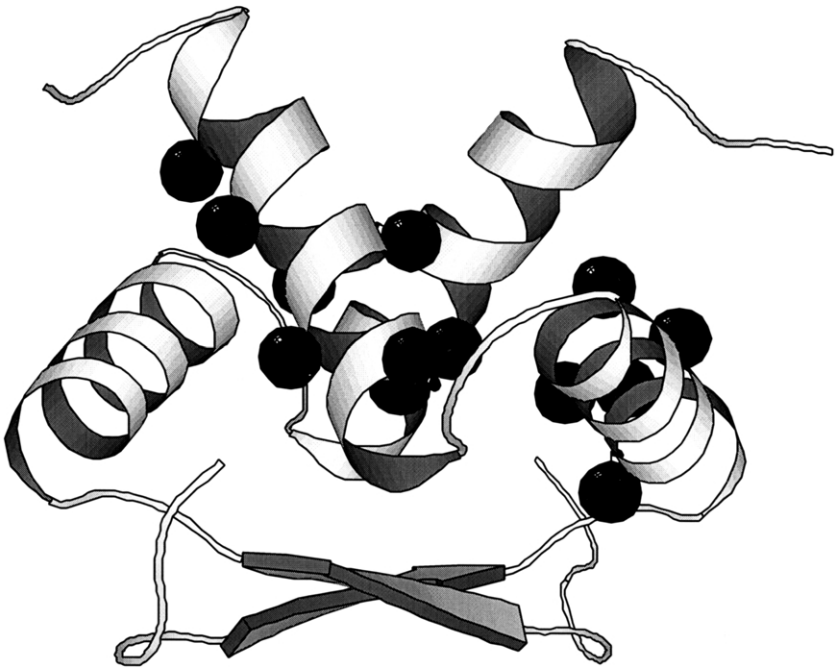

Fig. 1. View of the Arc-MYL dimer as a backbone ribbon trace. The sites in one subunit at which amino acid substitutions were introduced are highlighted as black spheres. This figure was prepared using the coordinates of an Arc dimer (1MYL) [2] and MOLSCRIPT [13].

parent (data not shown). Thus, these mutations do not appear to alter the native Arc-MYL structure in any dramatic fashion. Two mutants—YA36 and YA38—had CD spectra similar to Arc-MYL but showed lower intensity in their fluorescence spectra, presumably reflecting the reduced number of aromatic residues in these mutants. As a more detailed probe of structure, two-dimensional NMR HSQC experiments were performed for the LA19, VA33 and IV37 mutants in the Arc-MYL background. In each case, the mutants showed good chemical-shift dispersion, indicative of stably folded tertiary structures. Relative to Arc-MYL, there were small shifts in the resonances of approximately half of the protons in the mutant HSQC spectra, but similar shifts were also observed between wild-type Arc and the same mutants in otherwise wild-type backgrounds (data not shown). It seems likely that these resonance shifts reflect relatively minor structural changes in both the wild-type and MYL backgrounds.

The equilibrium denaturation of each mutant was assayed and data were fit to obtain the free energy of dimer dissociation and unfolding (ΔG_u) at 3.2 M urea and the *m*-value or change in ΔG_u with denaturant concentration (Table 1). Fig. 2 shows representative denaturation curves. Most of the mutants destabilize the Arc-MYL dimer by approximately 0.8–3.8 kcal/mol-dimer but the IA37 mutant was too unstable to study and the VA25 mutation had no significant effect on stability (Table 1). $\Delta\Delta G_u$ values for each mutation in the MYL and Arc backgrounds are also shown in Table 1. Interestingly, the VA22 and FA45 substitutions in the hydrophobic core were at least 2 kcal/mol more destabilizing in the wild-type Arc context than in the Arc-MYL background, whereas the SA32 mutation, which removes N-cap hydrogen bonds in helix B, was 1.3 kcal/mol more destabilizing in Arc-MYL.

