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Detection of initiation sites in protein folding of the four helix bundle ACBP by chemical shift analysis

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Abstract A simple alternative method for obtaining "random coil" chemical shifts by intrinsic referencing using the protein's own peptide sequence is presented. These intrinsic random coil backbone shifts were then used to calculate secondary chemical shifts, that provide important information on the residual secondary structure elements in the acid-denatured state of an acyl-coenzyme A binding protein. This method reveals a clear correlation between the carbon secondary chemical shifts and the amide secondary chemical shifts 3–5 residues away in the primary sequence. These findings strongly suggest transient formation of short helix-like segments, and identify unique sequence segments important for protein folding.

Keywords: Protein folding; NMR; Chemical shift; Intrinsic random coil chemical shifts; Residual dipolar coupling; Unfolded states

1. Introduction

The processes leading from the unfolded state of a polypeptide chain to the formation of the stable folded state of a native protein are crucial for the function of any living organism. The molecular details of these interactions hold the key to understanding the origin of the protein folding processes, which were selected for during the early evolution of life to form the globular folds of proteins. A search for generic molecular mechanisms for protein folding may hold the key to an ab initio understanding of the relationship between primary and tertiary structure based on understanding the processes of formation of structure rather than predictions based on the correlation between the known primary structures and the end product of protein folding, the three-dimensional structures of proteins.

The spontaneous processes of protein folding often occur in a micro- to millisecond time range. It is therefore technically demanding to measure the kinetic details and relevance of the multitude of processes, which are involved in the folding of a protein. Alternatively, it is possible to study the dynamic processes, which occur in the unfolded state of the protein by monitoring the equilibrium between different states of unfoldedness. By combining such studies with measurements of protein folding kinetics and the effects of site specific mutagenesis on these, it may become possible to examine the nature of the interactions that lead to productive folding.

One model system of protein folding that has been studied extensively in this respect is the four helix protein acyl-coenzyme A binding protein (ACBP). Originally the folding of ACBP was identified as a "two state process" [1], but subsequent studies have identified intermediate processes both in the folding and in the unfolding pathway [2,3]. Interactions between a number of conserved hydrophobic residues were identified to be rate determining in the folding process [4,5]. These residues are found in three segments of the peptide chain, which in the native state stabilize the three of the four helices (helix 1, 2 and 4). Further, kinetic studies of the protection towards amide hydrogen exchange have shown that residues, particularly in the C-terminal helix, are already strongly protected in the burst phase of folding suggesting that local helix formation precedes the formation of persistent structure [6]. This is in good agreement with equilibrium residual dipolar coupling (RDC) measurements of the H^N–^{15}N coupling in the unfolded state of ACBP, which suggested a significant propensity to form helical conformations for the same segments in ACBP [7]. This effect was particularly strong for residues in the C-terminal part of the sequence involved in helix formation in the folded state. Experimental studies of ACBP have the advantage that the unfolding and folding processes at room temperature and unfolding conditions are in slow exchange [8]. This permits separate observations of the signals from the folded and unfolded forms, and this has had distinct advantages for the NMR studies of the unfolded form.

Nitroxide induced paramagnetic relaxation studies have shown, that the two segments forming helix 2 and helix 3 in the folded state are more likely to form interactions than any other part of the chain in the unfolded form of ACBP [9,10]. Other native-like structures in the unfolded state were formed less frequently, in keeping with the observation that in the folding process the formations of native like interactions

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Abbreviations: ACBP, acyl-coenzyme A binding protein; RDC, residual dipolar coupling

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between residues in the N- and C-terminal part of the sequence were rate limiting. Isoleucine 27 in the ACBP sequence is known to form important interactions in the rate determining step of folding, and the mutation of this residue to alanine decreases the folding rate by a factor of five. Analysis of the effect of the same mutation on the RDCs of the unfolded state showed clearly that residues were involved not only in the sequential vicinity of the mutation site, but also at sites known to be interaction partners in the folded state [7]. This suggests that long range native like interaction formed in the unfolded state are important for the measured RDCs and indicates that these long range interactions may stabilize local helix-like conformations in the peptide segments.

It therefore appears that both short range and long range interactions forming in the unfolded state of ACBP as observed by NMR spectroscopic methods are important for the formation of the rate-limiting structures. The ability of NMR spectroscopy to observe features in atomic detail means that NMR spectroscopy of the unfolded state can provide information about the structure formation and the dynamics of the processes leading to the folding of ACBP.

