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Relaxation of Structural Constraints during Amicyanin Unfolding

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Abstract

We study the thermal unfolding of amicyanin by quantifying the resiliency of the native state to structural perturbations. Three signatures characterizing stages of unfolding are identified. The first signature, lateral extension of the polypeptide chain, is calculated directly from the reported crystallographic data. Two other signatures, the radial displacement of each residue from Cu(II) and the angular spread in the chain as the protein unfolds, are calculated using crystallographic data in concert with a geometrical model we introduced previously (J.J. Kozak, H. B. Gray, R. A. Garza-López, *J. Inorg. Biochem.* 155(2016) 44–55). Particular attention is paid to the resiliency of the two beta sheets in amicyanin. The resiliency of residues in the near neighborhood of the Cu center to destabilization provides information on the persistence of the entatic state. Similarly, examining the resiliency of residues intercalated between structured regions (beta sheets, the alpha helix) provides a basis for identifying a “hydrophobic core.” A principal focus of our study is to compare results obtained using our geometrical model with the experimental results (C. La Rosa, D. Milardi, D. M. Grasso, M. P. Verbeet, G. W. Canters, L. Sprtelli, R. Guzzi, *Eur. Biophys. J.* 30(8), (2002) 559–570) on the denaturation of amicyanin, and we show that our results support a classical model proposed by these authors.

Graphical Abstract

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Native structure of amicyanin (PDB # 1aac) showing the beta sheets and the residues in the neighborhood of the Cu (II) ion (in deep sky blue).

I. Introduction

Blue copper proteins (BCPs), their structures, properties, and roles in photosynthesis, in respiration, in oxidative deamination of primary amines, and in reduction of nitrite reductase have long attracted the interest of a large community of chemists. Fundamental advances in our understanding of these proteins have been discussed in seminal papers and influential reviews [1–9]. A physical chemical property underlying the structures and functions of these proteins is their structural stability. The intent of this study is to focus on one blue copper protein, amicyanin, and assess its response when the native state is systematically disrupted.

We have developed a geometrical model to follow quantitatively the early stages in the unfolding of a native protein. The main objective of the present study is to make comparisons of results obtained using our geometrical model with experimental unfolding data for amicyanin from *T. versutus* (now *P. versutus*) reported by La Rosa et al. [10]. Our study is based on amicyanin coordinates obtained from *P. denitrificans* (PDB # 1AAC). We expect the difference (if any) between results obtained using PDB # 1AAC and the amicyanin studied by La Rosa et al. to be minor, as the alpha-carbon coordinates of both proteins are essentially the same.

Our geometrical model has been elaborated in earlier publications [11–17]. We emphasize that we have now extended our approach to account for the native structure of amicyanin (as well as those of other BCPs), as this structure features several beta sheets [See Fig. 1]. Fundamental to our overall approach is that *first-nearest-neighbor* repulsive and attractive interactions between each residue *i* and its two nearest-neighbors are kept intact by introducing a triplet modular unit centered on residue *i*. The geometry of each triplet is determined by, and our subsequent analysis rests on, crystallographic evidence reported for this protein: See Refs. [18–21] for amicyanin residue and metal ion coordinates (105

residues). By enforcing the constraint that nearest-neighbor interactions remain unchanged, we conserve the locally optimized structure of the protein.

In a globally optimized native state, based on crystallographic data, geometrical constraints delimit sterically the number of possible configurations accessible to a polypeptide chain, a fundamental insight that goes back to Ramachandran [22] (and can be seen in phi/psi plots). In our approach, structural perturbations of the native state are studied by relaxing steric (geometrical) constraints (*only*) between and among *non-nearest* neighbors.

Among the myriad of configurations that can be adopted by a segment of a polypeptide chain as the native state is disrupted, we adopt one configuration as representative of the many that can result upon destabilization, viz., a maximally extended, linear sequence of triplets. By choosing this configuration for all proteins in a given family, or for proteins of different families with very different physiological functions (e.g., the cytochromes [11], or microbial proteins [14]), unfolding processes can be systematically compared (as the reference state is fixed).

II. Computational Details

To study the structural stability of the native state of a protein, we calculate three signatures (defined below) that characterize stages of unfolding of the native state. The first signature, the lateral displacement, is calculated directly from reported crystallographic data. Two signatures, the radial displacement of each residue relative to the metal center (in our case the copper of amicyanin) and the angular spread in the chain as the protein unfolds, are calculated using crystallographic data in concert with our geometrical model.