Urea-jump experiments were used to determine refolding and unfolding rate constants for the

Table 1
Equilibrium stabilities of mutants (3.2 M urea, pH 7.5, 250 mM KCl, 25 °C)

Mutation in MYL	ΔG_u (kcal/mol)	m_u	$\Delta\Delta G_u$ in MYL (kcal/mol)	Mutation in Arc	$\Delta\Delta G_u$ in Arc (kcal/mol)
LA12	8.60	1.29	1.32	LA12	1.41
VA18	9.13	1.43	0.79	VA18	0.75
LA19	9.10	1.51	0.82	LA19	1.55
VA22	6.90	1.21	3.02	VA22	>5.00
VA25	9.96	1.13	−0.04	VA25	−0.65
GA30	8.63	1.56	1.29	GA30	1.95
MA31	6.75	1.28	3.17	RA31[a]	2.88
SA32	6.16	1.01	3.76	SA32	2.42
VA33	8.15	1.44	1.77	VA33	2.19
YA36	7.21	1.00	2.71	EA36[a]	>5.00
IA37	<5.00	–	>5.00	IA37	>5.00
IV37	8.55	1.43	1.37	IV37	1.56
YA38	6.92	1.00	3.00	YA38	3.16
LA40	8.61	1.34	1.31	RA40[a]	2.04
VA41	6.21	1.15	3.71	VA41	>5.00
MA42	6.95	1.13	2.97	MA42	3.05
FA45	7.22	1.07	2.70	FA45	>5.00

ΔG_u and m_u for Arc-MYL under these conditions are 9.92 kcal/mol and 1.21 kcal/M×mol, respectively. $\Delta\Delta G_u$ values for mutations in wild-type Arc are from Milla et al. [9].

[a] These mutants represent positions where Arc-MYL and wild-type Arc have different residues.

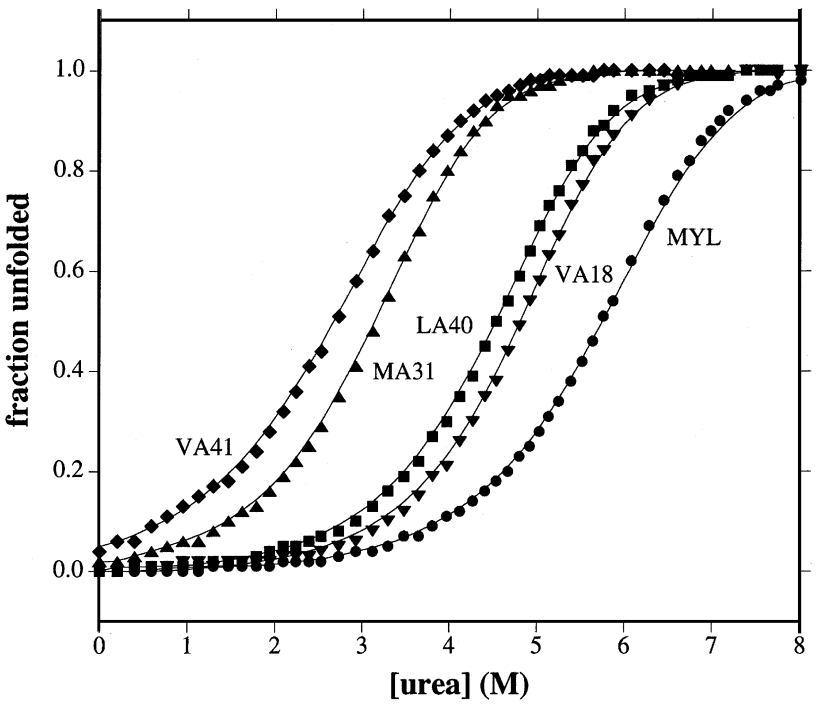

Fig. 2. Urea denaturation of Arc-MYL-st11 and selected mutant proteins.

