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Investigation of an Anomalous Accelerating Substitution in the Folding of a Prototypical Two-State Protein

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Abstract

The folding rates of two-state, single-domain proteins are generally resistant to small-scale changes in amino acid sequence. For example, having surveyed here over 700 single-residue substitutions in 24 well characterized two-state proteins, we find that the majority (55%) of these substitutions affect folding rates by less than a factor of 2, and only 9% affect folding by more than a factor of 8. Among those substitutions that significantly affect folding rates, we find that accelerating substitutions are an order of magnitude less common than those that decelerate the process. One of the most extreme outliers in this data set, an arginine to phenylalanine substitution at position 48 (R48F) of chymotrypsin inhibitor 2 (CI2), accelerates the protein's folding rate by a factor of 36 relative to that of the wild-type protein and is the most accelerating substitution reported to date in a two-state protein. In order to better understand the origins of this anomalous behavior we have characterized the kinetics of multiple additional substitutions at this position. We find that substitutions at position 48 in CI2 fall into two classes: substitutions such as isoleucine or phenylalanine, which ablate the charge of the wild-type arginine while retaining the hydrophobic component corresponding to its alkane chain, accelerate folding at least tenfold, but all other substitutions produce folding rates within a factor of two of the wild-type rate. A significant positive correlation between hydrophobicity and folding rate across all of the residues we have characterized at this position suggests that the hydrophobic methylene units of the wild-type arginine play a significant role in stabilizing the folding transition state. Likewise, studies of the pH dependence of the histidine substitution indicate a strong correlation between folding rate and charge state. Thus, mutations that ablate the arginine's positive charge whilst retaining the hydrophobic contacts of its methylene units tend to dramatically accelerate folding. Previous studies have suggested that arginine-48 plays an important functional role in CI2, which may explain why it is highly conserved despite the anomalously large deceleration it produces in the folding of this protein.

Keywords

transition state; protein folding; point substitution; unfolding kinetics; mutations

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Introduction

When Anfinsen demonstrated that a protein's native structure is defined by its sequence¹, it followed that protein folding mechanisms are also defined by protein sequence. Conversely, differing polypeptide sequences are likely to fold via different routes and with different rates; even the set of simple, single-domain proteins that fold via apparently two-state kinetics exhibit a million-fold range of folding rates^{2,3}. But these observations leave open a subtler question: to what extent are protein folding and unfolding rates defined by the finest details of protein sequence? That is, how sensitive are protein folding kinetics to small-scale sequence substitutions? The earliest efforts to apply the techniques of protein engineering to the study of protein folding resulted in the discovery that point substitutions can affect folding rates by an order of magnitude^{4,5}. Likewise, early protein-engineering studies emphasized the surgical precision with which individual functional groups in a side chain contribute to the thermodynamics of the folding transition state⁶.

In contrast to some of the above observations, a more global view of protein folding suggests that the folding kinetics of at least two-state proteins may be relatively insensitive to the fine details of the protein's sequence. For example, the order-of-magnitude decelerations and accelerations typically associated with single residue substitutions are dwarfed by the six-orders-of-magnitude range spanned by the folding rates of even two-state proteins⁷. Likewise, others have pointed out that ϕ values (the ratio of the extent to which a substitution alters the free energies of the transition and native states relative to the denatured state⁵) appear to be defined more by the position of a residue in the protein's sequence (*i.e.*, all mutations at a given position produce the same ϕ value) than by the precise nature of the functional groups deleted or appended by the substitution^{8–11}. Finally, it is also known that several gross measures of a protein's overall topology strongly correlate with the folding rates of two-state proteins (reviewed in Makarov and Plaxco¹²), an observation that is perhaps difficult to reconcile with strongly sequence-dependent kinetics as such likely swamp any signals arising from more global parameters (reviewed in Gillespie and Plaxco¹³). In this light we again ask: to what extent do the fine details of a protein's sequence define its folding kinetics? Here we address this question by (1) surveying the literature to better characterize the extent to which single substitutions affect two-state folding and unfolding rates, and (2) performing a detailed experimental examination of the single substitution in our survey that most dramatically accelerates the folding rate of a two-state protein.