To illustrate the calculation of these signatures using our geometrical model, consider the second stage of unfolding for the $n=7$ residue segment, Gly 86 to Cys 92, centered on Asp 89. This segment of amicyanin is H-bonded to the His 95 to Val 104 segment, forming one “ladder” of a beta sheet [23–24]. We specify the origin of the coordinate system to be the metal ion [Cu(II)]; and all unfolding processes are calculated relative to this fixed point.

The following distances can be calculated from the crystallographic data using the Theorem of Pythagoras:

$$D(\text{Cu to R86}) = 23.83 \text{ \AA}$$

$$D(\text{Cu to R92}) = 4.571 \text{ \AA}$$

$$D(\text{R86 to R92}) = 20.10 \text{ \AA}$$

Using these values and the Law of Cosines (Proposition 12 in Euclid’s *Geometry*), the angles α , β , and γ are:

$$\beta = 31.93^\circ$$

$$\alpha = 141.16^\circ$$

$$\gamma = 6.910^\circ$$

Using the Law of Cosines, we determine the angles between residue 86 and 88, residues 88 and 90, and residues 90 to 92:

$$\beta(86 \text{ to } 88) = 4.623^\circ$$

$$\beta(88 \text{ to } 90) = 7.910^\circ$$

$$\beta(90 \text{ to } 92) = 20.30^\circ$$

The sum of these three angles, which characterizes the angular extension of a fully extended segment centered on Asp 89, is 32.83° , a value that can be compared with the angular extension between residues 86 and 92 in the native state calculated above, viz., 31.93° . The ratio of these two values, 1.03, gives a measure of the angular extension of the fully extended, seven-residue segment centered on Asp 89 relative to the angular extension of the same segment in the native state.

Using crystallographic data, we next calculate the distance between the alpha carbons of the two “terminal” residues in the seven-residue segment, Gly 86 to Cys 92, in the native state:

$$R(86 \text{ to } 92) = 20.10 \text{ \AA}$$

Again using crystallographic data, we determine the distance T_{89} for the fully extended configuration displayed in Fig 2,

$$T_{89} = R_{86 \text{ to } 88} + R_{88 \text{ to } 90} + R_{90 \text{ to } 92} = 20.23 \text{ \AA}$$

The ratio of these two distances,

$$T_{89}/R(86 \text{ to } 92) = 1.01$$

gives a measure of the lateral extension of the seven-residue segment, Gly 86 to Cys 92, in the fully extended state relative to the native state.

Finally, using the distance T_{89} in conjunction with the Law of Sines, we calculate the change in the radial distance of the alpha carbon of Asp 89 in the native state versus the seven-residue, fully extended state. The distance R (Cu to Asp 89) in the native state is 14.70 Å and in the fully extended state is 22.25 Å. The ratio of these two values defines the radial-extension metric f_{89} for Asp 89:

$$f_{89} = R(\text{Cu to Asp 89})_{\text{extended state}} / R(\text{Cu to Asp 89})_{\text{native state}} = 1.51$$

As evident from the above calculations, whereas the relative change in the angular spread and in the lateral extension between the native state and the fully extended segment is small, the change in extension metric is more pronounced. It is important to note, however, that the values of these signatures depend on the environment in which a particular residue is situated, a beta sheet (the above case), an alpha helix or, for example, a hydrophobic region: See, for example, Figs. (3–5), where the three signatures are displayed for all amicyanin residues in the second stage of unfolding.

Values of the above metrics have been calculated for each amicyanin residue and for each of the first six stages of unfolding. For example, see Table 1, where we display the signatures for the first six stages of unfolding for residues within ≈ 10 Å of Cu(II), several of which are associated with the protein entatic state. These three signatures change synchronously as amicyanin unfolds. To illustrate the simultaneous configurational changes in amicyanin as a whole as the native structure is thermally destabilized, see Figs. (6a) and (6b), which display the third and sixth stages of unfolding calculated using our geometrical model.

III. Resiliency of Beta Sheets

To explore the resiliency of amicyanin beta sheets to structural perturbations we again focus on alpha-carbon to alpha-carbon distances, as determined from crystallographic data. Here, however, we consider residues in adjacent segments H-bonded to each other. To illustrate, the data show that the following residues are “coupled,” forming one “ladder” of a beta sheet (H-bond distances in parentheses):

Thr 42 to Thr 81 (2.744 Å)

Val 43 to Leu 80 (2.815 Å)

Thr 44 to Ser 79 (2.769 Å)

Trp 45 to Tyr 78 (2.910 Å)

Ile 46 to Ala 77

Asn 47 to Gln 76 (2.934 Å)

The lengths of the H-bonds between paired residues in the native state (except for residues 46 to 77) were obtained from Chimera. [NOTE: “Conventional” hydrogen bonding distances were obtained from Chimera for PDB # 1AAC. These values match several experimental studies such as by Sukumar et al., [24] and Dudley et al. [23]. Two of the Chimera values can be checked with values reported in the original crystallographic study of Cunane et al. [18] and are in good agreement.] See Fig. 7 and Table SI–1. Figures (7–9) show the native-state structure and H-bond distances between paired residues in the beta sheets of amicyanin.