VA18, LA19, VA22, VA25, MA31, VA33, YA36, LA40, VA41, MA42 and FA45 mutants. The kinetics appeared two-state by two criteria. First, the kinetic amplitude was the same or close to the value expected from equilibrium experiments. Second, the kinetic data were fit well by a two-state relaxation model using the equilibrium constant for unfolding and dimerization. Plots of $RT{\cdot}\ln(k_u)$ and $RT{\cdot}\ln(k_f)$ vs. [urea] are shown in Fig. 3 for the mutants and for Arc-MYL. Table 2 lists values of k_f and k_u for each mutant at 3.2 M urea (25 °C, pH 7.5 and 250 mM KCl). At this denaturant concentration, refolding and unfolding rates can be compared under conditions where both rates are significant and extrapolation errors are minimized. Relative to Arc-MYL, most of the mutations changed both the refolding rate constant and the unfolding rate constant. For the VA18 and LA40 mutants, however, the refolding rate constants were reduced but there were no significant changes in the unfolding rate constants.

Based on m_f/m values (Table 2), approximately 57% of the surface area buried in native Arc-MYL is solvent inaccessible in the transition state at 3.2 M urea. The mutant MYL variants had m_f/m values between 0.47 and 0.71 with an average value of 0.60 ± 0.07. For comparison, the m_f/m values for a set of 44 alanine-substitutions mutations in the wild-type Arc background ranged from 0.69 to 0.92 with an average value of 0.78 ± 0.05 [8]. Hence, as would be expected from the relative solvent exposure of the transition states of the parent molecules, the transition states of the Arc-MYL mutants are more solvent exposed than the transition states of mutants in the wild-type background.

Table 3 lists the rate constants for refolding and unfolding of each alanine mutant relative to the same constants for Arc-MYL and also shows Φ_F values, which represent the fractional extent to which the mutation exerts its energetic effect before or after the transition state. Φ_F values of 1 and 0 indicate effects solely on the refolding reaction or solely on the unfolding reaction, respectively; a Φ_F value of 0.5 suggests that half of the energetic effect of the mutation occurs before the transition state and half after the transition state. Of the 11 mutants for which kinetic studies were performed, three (VA18, LA19, and LA40) had Φ_F values between 0.75 and 0.99 and seven (VA22, MA31, VA33, YA36, VA41, MA42 and FA45) had Φ_F values between 0.16 and 0.35. The remaining mutation (VA25) did not alter the stability or kinetics sufficiently to permit calculation of a meaningful Φ_F value.

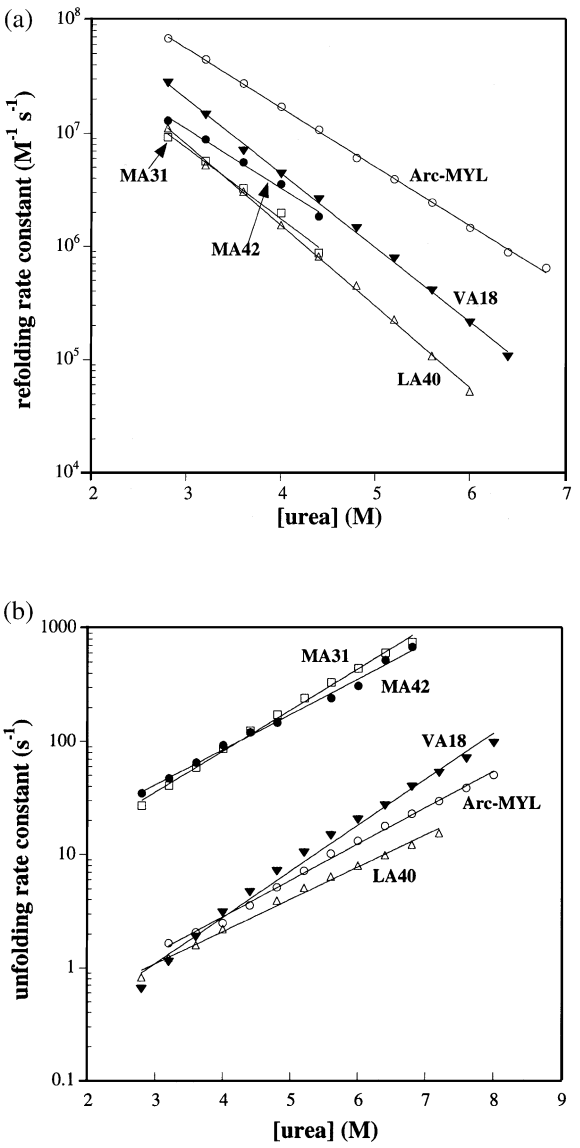

Fig. 3. Urea dependence of the rate constants for refolding (a) and unfolding (b) of Arc-MYL and selected mutants.