Results

In order to better assess the frequency with which small-scale sequence substitutions lead to significant kinetic folding effects we have compiled a data set comprised of the folding kinetics of 746 single site substitutions in 24 of the most exhaustively characterized two-state proteins described in the literature to date (Table 1, Figure 1, and supporting material). The proteins in the data set include helical, sheet, and mixed helix/sheet structures and fold with (wild-type) rates ranging from $169,000 \text{ s}^{-1}$ for 1prb_{7–53} [ref ¹⁴] to 0.24 s^{-1} for acylphosphatase³. We note, however, that while this data set reflects a broad sampling of two-state proteins – and substitutions therein it is – not without potentially important biases. For example, many of the data points compiled in this survey were collected to support ϕ -value analyses, and thus it is common to select minimally disruptive substitutions for these studies^{6,10}. In contrast, however, solvent-exposed positions are only rarely selected for ϕ -value analysis (since, it is generally assumed, these are less likely to participate in stabilizing the transition state¹⁵), and thus the data set may be skewed to report more disruptive substitutions than a random sample. Also, since protein engineering approaches often

involve substitution to alanine, the data set may be skewed to contain many cavity creating variants, which likely decelerate folding since they typically destabilize^{16–22}. Finally, substitutions resulting in highly destabilized proteins may be difficult to characterize experimentally and thus may also be missing from the literature (and our data set). Nevertheless, with over 750 entries, including numerous sequence variants that were generated for reasons other than ϕ -value analysis, we believe that our data set is sufficiently representative to provide insight into the extent to which point substitutions affect folding rates.

We find that, as previously noted (*e.g.*, Plaxco *et al.*⁷), point substitutions generally do not substantially affect the folding rates of two-state proteins (Figure 1a). For example, 55% of the modified proteins in our data set fold with rates within a factor of two of that of the equivalent wild-type protein (that is, between half and twice the wild type folding rate), and 91% of all the variants in our data set fold with rates within a factor of 8 of the wild-type sequence. Perhaps not surprisingly the large majority (91%) of the variants that affect the folding rate by a factor of two or more decelerate folding, and only 4% of the total substitutions compiled in our survey accelerate folding by more than a factor of two. Put another way, mutations that significantly (>2-fold) decelerate folding are an order of magnitude more common than those that significantly accelerate folding. Indeed, despite the potential bias produced by the disproportionate number of alanine substitutions in our data set, we find that, among substitutions to residues other than alanine, significant (more than two-fold) acceleration is five times less common than significant deceleration. Consistent with this, the kinetic effects (k_f'/k_f) of alanine and non-alanine substitutions are quite similar, with median values of 0.57 and 0.68, respectively.

In contrast to the generally limited affect they have on folding rates, point substitutions often alter *unfolding* rates quite dramatically (Figure 1b). Across our data set (save for approximately four dozen substitutions for which unfolding data were not reported) we find that, on average, point substitutions change unfolding rates by a factor of 50, with the most dramatic effect being a four orders-of-magnitude acceleration. Consistent with this, fully 69% of the unfolding rates are affected at least twofold: of these, 94% are accelerated, and 6% decelerated. This means that substitutions that significantly speed up unfolding are 15 times more common than those that significantly slow unfolding. That substitutions affect unfolding rates more than they affect folding rates is consistent with the observation that the folding transition state is expanded and less structured than the tightly packed, fully-folded native state^{23–25}. That is, the folding barrier is less sensitive to mutations because its height is defined by interactions in the (expanded, relatively unstructured) transition state and the (even more expanded, still less structured) unfolded state, where substitutions are less likely to lead to significant energetic consequences. In contrast, the *unfolding* barrier is defined by the thermodynamics of the (well packed, well structured) native state, and thus unfolding rates are much more sensitive to the fine details of sequence. This argument is consistent with the observation that folding ϕ values are typically low²⁶: the median value of the reported ϕ values in our data set is 0.24, implying, by the usual interpretation of such data²⁶, that the structure gained from the unfolded state to the transition state is significantly less than the structure gained from the transition state to the native state.