The chemical shift is probably the most sensitive nuclear magnetic resonance probe of local structure in proteins, even though the many contributions render the information content hard to disentangle [12–18]. We describe, here, the measurement and analysis of the chemical shifts of four types of nuclei, H^N, ^{15}N, $^{13}C^{\alpha}$ and $^{13}C'$, in the residues throughout the peptide backbone to study the unfolded state of ACBP. Our results show a striking profile of chemical shift variations that reveal transient formation of helical segments in the unfolded state. The implications for the folding processes of ACBP are discussed.

2. Results and discussion

The C^{α}, C, N and H^N shifts of bovine ACBP were recorded at pH 2.3, 25 °C and six different urea concentrations between 0 and 5 M, Fig. 1. At these conditions ACBP is fully denatured as demonstrated by equilibrium pH titrations [8] using NMR spectroscopy.

The urea dependence of the chemical shifts of the backbone atoms indicates that local conformational preferences become more random with increasing urea concentration. Examination of the changes of the chemical shifts as the urea concentration is varied reveals an obvious correlation between those residues in the amino acid sequence, which are involved in helix formation in the folded state and the residues, whose chemical shift values change the most (Fig. 1). Another interesting observation is that there is a regular variation along the sequence and that certain residues, particularly those in the helical sequence segments, have a very strong dependence of the urea concentration with well-defined local minima or maxima at specific residues as shown in Fig. 1. For C^{α} the local minima are observed in the helical regions as indicated in Fig. 1, suggesting that residual structural elements which are responsible for the residual chemical shift are being affected and most likely abolished by urea. The C^{α} chemical shift of a number of residues in the sequence segments between the helical segments show very little effect of urea indicating that little residual structure is present at these residues in the unfolded state at pH 2.3 and that the effects of urea binding to the unfolded peptide backbone are very small. However, for certain residues in these segments there is a positive change in chemical shift with increasing urea concentration. The origin of this behaviour is not known. However, we notice that this is true for all glycine residues and for residues in the vicinity of proline residues in the sequence.

In order to quantify the chemical shift information a secondary shift analysis was performed. The definition of the secondary chemical shift is $\Delta\delta = \delta - \delta_{rc}$, where the chemical shift δ is referenced to a random coil shift δ_{rc}. It has been shown in several studies that $\Delta\delta$ can be a strong indicator of secondary structure type in folded proteins [14,19]. The secondary chemical shifts are also used as a standard tool in the search for residual secondary structure in non-native protein states [7,20–25]. The main challenge is the definition of δ_{rc}, which has received a great deal of attention over the years. In the

