FOR THE RECORD

Partial NMR assignments and secondary structure mapping of the isolated α subunit of *Escherichia coli* tryptophan synthase, a 29-kD TIM barrel protein

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Abstract

The α subunit of tryptophan synthase (α TS) from *S. typhimurium* belongs to the triosephosphate isomerase (TIM) or the $(\beta/\alpha)_8$ barrel fold, one of the most common structures in biology. To test the conservation of the global fold in the isolated *Escherichia coli* homolog, we have obtained a majority of the backbone assignments for the 29-kD α TS by using standard heteronuclear multidimensional NMR methods on uniformly ¹⁵N- and ¹⁵N/¹³C-labeled protein and on protein selectively ¹⁵N-labeled at key hydrophobic residues. The secondary structure mapped by chemical shift index, nuclear Overhauser enhancements (NOEs), and hydrogen-deuterium (H-D) exchange, and several abnormal chemical shifts are consistent with the conservation of the global TIM barrel fold of the isolated *E. coli* α TS. Because most of the amide protons that are slow to exchange with solvent correspond to the β -sheet residues, the β -barrel is likely to play an important role in stabilizing the previously detected folding intermediates for *E. coli* α TS. A similar combination of uniform and selective labeling can be extended to other TIM barrel proteins to obtain insight into the role of the motif in stabilizing what appear to be common partially folded forms.

Keywords: 3D NMR; nuclear magnetic resonance; ¹⁵N- selective labeling; H-D exchange; folding intermediates

The $(\beta/\alpha)_8$, or triosephosphate isomerase (TIM) barrel structure, is one of the most common motifs in proteins, comprising ~10% of the open reading frames in all three super kingdoms (Farber and Petsko 1990; Orenga et al.

1997; Nagano et al. 2001). The ubiquitous nature of the TIM barrel is probably related to its utility as a platform for enzyme catalysis; representatives are found in five of the six primary functional classes (Pujadas and Palau 1999; Nagano et al. 2002). A defining feature of the canonical TIM barrel is the presence of eight parallel β -strands, forming a closed cylinder secured by hydrogen bonds between consecutive strands; hydrogen bonds between the N- and C-terminal strands seal the barrel. The amphipathic α -helices, which alternate in sequence with the strands, form an amphipathic shell covering the hydrophobic β -barrel and, thereby, enhance its solubility. Loops between the C termini of the strands and the N termini of the helices comprise the active site.

The α subunit of the tetrameric bifunctional enzyme tryptophan synthase from *Salmonella typhimurium* adopts a

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Abbreviations: α TS, the α subunit of tryptophan synthase from *Escherichia coli*; CSI, Chemical shift index; DTE, dithioerythritol; H-D, hydrogen-deuterium; HSQC, heteronuclear single quantum coherence; *I*1, equilibrium intermediate of α TS maximally populated at ~3 M urea; *I*2, equilibrium intermediate of α TS maximally populated at ~5 M urea; K₂EDTA, ethylenediaminetetraacetic acid, dipotassium salt; NOE, nuclear Overhauser enhancement; NOESY, nuclear Overhauser enhancement spectroscopy; ppm, parts per million; TIM, triosephosphate isomerase.

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TIM barrel fold in the $\alpha_2\beta_2$ complex (Hyde et al. 1988). The hydrophobic core of the protein is largely defined by side-chains from the eight β -strands; the 11 α -helices, including one that caps the N terminus of the barrel, are interspersed with the β -strands and are solvent exposed. Several residues in the α subunit are involved in side-chain/side-chain and side-chain/main-chain hydrogen bond interactions with β subunit residues in the $\alpha_2\beta_2$ complex. The α subunits from *S. typhimurium* and *Escherichia coli* are 85% identical in sequence, and both α subunits are stable and stimulate the activity of both β subunits (Nichols and Yanofsky 1979). Although these properties imply that the structures are highly conserved, high-resolution structural information that can test this presumption is not available. We have used standard multidimensional NMR methods at pH 7.8 and 25°C on uniformly ^{15}N - and $^{13}\text{C}/^{15}\text{N}$ -labeled protein and selective ^{15}N -isotopic labeling of a few key hydrophobic residues to obtain assignments for a significant majority of the backbone ^1H - ^{15}N heteronuclear single quantum coherence (HSQC) cross-peaks. The secondary structure predicted by chemical shift indices and by several abnormal chemical shifts is consistent with the placement of helices and strands expected for the TIM barrel structure and the conservation of the global fold in the isolated *E. coli* homolog. To the best of our knowledge, this effort represents the first example of sequence specific backbone assignments for a TIM barrel protein.