Using our geometrical model, we can calculate the changes in α -carbon to α -carbon distances between paired segments as amicyanin unfolds. These distance changes are the metrics that monitor the resiliency of H-bonded residues in a beta sheet to structural perturbation. As noted above, the H-bond distance between the α -carbons in Thr 42 and Thr 81 in the native state is 2.744 Å. As the protein unfolds, this distance changes, as does the distance between alpha carbons in the native state.

Denoting by $\mathbf{R}$ the distance between the origin (Cu) and a given alpha-carbon in the native state and by $\check{\mathbf{R}}$ the corresponding distance in the extended state (calculated using our geometrical model), the change in distance between H-bonded residues in the extended state versus the native state can be determined. For example, for the above residues (Thr 42 and Thr 81) in the second extended state, the distance Δ is:

$$\Delta = \sqrt{((R_{42x} + (R_{42x} - \check{R}_{42x})) - (R_{81x} + (R_{81x} - \check{R}_{81x})))^2 + ((R_{42y} + (R_{42y} - \check{R}_{42y})) - (R_{81y} + (R_{81y} - \check{R}_{81y})))^2 + ((R_{42z} + (R_{42z} - \check{R}_{42z})) - (R_{81z} + (R_{81z} - \check{R}_{81z})))^2} = 5.983 \text{ Å}$$

The data presented in Table SI–1 document distance changes between paired residues of a beta sheet, relative to the native state, as amicyanin unfolds. Further, the distance between residues in a given beta sheet and “other” residues in the protein also change. In a later section, we shall contrast distance changes calculated for H-bonded “ladder” residues, versus “ladder” residues H-bonded to “other” residues of the protein. In our geometrical model, we find that H-bonds between ladder residues (which are extended already) are more stable than H-bonds between ladder and non-ladder residues.

IV. Resiliency of the Rack-Induced (Entatic) State

The “rack-induced” or “entatic” state has been discussed extensively in the literature on blue copper proteins [2–9,12]. In this study we take an operational approach and use crystallographic data to identify (all) residues whose α -carbon is within ≈ 10 Å of the Cu center. The choice of 10 Å is totally arbitrary; it is the distance metric adopted in our study of intelectin [16], where the stability of regions in the near neighborhood of three Ca ions was quantified. Adopting the same metric allows an assessment of the stabilization induced by different metal ions.

Data for the three signatures, T, β and f for the above region are set out in Table 1. Residue segments within ≈ 10 Å of the Cu center that are relatively resilient to structural change as amicyanin unfolds through the first six stages of destabilization are:

Ile 25, Ala 26, Lys27, Met 28, Lys 29, Tyr 30

and His 91, Cys 92, Thr 93, Pro 94, His 95, Pro 96, Phe 97, Met 98, Arg 99 (residues highlighted in red are common to the entatic region of several BCPs.) Note that the latter region is a loop that contains 3 of the 4 Cu(II) ligands (residues 95 to 104); this loop likely anchors the “rack” during amicyanin unfolding. The relative immobility of these 3 residues (His 95, Cys 92 and Met 98) with respect to Cu(II) as the protein unfolds through six stages of destabilization is a remarkable illustration of the structural stability of the polypeptide chain in the region of the active site [38–40].

As noted previously, the three signatures reported in Table 1 change synchronously as amicyanin is destabilized. It is instructive to display their concerted behavior graphically. We present in Fig. (6a) the behavior of protein “rack” residues in the first stage of unfolding. Then, in Fig. (6b), this region is displayed for the same residues in the sixth stage of unfolding. The “rack” structure in the native state is formed by four strong bonds involving the residues His53, Cys92, His95 and Met98. In the crystal PDB# 1AAC, the distances to Cu ion are 1.89, 2.09, 1.97 and 2.92 Å, respectively. As pointed out by a referee, aside from H-bonding considerations, the structure surrounding the copper center may appear to be more resilient to unfolding because many of the structural elements already are nearly fully extended.