While the large majority of characterized single-site substitutions do not significantly alter folding rates, a few notable exceptions are apparent. For example, a number of substitutions that involve the truncation of large hydrophobic residues significantly decelerate folding, including the F15A variant of Im9, which does so by a factor of 190 [ref ²⁷]. Substitutions that accelerate folding, while much less common, are also present in our data set. One example, the D48G substitution in the α -spectrin SH3 domain, accelerates folding 20-fold, reportedly due to relief of backbone strain arising from unfavorable Ramachandran angles in

the native state^{28,29}. The most extreme acceleration due to a single substitution is found in chymotrypsin inhibitor 2 (CI2; residue numbering as in Itzhaki *et al.*³⁰), the first protein reported to fold in an apparently two-state fashion³¹ and the most exhaustively characterized protein to date in terms of the number of positions that have been investigated via substitution. Indeed, 12% of the approximately 750 substitutions in our data set are in this 64-residue protein. Among these is the R48F variant, which is reported to produce a 40-fold increase in folding rate³², the single most accelerating substitution reported to date in a two-state folding protein (Figure 2).

Because substitutions that accelerate folding are much rarer than those that decelerate folding, the R48F substitution in CI2 appears particularly anomalous. In order to better understand the mechanisms underlying this unusual effect we have characterized the kinetic effects of other substitutions at the same position. To guide these studies we first noted that two mechanisms have been proposed for the acceleration associated with the R48F substitution: 1) the arginine to phenylalanine substitution alleviates unfavorable charge interaction in the transition state between arginine-48 and arginine-46 (Figure 2), and 2) like the three methylene units in the arginine side chain, phenylalanine-48 makes favorable hydrophobic transition state contacts with residues isoleucine-30, leucine-32 and phenylalanine-50 [ref³²]; at least two of these residues exhibit statistically significant non-zero ϕ values³⁰, indicating that these residues play an important role in stabilizing the folding transition state. To test these putative mechanisms, we selected a representative sampling of substitutions that included hydrophobic, charged, polar, large, small, and β -branched residues (Table 2). The relevant thermodynamic and kinetic parameters describing each substituted protein were obtained, as is now standard³³, using fits of the linear dependence of the natural log of the observed folding and unfolding relaxation rate constants on denaturant concentration (Figure 3).

The wild type folding rate constant we obtained for CI2 is, at $44 \pm 3 \text{ s}^{-1}$ (error bars represent standard errors calculated as per Ruczinski *et al.*³⁴), quite close to previously reported values, which range from 48 s^{-1} to 59 s^{-1} under the conditions employed here^{30–32, 35,36}. Likewise consistent with previous reports, we observe dramatically accelerated folding for the R48F variant, although the $1570 \pm 110 \text{ s}^{-1}$ rate we observe is experimentally significantly slower than the previously published value of $2300 \pm 200 \text{ s}^{-1}$. In contrast to the behavior of the phenylalanine substitution, most of the other substitutions we have investigated at position 48 in CI2 produce only small (less than 2-fold) changes in folding rate, with the polar substitutions generally decelerating folding (Table 2). For example, substitution to asparagine and glutamine reduces the folding rate by 30% and trivially, respectively. Substitution with histidine, in contrast, accelerates the folding of the wild type protein by almost 2-fold at neutral or alkaline pH. As the pH is reduced, however, and the histidine presumably becomes charged (and less hydrophobic), the folding rate of this substitution decelerates substantially relative to that of the wild type at the same pH (Figure 4a), supporting the aforementioned charge repulsion hypothesis. In light of this charge repulsion hypothesis, one might expect that substitution of arginine-48 with a negatively charged glutamate would accelerate folding. It, however, does not, presumably because the shorter side chain of this residue places a charged group in a buried position and thus the dependence of folding rate on hydrophobicity takes precedent.