4. Discussion

The Arc-MYL results presented here combined with previous studies of the effects of alanine-substitution mutations in the wild-type Arc background [8,9] permit comparisons of the same amino acid substitutions in two proteins with virtually identical structures. For nine mutations (LA12, VA19, LA19, GA30, VA33, IV37, YA38, LA40 and MA42), the destabilizing effects in the wild-type and Arc-MYL backgrounds were approximately similar. For example, these nine mutations caused an average destabilization of 1.63 kcal/mol in Arc-MYL and 1.96 kcal/mol in wild-type Arc. These differences are within the experimental error, approximately 0.5 kcal/mol, of these measurements.

For the VA22, SA32, VA41 and FA45 mutations, significant stability differences were observed in the wild-type and MYL contexts. At the hydrophobic-core positions 22, 41 and 45, the alanine-substitution mutations were more destabilizing in the wild-type context by 1.3 to more than 2.3 kcal/mol. Why does Arc-MYL tolerate the deleterious effect of these core mutations better than wild-type Arc? Studies of T4 lysozyme mutants suggest that mutations in the protein core are less destabilizing if the conformations of nearby side chains can adjust to fill as much of the mutational cavity as possible [10]. One possibility, therefore, is that Arc-MYL is able to rearrange its core to minimize the cavities created by the VA22, VA41 and FA45 mutations, whereas the wild-type protein is unable to fill these voids without disrupting the geometry of the R31-E36-R40 salt-bridge hydrogen bond network. Because this polar network is largely solvent inaccessible, even small distortions

Table 3
Rate constants for mutants relative to Arc-MYL parent

Mutation in MYL	k_f^{MYL}/k_f^{ala}	k_u^{ala}/k_u^{MYL}	Φ_F
Helix A			
VA18	3.91	1.01	0.99
LA19	2.92	1.41	0.75
VA22	6.05	28.10	0.35
VA25	1.16	0.84	–
Turn			
MA31	6.35	36.01	0.34
Helix B			
VA33	2.31	8.97	0.28
YA36	2.12	48.13	0.16
LA40	8.52	1.10	0.96
VA41	4.87	111.56	0.25
MA42	3.73	41.99	0.26
FA45	2.41	42.27	0.19

Table 2
Kinetic properties of MYL mutants

Mutant protein	k_f (s^{-1} M^{-1})	k_u (s^{-1})	m_f (kcal/mol×M)	m_u (kcal/mol×M)	m_f/m
Arc-MYL	4.44E+07	1.12	0.69	0.52	0.57
Helix A					
VA18	1.14E+07	1.13	0.93	0.51	0.65
LA19	1.52E+07	1.58	0.71	0.79	0.47
VA22	7.34E+06	31.53	0.63	0.58	0.52
VA25	3.82E+07	0.94	0.63	0.50	0.56
Turn					
MA31	6.99E+06	40.39	0.78	0.49	0.61
Helix B					
VA33	1.92E+07	10.06	0.98	0.47	0.68
YA36	2.09E+07	53.99	0.59	0.41	0.59
LA40	5.21E+06	1.23	0.95	0.39	0.71
VA41	9.12E+06	125.14	0.71	0.44	0.62
MA42	1.19E+07	47.10	0.71	0.42	0.63
FA45	1.84E+07	47.42	0.57	0.50	0.53

of the hydrogen bond angles or distances that accompanied local core repacking in response to cavities could be very destabilizing.