V. Resiliency of Hydrophobic Regions

Residues intercalated between regions identified as beta sheets or alpha helix constitute a “hydrophobic core,” providing (additional) structural stability to the native protein [25]. Measures of hydrophobicity, while invaluable, are not sufficient to determine unequivocally which regions may constitute “core” stabilization. In the case of amicyanin, we have calculated the signatures T, β and f for polypeptide segments of five residues or more that intercalate structured regions (see Table A in the Appendix).

We find hydrophobic/hydrophatic regions of five or more residues to be relatively resilient to structural changes as amicyanin unfolds:

Val 16, Ala 17, Asp 18, Gly 19, Ala 20 (0.78)

Ala 26, Lys 27, Met 28, Lys 29, Tyr 30, Glu 31, Thr 32, Pro 33 (– 1.40)

Val 58, Ala 59, Gly 60, Val 61, Leu 62, Gly 63, ... (2.2)

The number in parenthesis following each sequence is the average hydrophobic value, calculated using the scale reported in [26]. Using this index, the following ordering (highest to lowest hydrophobicity) is established:

$$\text{residues } 58 - 63 > \text{residues } 16 - 20 > \text{residues } 26 - 33$$

We conclude that since the 58–63 residue region remains more-or-less intact with increasing destabilization, it is the amicyanin hydrophobic core.

VI. Discussion

Several experimental techniques for studying amicyanin unfolding have been reported [10,18,23–24]. In one study [10], differential scanning calorimetry, fluorescence, optical density, circular dichroism and electron paramagnetic resonance are used to study the thermal unfolding of the protein [27–32]. Based on an analysis of experimental results, La Rosa et al. [10] proposed a classical model for amicyanin thermal denaturation. In the present investigation, we set out to determine whether and to what extent the results obtained using our geometrical model accord with the aforementioned classical model [10].

Our geometrical model is implemented using the coordinate data reported in the X-ray crystallographic study of native amicyanin (from *P. denitrificans*) refined at 1.31 Å resolution (PDB # 1AAC) [17]. The overall structure of the protein features two sheets of 5- and 4-beta strands. We concentrated on the beta strands reported in PDB # 1AAC that contain more than 3 residues: See Fig.1 where the blue, red and green ribbons form one sheet and the yellow, magenta and orange ribbons are the beta strands forming the other sheet.

The experimental study of La Rosa et al. [10] reveals two features that characterize the response of amicyanin to an increase in temperature. The first is the geometry of residues in the near neighborhood of Cu(II) in the native state and, as the protein unfolds, the associated structural rearrangements. The second feature involves the single Trp 45 residue. La Rosa et al. [10] exploit this buried “built-in” fluorescent probe to monitor structural changes as the protein unfolds: See Fig. 10. Later studies comparing native amicyanin to the apo protein [23,33–34] have shown, however, that Trp fluorescence can be quenched by copper.

La Rosa et al. [10] propose two mechanisms to account for the experimentally observed thermal unfolding behavior of amicyanin, both involving a reversible step followed by two irreversible steps. They conclude that Model B reproduces the experimental data with a high degree of accuracy over the range of temperatures studied.

In our geometrical model we focus on the early stages in the denaturation of the protein, where thermal fluctuations can disrupt the native structure. Except as noted later, our approach does not cover the whole range of temperatures studied [10]. Further, our methodology is not applicable to higher temperature ranges under anaerobic conditions. Our calculations are most relevant to Model A, which accounts for the experimental findings when the temperature is increased to the point where the partially unfolded protein can return reversibly to the native state, thereby establishing an equilibrium between the two states. The six extended states considered (for which calculations have been carried out) are regarded as “early stages of unfolding”. We do not consider the limiting case, irreversible melting of the polypeptide chain, as proposed in Model B of La Rosa et al. [10].

The first step in the mechanism of both models [10] establishes an equilibrium between the native state N and an unfolded state U. Some researchers have suggested this reversible unfolded state might correspond to the dissociation of copper and its rebinding prior to the irreversible further unfolding [11]. In our approach, the most probable first stage of unfolding is the first, $n=5$ residue extensions. However, owing to thermal fluctuations, it is possible that the protein may transit directly to the second stage ($n=7$ residue extensions), ..., or to the sixth stage ($n=15$ residue extensions), but such an event is much less probable energetically. Scenarios can be proposed in which the six-stage unfolding scheme quantified here can be morphed into a version of Model B.