In contrast with the above results, but consistent with the accelerating effects of the phenylalanine substitution, substitution of arginine-48 with other large hydrophobic residues leads to significantly accelerated folding. Substitution by isoleucine, for example, accelerates the folding rate by a factor of 50, which is significantly greater than the 36-fold acceleration observed for the phenylalanine substitution. More generally there is a significant correlation between hydrophobicity and folding rate across all of the residues we

have characterized at position 48 (Figure 4b). That is, while there is no single, universal scale of hydrophathy, using four commonly employed scales –Kyte-Doolittle³⁷, Hopp-Woods³⁸, Fauchère³⁹, and Roseman⁴⁰ – we find that there is a statistically significant correlation between a substitution's impact on folding rate and its hydrophathy value, with r^2 values ranging from 0.57 to 0.77 (shown in Figure 4b is the Roseman scale). Similar relationships between folding rate and the hydrophathy of substitutions – or entire proteins – have been seen in other proteins. For example, incremental ablation of non-hydrogen atoms at six different positions in the amino-terminal domain of the ribosomal protein L9 (NTL9), all of which are at least 70% buried, produces a statistically significant correlation between folding rates and the hydrophathy of the altered residue at all six positions⁴¹. A similar relationship is observed in the protein prb₇₋₅₃ in which a hydrophobic measure of various amino acid substitutions correlates strongly ($r^2 = 0.93$) with folding rate¹⁴. Finally, a significant correlation has been reported between sequence-averaged hydrophobicity and folding rate across a set of several single-domain proteins in the acylphosphatase structural superfamily⁴².

Perhaps consistent with the anomalously large acceleration associated with the introduction of large hydrophobic residues at position 48 in CI2, the rather divergent folding kinetics for various substitutions at this single site differs from the more homogeneous behavior observed for other two-state proteins that have been investigated via multiple substitutions at a single site (Figure 5). For example, eight distinct positions in two different SH3 domains have been characterized via multiple substitutions^{8,9,11,43-45}, and in each case the distribution of folding rates is relatively narrow (the most rapid variant in each set folds at most 31-times more rapidly than the slowest) and unimodal (the folding rates of all substitutions cluster around the mean value). This contrasts sharply with substitutions at position 48 in CI2, which are at least three times more divergent (they span an almost 100-fold range of rates) and are clearly bimodal: while the folding rates of the larger hydrophobic variants cluster within a two-fold range around 1100 s⁻¹ those of the other variants cluster within a factor of two of the wild-type value of 44 s⁻¹.

Discussion

The vast majority of single-site substitutions in our data set affect folding rates by less than twofold, and over 90% of all substitutions affect folding rates less than 8-fold (Figure 1a). These observations are consistent with the previous observation that homologous proteins tend to fold with similar rates (see Plaxco *et al.*, and references therein⁷), and that even very extensive sequence modifications leave folding rates largely unchanged⁴⁶⁻⁴⁸. We also find that point substitutions decelerating folding by more than two-fold are 10-times more common than point substitutions that accelerate folding by at least a similar amount. Finally, in contrast to folding rates, unfolding rates appear highly sensitive to the effects of point substitutions: on average we observe a 50-fold change in unfolding rate associated with the substitutions in our data set, with single-site substitutions accelerating unfolding by as much as four orders of magnitude (Figure 1b).