The SA32 mutation was 1.3 kcal/mol more destabilizing in Arc-MYL than in wild-type Arc. In both structures, the side-chain hydroxyl group of serine 32 forms N-cap hydrogen bonds with the backbone amide and side-chain hydroxyl of serine 35. The S32 Oγ→S35 N distance is 3.15 ± 0.14 Å ($n=4$; 1PAR) in wild-type Arc and 3.33 ± 0.16 Å ($n=6$; 1MYL) in Arc-MYL. The S32 Oγ→S35 Oγ distance is 3.35 ± 0.22 Å ($n=4$; 1PAR) in wild type but 2.70 ± 0.13 Å ($n=5$; 1MYL) in five of the six Arc-MYL subunits in the crystallographic asymmetric unit [2]. Assuming that shorter hydrogen bonds are stronger, the SA32 mutation may more destabilizing in Arc-MYL because it removes one strong hydrogen bond and one moderately strong hydrogen bond, whereas the same mutation removes two moderately strong hydrogen bonds in wild-type Arc. We presume that small structural perturbations resulting from the MYL substitutions, which flank serine 32 and 35, allow the stronger interaction in Arc-MYL.

Previous studies have shown that the transition state for the folding of wild-type Arc is less solvent exposed than the transition state for Arc-MYL [3]. This same trend is observed for the Arc-MYL mutants, which show an average m_f/m value of 0.60 ± 0.07 compared to a value of 0.78 ± 0.05 for the same mutants in wild-type Arc [8]. We note that Waldburger et al. [3] reported an m_f/m value of 0.40 for Arc-MYL, whereas the value determined here was 0.57 (Table 2). This difference is caused by our use of higher urea concentrations in refolding studies. Curvature in plots of $\ln(k)$ vs. denaturant shifts the transition state closer to the native state for both Arc and Arc-MYL as the denaturant concentration increases [11].

The effects of the Arc-MYL mutations on the kinetics of unfolding and refolding probe the role of the mutated residues in the transition state of this Arc variant. Except for VA25, all of the mutations studied reduced the refolding rate by at least twofold, indicating that almost all of the mutated positions, which includes most of the hydrophobic core, participate in some interactions in the MYL transition state. The VA18, LA19 and LA40 mutations had most or all of their energetic effects on the refolding reaction, indicating that the interactions perturbed by these mutations form early in folding and are energetically nearly complete in the transition state. This, in turn, suggests that the first turn of helix A and central region of helix B may be reasonably well structured in the transition state of Arc-MYL. By contrast, none of the alanine-substitution mutations in otherwise wild-type Arc affected the refolding rate more than the unfolding rate [8]. As a result, some side chains appear to play rather different energetic roles in the transitions state of Arc-MYL and wild-type Arc.

The VA22, MA31, VA33, YA36, VA41, MA42 and FA45 substitutions in Arc-MYL altered both the refolding and unfolding rates with the latter being affected most strongly (Φ_F values of 0.16–0.36). Bronsted plots of $\Delta\Delta G_u/RT$ vs. $\ln(k_u)$ and $\ln(k_f)$ for these mutants were reasonably linear with slopes of -0.69 ($r=0.86$) and 0.34 ($r=0.69$), respectively. As a result, these core residues appear to be involved in loosely formed interactions that are approximately 30% complete in the transition state [12]. For wild-type Arc, Bronsted analyses suggested that approximately 20% of side-chain interactions involving core residues were made in the transition state [8]. Because the transition state of Arc-MYL is more solvent exposed than that of the wild type, it seems counterintuitive that the core interactions seem to be slightly better formed in the transition state of Arc-MYL. Nevertheless, the presence of the R31, E36 and R40 side chains in wild-type Arc may prevent early consolidation of other core packing interactions, whereas the M31, Y36 and L40 side chains in Arc-MYL promote interactions that lead to better core interactions without significant core compaction.

In conclusion, replacing the R31-E36-R40 salt bridge in wild-type Arc with the M31-Y36-L40 packing and hydrophobic interactions in Arc-MYL has significant effects both on the tolerance of the Arc fold to mutations and on the properties of the folding transition state. Most significantly, the energetics of certain side-chain interactions appear to be more complete in the transition state of Arc-MYL despite the fact that this transition state

appears to be less structured that that of wild-type Arc. This observation is inconsistent with the idea that the less structured transition state of Arc-MYL contains a simple subset of the interactions present in the wild-type transition state.

Acknowledgments

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