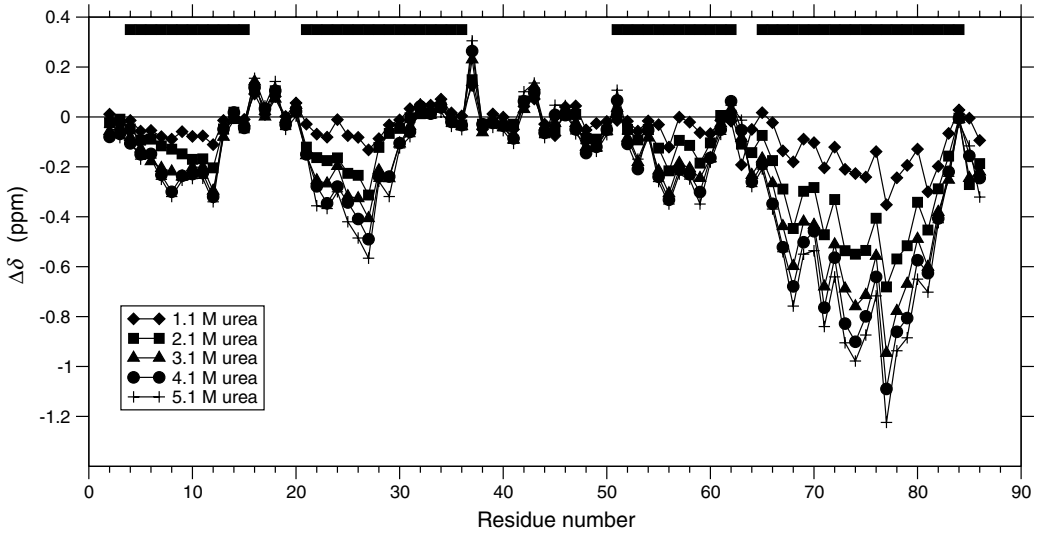

Fig. 1. The difference between C^{α} chemical shifts of a 60 μM ACBP sample at pH 2.3 and 0 M urea and samples of ACBP at pH 2.3 and 1.1, 2.1, 3.1, 4.1 and 5.1 M urea, respectively. The symbols are explained in the insert. The black horizontal bars indicate the positions of the α-helices in the folded state.

standard procedure, δ_{rc} is measured on the central residue in short linear peptides that are assumed to have a random coil state [1,12,17]. To achieve a greater accuracy, the effect of differing nearest neighbours can be taken into account [12,16,17]. There are, however, a few obstacles in the standard procedure. First, it is hard to find a general definition of a random coil state for a given amino acid sequence. In principle, the random coil is a state where the dihedral angles of each residue is independent of the conformation of the neighbouring residues [26]. It is, however, very hard to imagine such a state for a complex peptide chain where each of the 20 amino acids have distinct steric properties [27]. Second, it is not evident a priori that the short peptides capture the conformational space of a true random coil, even if we could define such a state. Third, short-lived long-range hydrogen bonds (not included in the nearest neighbour correction), solvent binding and ring current effects will add to δ_{rc} in an complex manner.

Here, we tackle these problems by calibrating δ_{rc} "intrinsically": the backbone chemical shifts of the acid-denatured state of ACBP at pH 2.3 are referenced to the chemical shifts obtained at pH 2.3 at a high urea concentration. Thus, we adhere to a pragmatic description of the "random coil" state, where the allowed (ϕ, ψ)-space is given by the intrinsic properties of the specific peptide chain in question. The chemical shifts were fitted as a function of urea concentration, using standard equations for fitting equilibrium denaturation curves [28], in order to obtain the intrinsically referenced "random coil" shifts δ_{irc}. These shifts are reported in the Supplementary material for all four types of atom types and compared to those calculated using the procedures described in [11,12].

Using the intrinsic random coil shift for ACBP to calculate the sequence specific secondary shifts results in the sequence variation seen in Fig. 2 for C^{α}, C' and N. The overall trends of the variations were already observed in the analysis of the urea dependence of the C^{α} shift shown in Fig. 1. The predominant positive secondary shifts for the C^{α} and C' and the negative secondary shift for N, and the presence of contiguous sequence segments with well defined maxima and minima in the regions that form helices in the folded form are strong indications that the residues concerned may be involved in a conformational equilibrium between a helical and an extended structure. Importantly, it is seen that the variations of the secondary shifts of the N and the H^N nuclei (the latter not shown) appear to be shifted about four residues relative to the position of the C^{α} and C' secondary shifts. The linear correlation coefficients between $\Delta_{irc}\delta$ of the carbon atoms and the amide atoms are shown in Fig. 3 (top panel). Indeed, the correlation is maximal when the separation is 3–5 residues. The effect is most dramatic for the atoms that may be directly involved in hydrogen bond formation, CO–HN, three or four residues apart. While the intra-residue shifts are almost completely uncorrelated ($r = -0.16$), the shifts are highly anti-correlated when the separation is four residues ($r = -0.80$). (For more details see the Supplementary material.) Thus, a conformational equilibrium between helical or turn structures, with hydrogen bonds between residues i and $i + 3/i + 4$, and extended structures would induce a correlation between the carbon and amide shifts over that distance of residues. It is of interest to note that when the traditional random coil shift methods [11,12,17,18] were used to calculate the secondary shifts these correlations were only vaguely observable, Fig. 3 (bottom panel). A table reporting the random coil shifts for each residue