While point substitutions that accelerate folding are rare, the effects of some can nevertheless be dramatic, although still small relative to the million-fold range of known two-state folding rates. The most accelerating of the previously reported substitutions, R48F in CI2, folds 36 times more rapidly than the wild type sequence from which it was derived; investigating this effect in detail, however, we find that substitution of this position to a large hydrophobic residue accelerates folding 13- to 51-fold (Table 2). In contrast, substitution to smaller and/or charged residues at this position in CI2 produces folding rates that remain within a factor of two of the wild type rate. This strongly biphasic behavior differs from other single sites that have been investigated via multiple substitutions, for

which the folding rates of all substitutions cluster fairly tightly around the mean value^{8,9,11,43–45}. This pattern of kinetic effects is consistent with the hypothesis put forth by Fersht and coworkers³², who proposed that the R48F substitution in CI2 alleviates unfavorable charge interactions in the transition state (arising due to neighboring arginines 46 and 48, which are both relatively solvent inaccessible) whilst simultaneously increasing favorable interactions with nearby hydrophobic residues. Specifically, the correlations we have observed between charge and folding rate – in which the pH dependence of folding of an R48H variant is evident (Figure 4a) – and hydrophobicity and folding rate (Figure 4b) support this model.

The hypothesis of Fersht and coworkers suggests that the anomalously rapid folding of the R48F substitution arises because of the amphipathic nature of arginine: with a positively charged guanidinyll group and three hydrophobic methylene units in its side chain, this residue is capable of simultaneously supporting both hydrophobic interactions (which, in this context, are favorable) and electrostatic interactions (here unfavorable). Nevertheless, the significant acceleration – as much as 51-fold – associated with substitutions of this specific arginine appears to be limited to the specific physics of position 48 in CI2. That is, even among other, similar substitutions in which a partially buried (86% for arginine-48) charged residue is substituted to a hydrophobic residue, site 48 in CI2 remains anomalous. For example, substitution of two buried lysines to isoleucine or valine results in 2-fold and 3-fold rate increases, respectively, for the folding of the protein 1prb_{7–53} [ref¹⁴]. Likewise, single and double substitutions of buried lysines for norleucine results in a maximum acceleration of only 6-fold in a subdomain of the chicken villin headpiece⁴⁹.

The anomalously large extent to which substitutions of arginine-48 in CI2 accelerates folding raises a question regarding why evolution has placed an arginine in this position not only in CI2 but in the large majority of its known homologs. Indeed, among 17 readily identified, diverse homologs of CI2, all but four contain arginine in this position (Figure 6). This conservation appears to arise due to functional constraints linked to the stabilizing effect of a network of hydrogen bonds formed between the scissile bond loop and other internal residues, which is thought to confer resistance to proteolysis⁵⁰. Consistent with this, the homologs lacking an arginine at position 48 are at least an order of magnitude poorer protease inhibitors than the other functionally characterized members of this set of proteins^{50–53}. Further support of this argument is provided by Koshland and coworkers, who measured the inhibitory activity of an R48A variant of CI2 and found that its activity was also compromised, with an inhibition constant more than two orders of magnitude poorer than that of the wild type protein⁵⁰. More generally, while several authors have argued that rapid folding itself provides a selective advantage^{54,55}, functional selections appear to have maintained the more slowly folding sequences containing arginine at position 48. Perhaps tellingly, the four non-arginine residues observed in our data set of low-redundance CI2 homologs are isoleucine, phenylalanine, or tryptophan, all of which accelerate the folding of CI2.

The rapid folding associated with large, hydrophobic substitutions at position 48 in CI2 indicates that, even if they are rare, some point substitutions accelerate folding rather dramatically, an observation that is seemingly at odds with previous reports that global measures of topology accurately predict folding rates⁵⁶ (see also Makarov and Plaxco¹²). We note, however, that even the most rapidly folding substitution we have identified, R48I, folds only 11 times more rapidly than the 200 s⁻¹ predicted⁵⁷ using the contact order of the wild type protein (PDB code 2CI2). That is, while this variant is one of the most significant upward outliers on a contact order/rate plot, its deviation from the predicted value remains modest relative to the million-fold range of known two-state folding rates. As we have previously argued, this may be because topological metrics such as contact order capture the

entropic cost of achieving a grossly correct folded state, and thus reflect a sequence-independent component of the folding rate barrier insensitive to substitutions¹². A test of this argument would be to systematically attempt to increase folding rates via the judicious selection of multiple, accelerating substitutions, an approach that, to the best of our knowledge, has seen only limited exploration to date (see, perhaps, refs^{14, 44, 47, 58, 59}).