The signature feature of a type 1 copper site is its intense blue color, which in amicyanin is attributable to ligand-to-metal charge transfer from Cys 92 to the copper ion. As reported by several investigators [2,7–10,12,30–32,35–37], Cu(II) in the native state is bound to four residues forming a distorted tetrahedron characteristic of type 1 centers: See Fig. 10 where the copper ion is coordinated by N atoms from His 53 and His 95 and S atoms from Cys 92 and Met 98 in a distorted tetrahedral geometry. As seen in the figure, the copper site is shielded from solvent; His 95 is the only ligand residue that is partially surface exposed. Upon thermal unfolding, the 3-D arrangement is disrupted. The parameters extracted from analysis of the EPR spectrum of denatured amicyanin are compatible with type 2 Cu(II) with two nitrogen and two oxygen donor atoms in square-planar coordination.

In implementing our geometrical model, we start with the alpha-carbon backbone of the native structure. As amicyanin unfolds, we observe disruption of the distorted tetrahedral arrangement around the Cu(II) center. Fig. 11 displays the four residues that form the native distorted tetrahedron when the first stage of unfolding is realized.

Change in the location of the Trp 45 residue.

A second feature that distinguishes native amicyanin from the unfolded state is the fate of Trp 45, a residue buried in a hydrophobic region of the protein. Because of its location, La Rosa et al. [10] used this residue to monitor structural changes in the protein as the temperature is increased. Specifically, they reported that at room temperature, Trp 45 exhibits a fluorescence maximum at $\lambda_{\text{max}} = 314$ nm, which indicates that the residue resides in a hydrophobic environment [41]. As noted previously, work comparing amicyanin and apoamicyanin has established that Trp fluorescence can be quenched by Cu(II) [33–34].

In our model, the Trp 45 alpha carbon in the native state is displaced, the distance depending on whether Trp 45 is a paired residue in a beta “ladder,” or whether it is H-bonded to a residue in a beta ladder. The latter displacement is significantly *larger*. See Fig. 12 and the following subsection. In so doing, Trp 45 moves from the native-state hydrophobic region into a more polar environment.

Several studies have focused on site-directed mutants of amicyanin. We recommend a review article by Choi and Davidson [42], where the effects of several mutations [M98Q, K68A, R99D, R99L, F97E, P94F, M51A and P94A] on protein properties are discussed. Of special interest is that these mutations impact the hydrogen bonding network as the protein unfolds [43].

We anticipate that if the crystal coordinates of amicyanin with a mutated residue are the same as (or very similar to) the protein in our study, the results calculated using our geometrical model would be very similar. For example, using Chimera, we examined the “match” between our protein [PDB# 1AAC] and reduced W45Y amicyanin [PDB# 4P5S] studied by Dow et al. [43]. The graphics reveal that the residue in question, Trp45, and the mutation, W45Y, overlap almost perfectly. To the extent that the two residues have essentially the same “native coordinates” for alpha carbons, we argue that the history of unfolding should be the same.

To summarize, the unfolding of rack residues defining the entatic state will not be greatly affected by site-directed mutagenesis as long as the coordinates of the alpha carbons of mutated residues are not significantly different from the non-mutated counterpart. Where differences will be observed is in the hydrogen-bonding network, since the hydrophobicity of the residue in question is changed upon mutation. Chimera can be used to identify newly formed hydrogen bonds, as well as to quantify the effect these bonds have on unfolding.

Beta “ladders” formed by hydrogen bonding of paired residues

As described in Section III, we monitor the change in H-bond distances between paired residues in a beta “ladder” by calculating the alpha-carbon to alpha-carbon distances between the same paired residues as amicyanin unfolds. Experimentally, the first calorimetric peak [10] is attributable mainly to β -turn denaturation, while β -sheets appear less affected.

Tables (SI-1A), (SI-1B) and (SI-1D) identify paired residues forming beta “ladders” as reported in the PDB file for amicyanin. Ladder residues are coded in RED. Tables (SI-1C) and (SI-1E) identify “ladder residues” H-bonded to “other” residues in the protein. In these tables, the second column from the left gives the H-bond distances obtained via Chimera. The third column from the left gives alpha-carbon to alpha-carbon distances between paired residues in the native state, as calculated directly from the coordinate data in the PDB file for amicyanin (PDB # 1AAC). The next column gives alpha-carbon to alpha-carbon distances between paired residues in the n^{th} extended state. The final column gives distances ($\text{\AA}$) between paired residues in the native and n^{th} extended states.