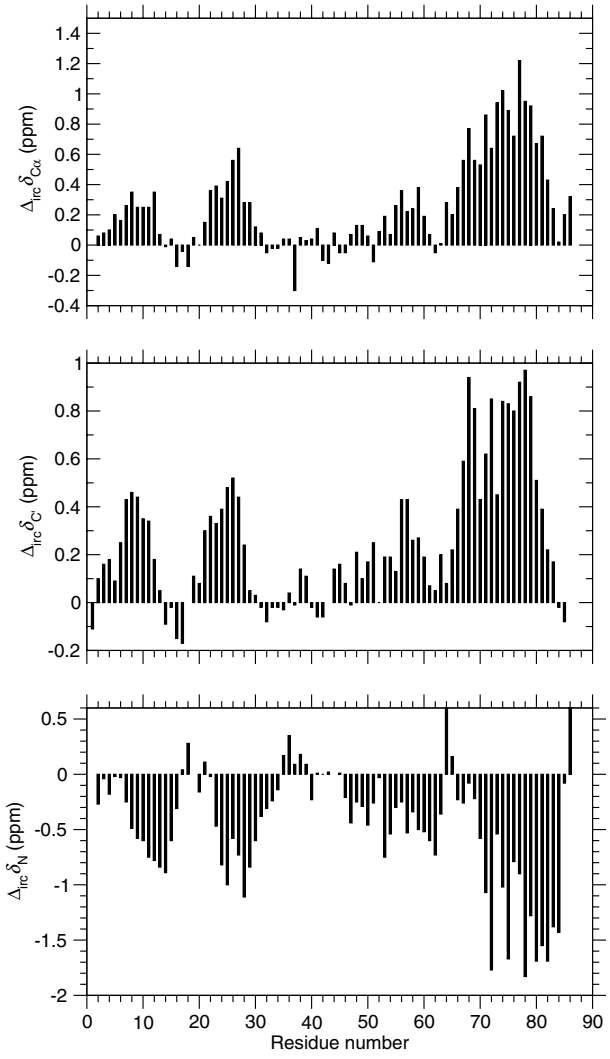

Fig. 2. Sequence dependence of secondary chemical shift of C^{α} (top), C' (middle) and N (bottom) of ACBP at pH 2.3 obtained by using protein and site specific random coil chemical shift references. (See Supplementary material for table of the secondary chemical shifts.)

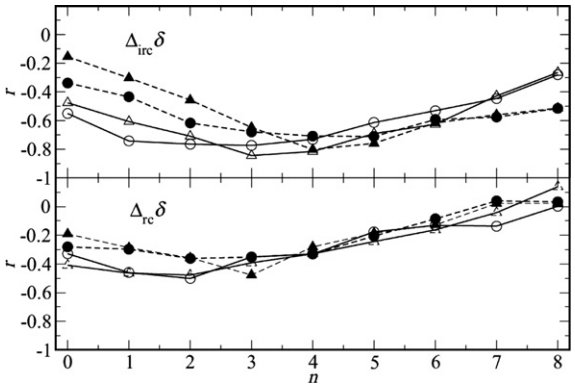

Fig. 3. The correlation coefficients, r, of $\Delta_{irc}\delta$ and $\Delta\delta_{rc}$ between $C^{\alpha}(i)$ and $N(i + n)$ (open circle, solid line), $C^{\alpha}(i)$ and $H^N(i + n)$ (filled circle, dashed line), $C'(i)$ and $N(i + n)$ (open triangle, solid line) and $C'(i)$ and $H^N(i + n)$ (filled triangle, dashed line). The values calculated using the intrinsic random coil reference are shown in the top panel and those calculated using a peptide random coil [11] are shown in the bottom panel. See Supplementary material.

obtained using traditional methods [11,12] and the procedure used here is shown in the Supplementary material.

Assuming that $i + 4$ hydrogen bonds are being formed in these local conformational equilibriums we have estimated the ΔG for the $i + 4$ hydrogen bond formation using the residue type specific average C^{α} chemical shifts for native folded helical structures as derived from the BioMagResBank [29], Fig. 4.

The positive ΔG for $(i + 4)$ hydrogen bond formation in all the residues throughout the entire sequence reveals that the equilibria are in overall favour of random coil conformations. However, a number of sites with relatively low ΔG values stand out as sites of a higher probability of $(i + 4)$ or $(i + 3)$ hydrogen bond formation than others. These sites are present in the regions of the peptide chain, which engage in contiguous helix formation in the folded protein. The higher probabilities of $(i + 4)$ or $(i + 3)$ hydrogen bond formation at these sites make them likely candidates of being nucleation sites for helix formation.