Materials and Methods

Expression and purification of CI2

The CI2 plasmid was generously donated by Dr. Yawen Bai. The CI2 gene was cloned into pET-17b (Novagen) at the NdeI and BamHI restriction sites. The plasmid was transformed into *E. coli* BL21 Gold (DE3) competent cells (Stratagene). Cells were grown to an OD₆₀₀ of 0.4, at which point expression was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG, Sigma). Cells continued growing for 4 to 6 hours, were harvested by centrifugation, and frozen. The pellet was resuspended in 50 mM Tris, pH 8.0 and lysed using a French press. Cell debris was removed by centrifugation. DEAE Trisacryl (Sigma) was used to nonspecifically scavenge proteins from the cleared lysate in batch adsorption. Size exclusion chromatography was carried out on a XK16/70 column with Superdex G75 resin (Amersham Biosciences) in 50 mM Tris, 150 mM NaCl, pH 8.6. CI2 was dialyzed extensively against water, flash frozen, and lyophilized.

Mutagenesis

Point mutations were made using the QuikChange II Site-Directed Mutagenesis kit (Stratagene). Purification for each variant was carried out as described above.

Kinetics measurements

Time resolved fluorescence measurements were performed on an Applied Photophysics stopped-flow fluorometer, model SX18MV. Unfolding reactions were initiated by rapid dilution of 25 μM protein, in 50 mM 2-(N-Morpholino)ethanesulfonic acid (MES, Sigma) at pH 6.25, with varying concentrations of guanidine hydrochloride (GuHCl, USB Corp), at pH 6.2. The protein:GuHCl ratio was 1:10. Unfolding reactions were done similarly except with the protein sample initially unfolded in 6.5 M GuHCl. Based on fluorescence at 354 nm the wild type protein is unfolded at this concentration (data not shown). λ_{ex}, was 280 nm (2 mm slit widths), and a 320 nm cut-off filter was used. The temperature was maintained with a water bath at 25° ± 0.2. Data collected were the average of five measurements. The fluorescence data were fitted to a single exponential with a sloped baseline using the Applied Photophysics software. The linear term corrects for a frequently observed baseline drift in the Applied Photophysics stopped flow fluorimeter^{60,61}. To obtain the kinetic parameters for folding and unfolding of CI2, the observed rate constants from kinetics experiments were fit to a “chevron” curve:

$$\ln(k_{obs}) = \ln(e^{\ln(k_{f,H_2O}) - m_{kf}[\text{GuHCl}]} + e^{\ln(k_{u,H_2O}) + m_{ku}[\text{GuHCl}]}) \quad (1)$$

in which k_{obs} is the measured relaxation rate in the folding/unfolding reaction, k_f and k_u are the folding and unfolding rate constants in water, respectively, and m_f and m_u are the free energy dependences on denaturant concentration for folding and unfolding, respectively.

pH jump experiments

For the measurements of the R48H variant at pH 4 and 8, a 25 μM sample of protein denatured in unbuffered pH 1.7 HCl was diluted (1:11) with buffered 50 mM MES to a final pH of 6.25, and fluorescence data were captured as described above.

Homology analysis

To assess the level of conservation in homologs of related and diverse species, we conducted a BLAST⁶² search against two databases – SwissProt⁶³ and RefSeq⁶⁴ – keeping the top ten hits from each. To further increase the diversity in this set, we used the CI2 sequence to query the conserved domain database (CDD) – a database of protein domain family hierarchies⁶⁵ – resulting in an additional 13 sequences in the potato inhibitor I family. This initial set of sequences was pared to 17 representative members by screening out those with a sequence identity above 70% to any other member of the set.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CI2	chymotrypsin inhibitor 2
GuHCl	guanidine hydrochloride

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Figure 1.