The H-bonded residue pairs exhibiting the greatest perturbations as the protein unfolds are shown in Figs. SI-1A to SI-5A. Calculated maximum and minimum displacements ($\text{\AA}$) are summarized in Table 2. In Fig. SI-1A for ladder 1, the H-bond that shows the largest displacement is between residues 43–80, reaching a maximum at the fifth extension, and a minimum by residues 47–60 in the third extension. In Fig. SI-2A for ladder 2, the H-bond that shows the largest displacement is between residues 46–25, reaching a maximum at the fourth extension (the H-bond between residues 46–23 reaches a minimum at the third extension).

In Fig. SI-3A for ladders 1 and 2 bonded to other residues, the H-bond that shows the largest displacement is between residues 45–90 at all extensions, reaching a maximum at the fifth extension, and one between residues 47–75 at all extensions, reaching a minimum at the fourth extension. In Fig. SI-4A for ladder 3, the H-bond that exhibits the largest displacement is between residues 96–99, reaching a maximum at the fifth extension, and one between residues 88–84, reaching a minimum at the third extension.

Note that in Fig. SI-7A for ladder 3 bonded to other residues, the H-bond that shows the largest displacement is between residues 90–45 at all extensions, with a maximum at the fifth extension, and one between 95–53, with a minimum at the same extension.

The inflections in the curves displayed in the above figures could be interpreted as evidence for Model B. The data in Table 2 give specific H-bonded residues and extensions where irreversible unfolding may be the preferred unfolding pathway.

It is noteworthy that Trp 45 forms an H-bond with non-ladder residue Tyr 90, which shifts significantly upon unfolding, whereas (as a ladder residue) Trp 45 behaves “normally.” The four residues His 53, His 95, Cys 92 and Met 98 behave “differently” as the protein unfolds. This behavior is readily seen in Figs. SI-1A to SI-5A.

Although the present contribution focuses on one amicyanin, namely, the native Cu(II) protein from *P. denitrificans*, refined at 1.31 $\text{\AA}$ resolution (PDB# 1AAC), in the future we plan to apply our geometrical approach to other variants [44–46], e.g., copper-free apoamicyanin [PDB# 1AAJ]. On comparing amicyanin with the apoprotein, there is little difference in the positions of the coordinating ligands with or without copper. The removal of the copper atom seems to cause few structural changes making these proteins essentially isomorphous. However, Davidson et al. [30,47] found that apoamicyanin exhibits apparent self-association at low ionic strength, not observed with the native protein. This finding was unexpected due to similarities in crystal structures. Davidson et al. [24,43,47] argue that the removal of copper may modify the charge distribution on the surface of the protein and that this in turn may affect its ability to bind to other proteins as well as to self-associate. Regarding the thermal stability of the protein, studies [23,30,47] show that this property also depends on the presence or absence of copper and its ligand geometry, but not the metal redox state.

We are in the process of extending this study to three more blue copper proteins: plastocyanin, azurin and rusticyanin. We have focused attention on regions of these proteins associated with entatic or “rack-induced” states, and residues in each protein that may define

a “hydrophobic core.” The resiliency of these regions to destabilization will be quantified and compared in each case. Results to date indicate that in all cases the copper center stabilizes residues constituting the cupredoxin fold, and that this stability persists for most (but not all) of the residues as the protein unfolds. Of relevance here is that copper-induced protein stabilization has been documented experimentally by Dow et al. [43].

VII. Conclusions

We have extended our geometrical model [11] to deal explicitly with beta sheets, the fundamental structural motif of amicyanin. Our determination of the resiliency of individual segments of the polypeptide chain are consistent with experimental data reported by La Rosa et al. [10] for early stages of amicyanin denaturation. Our results are aligned most closely with their Model A in which the native-state conformation is in reversible equilibrium with that of a single, partially unfolded state.

Both experimental results [10] and our calculations strongly indicate that there must be substantial changes in the geometry of residues surrounding Cu(II) as the protein begins to unfold. We also document the positional change in Trp 45, finding that, as in [10], the residue moves from a hydrophobic environment to an exposed one in the succession of states populated on increasing the temperature.

Aspects of amicyanin unfolding *not* reported in [10] also can be studied using our geometrical model. First, the model quantifies the disruption of H-bonds between paired residues in each of the three ladders comprising the two beta sheets of the protein, and between residues in a ladder and “other” residues. Our results show that the H-bonds between paired residues in ladders are more resistant to destabilization than H-bonds between ladder and non- ladder residues. Second, by studying the resiliency of segments intercalated between structured regions of the protein, we have identified residues that form the hydrophobic core: Val 58, Ala 59, Gly 60, Val 61, Leu 62, Gly 63.