The combined secondary chemical shift analysis using the intrinsic random coil chemical shift as a reference provides evidence for the existence of local turns and helical loops in the unfolded state of ACBP. This is in good agreement with studies of the rates of protection of amide hydrogen against solvent exchange during the folding of ACBP, which showed that the amide hydrogen atoms in four sites (Y28, L61, I74 and V77) were being protected even prior to the pulse labelling period [6].

Also, in previous work it was observed that positive H–N bond RDCs at unfolding conditions were predominantly seen in the sections forming helices in the folded protein [7]. Therefore, we examined the correlation between the secondary shifts at pH 2.3 and 0 M urea and the H–N RDCs, Fig. 5, and indeed it was found that the correlation coefficient r being 0.8 both for $\Delta_{\mathrm{irc}}\delta_{C\alpha} - D_{\mathrm{NH}}$ and $\Delta_{\mathrm{irc}}\delta_{\mathrm{CO}} - D_{\mathrm{NH}}$, but only -0.5 and -0.3 for $\Delta_{\mathrm{irc}}\delta_{\mathrm{N}} - D_{\mathrm{NH}}$ and $\Delta_{\mathrm{irc}}\delta_{HN} - D_{\mathrm{NH}}$, respectively. Thus, the positive H–N bond RDC appears to be generated by the same structural feature that appears to define $\delta_{C\alpha}$ and δ_{CO}, the latent hydrogen bond formation rather than the internal dihedral angle restrictions.

In order to generate an overview of all the results of the present and previous studies of the folding of ACBP, key sequence positions were identified for each type of study as shown in Fig. 6. The criteria for identifying key residues are mentioned in the Figure legend. For each of the seven different types of analysis the key residues have been marked in the sequence and all together the seven sets of data clearly identify a set of hot spot residues, which seem to play an important role in the folding processes of ACBP. A majority of the key residues are in the C-terminal helix 4. All the different experimental parameters point to this part of the sequence as being the key to the folding of ACBP. It is of interest to note that the residues, which initially were identified as key residues for the folding of ACBP, based on mutation studies and φ-value analysis are also identified as such by the kinetic, chemical shift and RDC analysis, all of which primarily measure local conformational preferences in the unfolded state.

It was previously shown that hydrogen bond formation preceded the formation of persistent structure in the folding process of ACBP. The present studies of ACBP in the unfolded state have shown that particular peptide segments have relatively higher propensity for local hydrogen bond formation than others and, as shown in Fig. 6, these segments coincide with the residues forming hydrogen bonds in the early states of folding. This implies that the key sites of the hydrogen bond formation seen both in the equilibrium and in the kinetic studies are important structure generators in the protein folding processes of ACBP. The observation that residues of these

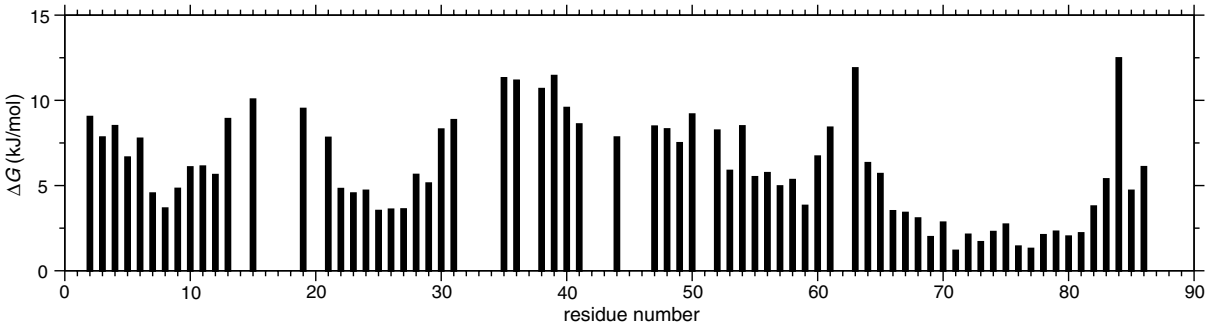

Fig. 4. The sequence dependence of ΔG for $i + 4$ peptide backbone hydrogen bond formation (the α-helix turn) for ACBP at pH 2.3 based on the C^{α} secondary chemical shift.