The effect of point substitutions on two-state protein folding and unfolding rates. We have collected a data set of the kinetic effects of 747 single site substitutions in 24 of the most well characterized single-domain, two-state proteins (Table 1), and find that, while folding rates are rather insensitive to fine changes in sequence, unfolding rates can vary dramatically with point substitutions. **(a)** The large majority (55%) of point substitutions do not affect folding rates by more than a factor of two, and 91% affect folding by less than a factor of 8. Consistent with this, the average acceleration or deceleration is only 4-fold. Also of note, decelerating substitutions are ten times more common than accelerating substitutions. Indicated is the R48F substitution in CI2, which is the most extreme accelerating outlier, corresponding to a ~40-fold increase in folding rate. **(b)** Unfolding rates, in contrast, are far

more sensitive to point substitutions, exhibiting an average rate constant change (acceleration or deceleration) of 50-fold.

Figure 2.

The structure of CI2 (PDB code 2CI2). Substitution of arginine-48 with large, hydrophobic residues accelerates the folding of this two-state protein by some 40-fold. This is thought³² to arise due to the alleviation of unfavorable electrostatic interaction with neighboring arginine-46 whilst simultaneously improving contacts with neighboring hydrophobic residues (I30, L32, and F50).

Figure 3.

Chevron plots for variants at site 48 in CI2. Whereas most substitutions do not significantly alter the protein's folding rate, substitution with the larger hydrophobic residues, Phe (F), Tyr (Y), Ile (I) or Trp (W), significantly accelerates folding. In contrast, *all* of the characterized substitutions significantly accelerate unfolding.

Figure 4.

The charge and hydrophobicity dependence of folding rates of CI2 substituted at site 48. **(a)** The folding rates of the histidine substitution at various pH values indicate that the folding rate of CI2 is a strong function of the charge at this position. At higher pH, where His48 is less positively charged, folding accelerates, presumably due to alleviation of charge repulsion with the neighboring arginine-46. Wild type, closed circles; R48H, open circles. **(b)** Likewise, the extent to which various substitutions at this position alter folding is well correlated ($R^2 = 0.77$) with the hydrophobicity of the substituted amino acid. This observation is consistent with the hypothesis that interaction with neighboring hydrophobic residues contributes to favorable transition state energy. The correlation improves ($R^2 = 0.82$), however, if charged residues are excluded (data not shown), suggesting once again that specific charge effects also play a role. The reported correlation coefficients are for the relationship between $\ln(k_f)$ and hydrophobicity as defined by Roseman⁴⁰.

Figure 5.

Bimodal behavior of substitutions at position 48. The distribution of rates associated with substitutions at position 48 in CI2 is bimodal and encompasses a much broader range of rates than other examples of multiple substitutions at a single site^{8,9,11,43–45}. That is, while sets of single site substitutions typically cluster around the mean value, the fastest CI2 variant folds 98 times more rapidly than the slowest, and the substitutions cluster into two groups.

Figure 6.

Conservation of arginine in CI2 homologs. The organism of origin is on the left, and the NCBI GI identifier is on the right. The vast majority of CI2 homologs contain arginine at the position corresponding to residue 48 in CI2. Four exceptions – in amaranth⁴⁸, barley⁹⁴, earthworm⁵¹, and rice (putative protein) – contain tryptophan, phenylalanine, or isoleucine. Both ATSI (amaranth) and CI-1 (barley) are reported to exhibit reduced inhibitory activity (see text). This suggests a tradeoff between rapid folding and specific biochemical function.

Table 1

proteins for which several substitutions' folding rates have been measured.