Importantly, our geometrical model can be used to track the unfolding of proteins whose structural integrity is primarily dependent on beta sheets as well as those that have extended alpha-helical and hydrophobic regions. The model takes explicit account of steric interactions (i.e., repulsive forces) among residues of the polypeptide chain. As in the modern theory of classical fluids, steric interactions dominate the structural features of the native state, with attractive interactions playing a supporting role in certain cases. Since we start with the coordinates of the native state, we know that optimal attractive and repulsive forces defining the globally optimized structure are taken fully into account. Our model quantifies the resiliency of the native state to steric/geometrical perturbations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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APPENDIX

Table A.

Signatures $\langle L \rangle$, $\langle f \rangle$ and $\langle \beta \rangle$ for non-helical region in amicyanin (PDB 1AAC). All-residue averages in parentheses. Number in parenthesis under residue range is average hydropathic character [26] of region. Number in parenthesis under n-unit gives the number of residues used in constructing the average.

Region	Residues	Extension	n-unit	$\langle L \rangle$	$\langle f \rangle$	$\langle \beta \rangle$
I	2–12 (−0.19)	Native	3	1.0	1.0	0.0
			1	1.958	1.342	12.78
			(10)	(2.000)	(1.301)	(16.44)
			2	2.938	1.366	22.36
			(9)	(2.998)	(1.290)	(28.39)
			3	3.914	1.397	29.96
			(8)	(3.995)	(1.329)	(36.90)
			4	4.894	1.365	33.08
			(7)	(4.995)	(1.311)	(42.48)
			5	5.746	1.423	22.36
			(6)	(5.995)	(1.357)	(44.17)
			6	6.712	1.411	31.80
			(5)	(6.992)	(1.286)	(45.81)
II	16–20 (0.78)	Native	3	1.0	1.0	0.0
			1	1.955	1.259	9.60
			(5)	(2.000)	(1.301)	(16.44)
			2	2.964	1.242	18.97
			(5)	(2.998)	(1.290)	(28.39)
			3	3.884	1.269	26.39
			(5)	(3.995)	(1.329)	(36.90)
			4	4.936	1.239	31.96
			(5)	(4.995)	(1.311)	(42.48)
			5	5.875	1.261	38.42
			(5)	(5.995)	(1.357)	(44.17)
			6	6.995	1.268	54.89
			(5)	(6.992)	(1.286)	(45.81)
III	26–33 (−0.14)	Native	3	1.0	1.0	0.0
			1	1.999	1.187	12.15
			(8)	(2.000)	(1.301)	(16.44)
			2	3.041	1.094	15.30
			(8)	(2.998)	(1.290)	(28.39)

Region	Residues	Extension	n-unit	<L>	<t>	< β >
IV	48–75 (–0.18)	Native	3	4.074	1.137	22.74
			(8)	(3.995)	(1.329)	(36.90)
			4	5.137	1.204	31.42
			(8)	(4.995)	(1.311)	(42.48)
			5	6.164	1.281	21.79
			(8)	(5.995)	(1.357)	(44.17)
			6	7.052	1.273	49.01
			(8)	(6.992)	(1.286)	(45.81)
			1	2.009	1.243	19.44
			(28)	(2.000)	(1.301)	(16.44)
			2	3.011	1.199	31.90
			(28)	(2.998)	(1.290)	(28.39)
			3	4.025	1.265	40.19
			(28)	(3.995)	(1.329)	(36.90)
			4	5.033	1.236	45.85
			(28)	(4.995)	(1.311)	(42.48)
			5	6.050	1.325	47.80
			(28)	(5.995)	(1.357)	(44.17)
			6	7.052	1.273	49.01
			(28)	(6.992)	(1.286)	(45.81)

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HIGHLIGHTS

- Model to study influence of steric factors on unfolding of amicyanin
- Predictions of model correlate with reported experimental data
- Change in geometry of “rack” residues and resiliency of beta sheets quantified
- Hydrophobic core regions identified and resiliency quantified

Figure 1.
Strands for amicyanin (PDB # 1AAC) considered.

Figure 2. Specification of the geometrical model for a seven residue segment in amicyanin. (See text).

Figure 3.

T vs residue number for second extended state. Residues in an alpha helical region are in blue, in a beta sheet in red and in all other regions in green. Horizontal line denotes overall average.

Figure 4.
 β vs residue number for second extended state. Residues in an alpha-helical region are in blue, in a beta sheet in red and in all other regions in green. Horizontal line denotes overall average.