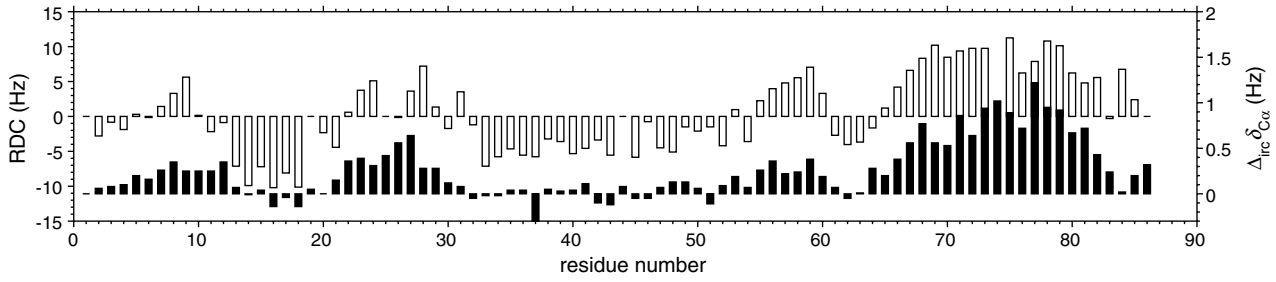

Fig. 5. Comparison of the sequence dependence of H–N RDCs (open bars) and the secondary chemical shifts of C^{α} (black bars) for ACBP at pH 2.3.

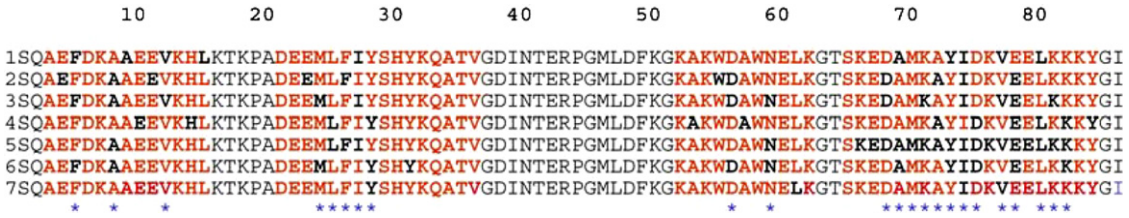

Fig. 6. Key residues identified by seven different types of measurements. The helix regions of folded ACBP are shown in red. The key residues are marked by bold black letters. The bottom line marks key residues identified in at least two different measurements. 1: Residues that form rate determining interactions as identified from a φ-value analysis. Residues highlighted have reduced folding rates by more than a factor of two [4,5]. 2: Local maxima of secondary chemical shift of C′. 3: Local maxima of secondary chemical shift of C^{α}. 4: Local minima of secondary chemical shift of N; 5: $\Delta G\ i + 3/i + 4$ hydrogen bond stability below 4.5 kJ. 6: Local maxima of H–N bond RDCs [7]. 7: Sites protected more than 15% in the 0 ms measuring point in the pulse labelling experiment [6].

peptide segments are rate determining in the global protein folding process suggests that the formation of local structures such as hydrogen bond formation is a necessary prerequisite for later steps in the protein folding processes. In this respect the transient formation of short helical segments in various fragments of the peptide chain may be important for the formation of the native like structures, which must be established in order for the protein to be folded.

3. Conclusions

Protein and sequence specific random coil reference values for the calculation of secondary shift of the unfolded form of ACBP have been shown to give a very clear and well defined sequence variation of the secondary shift for ACBP compared to results obtained by other reference methods. This has permitted a clear demonstration of a correlation between the size of secondary shift of C^{α} and C′ shifts with the size of secondary shift of the amide nuclei in the succeeding third or fourth residues in the unfolded state of ACBP. This observation has been used to calculate an apparent ΔG for helix formation for each of the residues with positive C^{α} secondary shift in the unfolded peptide chain. On the basis of this a number of key residues throughout the sequence of ACBP, which have stronger structure forming properties than other residues have been identified. A straightforward analysis of chemical shifts may, therefore, serve as a fast and simple measure for the primary identification of potential key residues of protein folding and assist in the selection of mutation sites for φ-analysis. It is also anticipated that an extension of this type of analysis to a large number of proteins may be the beginning of the establishment of a database of protein folding key sites that may serve as an auxiliary tool for prediction of the routes of protein folding. It must be emphasized that the use of intrinsic site specific random coil shifts as a reference for determining secondary shifts and the $(i + 3/i + 4)$ correlations of C^{α}, C′ and N chemical shifts has been very important for obtaining the results reported here. Hence we recommend this method of referencing in this type of analysis.