Protein name	Length	Number of mutations	Greatest acceleration/deceleration	Fold maximum change	acceleration (↑) or deceleration (↓)	Reference(s)
ACBP	86	30	L80A	19×	↓	66
α-spectrin SH3	62	52	V44T (D48G background)	59×	↓	29,67 <i>a</i> ,68, 69,70
λ repressor 6-85	92	7	A66G (G46A/G48A background)	19×	↓	71
acylphosphatase	92	24	Y11I	12×	↓	3
apo-azurin	128	10	L50A	32×	↓	72 <i>a</i> ,92
CI2	64	84	R48F	40×	↑	30,32 <i>a</i> , 36,73
CspA	69	13	F31S	4×	↓	74 <i>a</i> , 75
CTL9	92	3	H134Q	8×	↓	76
dnK SH3	59	8	T22G	7×	↑	8
fnIII	90	42	Y36A	42×	↓	77
FynSH3	59	86	I28S	37×	↓	9, 11, 78 <i>a</i> ,79, 80
Im9	85	25	F15A	189×	↓	27
Procarboxypeptidase A2	81	22	F65A	4×	↓	81
Protein G	57	31	D46A	18×	↓	82
Protein L	62	71	F22A	26×	↓	83 <i>a</i> , 84
S6	95	28	I8A	14×	↓	85
Sso7d	64	24	I30A	8×	↓	86
TI127 Ig	89	26	L60A	50×	↓	87
src SH3	57	82	S47A	14×	↓	84,88,89 <i>a</i>
NTL9	56	28	V3A	6×	↓	41
Ubiquitin	76	27	I23G	16×	↓	90
Iprb7-53	47	4	K39V	3×	↑	14

Protein name	Length	Number of mutations	Greatest acceleration/deceleration	Fold maximum change	acceleration (↑) or deceleration (↓)	Reference(s)
villin	35	11	L20V	2×	↑	91
azurin	128	17	Y108A	8×	↑	93

^aIndicates which reference contains the maximum acceleration/deceleration.

Table 2

kinetic parameters for single-site variants of CI2 at position 48.

Variant	k_f (s ⁻¹)	k_u ($\times 10^3$ s ⁻¹)	m_{kf} (kJ/mol/M)	m_{ku} (kJ/mol/M)	ΔG_u^a (kJ/mol)
WT	44 ± 3	0.3 ± 0.1	-4.86 ± 0.07	2.92 ± 0.12	29.6 ± 0.4
RK	23 ± 1	2.3 ± 0.2	-4.87 ± 0.07	2.95 ± 0.03	22.8 ± 0.1
RN	31 ± 2	3.5 ± 0.3	-4.87 ± 0.08	2.97 ± 0.05	22.5 ± 0.1
RE	36 ± 1	12.8 ± 0.5	-5.01 ± 0.04	2.64 ± 0.02	19.7 ± 0.1
RQ	47 ± 1	2.2 ± 0.2	-4.99 ± 0.04	2.83 ± 0.05	24.7 ± 0.1
RA	67 ± 2	4.2 ± 0.2	-4.88 ± 0.04	2.66 ± 0.02	24.0 ± 0.1
RH	80 ± 3	4.1 ± 0.3	-4.89 ± 0.05	3.01 ± 0.03	24.5 ± 0.1
RW	550 ± 11	5.7 ± 0.3	-4.82 ± 0.02	2.83 ± 0.03	28.5 ± 0.1
RF	1570 ± 110	0.3 ± 0.4	-4.52 ± 0.07	3.23 ± 0.5	38.0 ± 1.7
RY	2039 ± 102	1.7 ± 0.5	-4.58 ± 0.05	2.88 ± 0.12	34.8 ± 0.5
RI	2253 ± 113	0.1 ± 0.1	-4.49 ± 0.04	3.71 ± 0.36	42.9 ± 1.3

^a $\Delta G_u = RT \ln (k_f/k_u)$