Figure 5.

T vs residue number for second extended state. Residues in an alpha-helical region are in blue, in a beta sheet in red and in all other regions in green. Horizontal line in magenta denotes native state. Horizontal line in orange denotes overall average.

Figure (6a).

Highlighted are residues in the vicinity ($\approx 10 \text{ \AA}$) of the origin Cu(II) in amicyanin (1AAC). Displayed is Region III, PDB 91–99, in the native state (blue, rounded ribbon) and in the first extended state (red, edged ribbon). All other residues are in black (native state, flat ribbon) or in gray (first extended state, flat ribbon). The origin Cu(II) is in deep sky blue. The Region III amino acids are: His 91, Cys 92, Thr 93, Pro 94, His 95, Pro 96, Phe 97, Met 98 and Arg 99, where the Cu-ligated amino acids are identified in red.

Figure 7.
Ladder 1: residues 42 to 47 bonded to residues 76 to 81

Figure 8.
Ladder 2: residues 42 to 47 bonded to residues 21 to 25

Figure 9.
Ladder 3: residues 86 to 92 bonded to residues 95 to 104

Figure 10.
Distorted tetrahedral arrangement in the native structure of amicyanin.

Figure 11. Displacement of the four residues, His 53, His 95, Cys 92 and Met 98, from their position in the distorted tetrahedral structure of the native state (N, yellow) to their position in the unfolded state (U, red). Arrows show the direction of each displacement.

Figure 12.

Displacement of Trp 45 from its position in the native state (green) to its position in the unfolded state (magenta).

Figure (6b).
Region PDB 91–99 in the native state and in the *sixth* extended state.

Table 1.

Signatures $\langle L \rangle$, $\langle f \rangle$ and $\langle \beta^\circ \rangle$ for residues in vicinity (10 Å) of the **Cu(II)** ion in amicyanin (PDB 1AAC). All-residue averages in parentheses. Number in parenthesis under n-unit gives the number of residues used in constructing the average.

Region	Residues	Extension	n-unit	$\langle L \rangle$	$\langle f \rangle$	$\langle \beta^\circ \rangle$
I	25–30	Native	3	1.0	1.0	0.0
			1	1.999	1.190	15.78
			(6)	(2.000)	(1.301)	(16.44)
			2	2.941	1.008	12.32
			(6)	(2.998)	(1.290)	(28.39)
			3	3.980	0.994	7.384
			(6)	(3.995)	(1.329)	(36.90)
			4	4.952	1.060	7.950
			(6)	(4.995)	(1.311)	(42.48)
			5	5.961	1.171	2.431
			(6)	(5.995)	(1.357)	(44.17)
			6	6.924	1.146	1.913
			(6)	(6.992)	(1.286)	(45.81)
II	25–30	Native	3	1.0	1.0	0.0
			1	2.002	1.354	34.65
			(10)	(2.000)	(1.301)	(16.44)
			2	3.016	1.322	53.53
			(10)	(2.998)	(1.290)	(28.39)
			3	4.001	1.352	59.91
			(10)	(3.995)	(1.329)	(36.90)
			4	5.005	1.338	55.17
			(10)	(4.995)	(1.311)	(42.48)
			5	5.985	1.407	41.93
			(10)	(5.995)	(1.357)	(44.17)
			6	6.982	1.393	31.16
			(10)	(6.992)	(1.286)	(45.81)
III	91–98	Native	3	1.0	1.0	0.0
			1	2.004	1.255	25.47
			(8)	(2.000)	(1.301)	(16.44)
			2	3.030	1.182	32.48
			(8)	(2.998)	(1.290)	(28.39)
			3	4.068	1.157	24.32
			(8)	(3.995)	(1.329)	(36.90)
			4	5.123	1.093	10.53
			(8)	(4.995)	(1.311)	(42.48)
			5	6.182	1.172	–2.694
			(8)	(5.995)	(1.357)	(44.17)

Region	Residues	Extension	n-unit	$\langle L \rangle$	$\langle f \rangle$	$\langle \beta^\circ \rangle$
		6	15	7.418	0.975	-16.38
			(7)	(6.992)	(1.286)	(45.81)

Table. 2.

Maximum and minimum extension of H-bonds between paired residues in designated regions.

	Extension	Extreme maximum	Extension	Extreme minimum
A	5	43–80	3	47–76
B	4	46–25	4	46–23
C	5	45 –90	4	47–75
D	5	96–99	3	88–84
E	5	90–45	5	95–53