4. Materials and methods

4.1. NMR experiments

^{13}C, ^{15}N-labelled bovine wild-type ACBP was expressed and purified as described [30]. The C^{α}, C′, N and H^{N} shifts of ^{13}C and ^{15}N-labelled bovine ACBP were recorded at pH 2.3, 25 °C and six different urea concentrations (0, 1.1, 2.1, 3.1, 4.1 and 5.1 M), using standard ^{15}N-HSQC, HNCA, HN(CO)CA and HNCO experiments on a Varian Inova 800 MHz spectrometer. Since ACBP is known to form dimers at low pH and higher concentrations [31], the protein concentration was kept as low as 60 μM. The urea concentrations were determined from the refractive index of the solution [32]. The proton and carbon shifts were referenced internally to DSS and the nitrogen chemical shifts were referenced indirectly as recommended [33]. At intermediate pH values (~3), the two sets of peaks are present simultaneously, showing that the exchange N ↔ U is slow on the chemical shift time scale [8].

4.2. Fitting procedure

The chemical shifts were fitted as a function of urea concentration c in order to obtain the internally referenced "random coil" shifts δ_{irc}:

$$\delta(c) = p_{\mathrm{acid}}(c)\delta_{\mathrm{acid}} + (1 - p_{\mathrm{acid}}(c))\delta_{\mathrm{irc}} \quad (1)$$

where

$$p_{\mathrm{acid}}(c) = 1/\{1 + \exp[m(c - c_{1/2})/(RT)]\} \quad (2)$$

and δ_{acid} is the chemical shift in absence of denaturant. These are the standard equations for fitting equilibrium denaturation curves [28] but here we choose them because they conveniently represent the data and we do not interpret the values m and $c_{1/2}$. Fig. 7 shows an example of the fitting. In the cases where the errors of δ_{irc} were large, we put $\delta_{\mathrm{irc}} = \delta(5.1\ \mathrm{M})$. This happened primarily when the dependence of δ on c was weak.

4.3. Determination of the site specific fraction of helix/turn formation

The fraction of helix, F_i, at site i was determined as the ratio between the observed secondary chemical shift using intrinsically referenced "random coil" shifts δ_{irc} as reference, $\delta_{\mathrm{iobs}} - \delta_{\mathrm{irc}}$, and the difference between the average chemical shift value of the residue type when occurring in α-helices in native proteins $\delta_{\mathrm{RT}(\alpha)}$, as obtained from BioMagResBank [33] and the site specific intrinsically referenced "random coil" shifts δ_{irc}

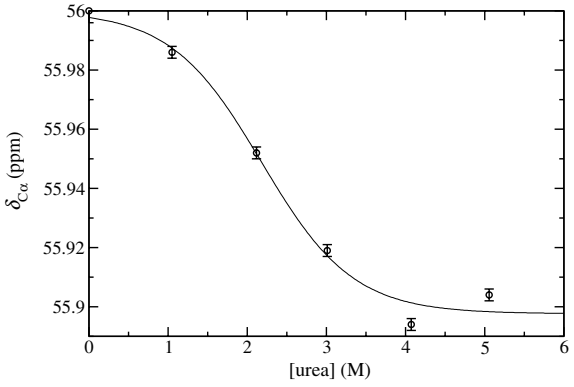

Fig. 7. Example of the fitting procedure used to obtain the intrinsic random coil shift for the C^{α} of Glu4.

$$F_i = (\delta_{\text{iobs}} - \delta_{\text{irc}}/(\delta_{\text{RT}(\alpha)} - \delta_{\text{irc}}))$$

It was decided to use the $\delta_{\text{RT}(\alpha)}$ as a reference and not the chemical shift of the α helical residues in the native state in order to avoid chemical shift contributions from tertiary structure elements, which are barely present in the ensemble of the unfolded forms of ACBP.

ΔG was calculated from

$$\Delta G = -RT \ln K = -RT \ln(F_i/1 - F_i)$$

where K is the equilibrium constant of the hydrogen bond formation.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2007.09.027.

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