Results and Discussion

Chemical shift assignments

A ^1H - ^{15}N HSQC spectrum of the α subunit of tryptophan synthase (αTS) at 25°C and pH 7.8 is shown in Figure 1. Of the expected 249 (268 residues less 19 prolines) backbone cross-peaks, ~215 are observed in the ^1H - ^{15}N HSQC spectrum. For a few very weak cross-peaks, no correlations are observed in several of the triple-resonance experiments used in this study. The inability to detect the remaining backbone peaks may reflect exchange broadening owing to conformational dynamics inherent to the TIM barrel and dynamics, resulting from the absence of the β subunit interactions for the isolated α subunit in solution. The side-chain ^1H - ^{15}N resonances of asparagine and glutamine have not been assigned; however, they can be identified from the ^1H - ^{15}N HSQC and three-dimensional (3D) ^1H - ^{15}N nuclear Overhauser enhancement spectroscopy (NOESY)-HSQC spectra (Fig. 1).

Three problems served to complicate the assignments of the cross-peaks:

- (1) The crowded region between 7.5 and 8.3 parts per million (ppm) and between ~117 and 119 ppm along the ^1H

and ^{15}N dimensions and the chemical shift degeneracy hindered the standard sequential assignments procedure.

- (2) The limited thermal stability of αTS (Matthews et al. 1980) precluded measurements at $\geq 40^\circ\text{C}$; key experiments like HNCACB, CBCA(CO)NH, which provide the characteristic $\text{C}\beta$ information, were ineffective and less efficient, respectively, at 25°C in fully protonated αTS .
- (3) The 19 prolines introduced numerous breaks in the sequential linking of the backbone atoms.

To resolve these problems, we explored the application of selective ^{15}N -labeling of key hydrophobic residues to reduce the spectral complexity and to obtain unambiguous residue-specific starting points. An auxotrophic bacterial strain was used to label individually the backbone nitrogens of the 27 leucine, 17 valine, 20 isoleucine, or 12 phenylalanine residues of αTS , which together constitute ~30% of the total residues. The number of cross-peaks observed is in very close agreement with the number of residues expected from the sequence: 27 of 27 leucines, 19 of 20 isoleucines, 17 of 17 valines, and 11 of 12 phenylalanines are observed in the ^1H - ^{15}N HSQC spectra (Fig. 2). With this simplification and various double- and triple-resonance experiments performed at 25°C (pH 7.8) on uniformly ^{15}N - and $^{13}\text{C}/^{15}\text{N}$ -labeled αTS , ~80% of the observed ^1H - ^{15}N cross-peaks have been assigned (Fig. 1). The difficulty in obtaining unambiguous assignments of the remaining peaks reflects, in part, the lack of dispersion in a protein with significant α -helix content (~50%).

Secondary structure in *E. coli* αTS

Chemical shift index (CSI) for the $^{13}\text{C}\alpha$ and $^1\text{H}\alpha$ resonances and hydrogen-deuterium (H-D) exchange data are summarized in Figure 3. The residues in the β -strands in general showed the expected downfield- and upfield-shifted $^1\text{H}\alpha$ and $^{13}\text{C}\alpha$ deviations from random coil values, respectively (Wishart et al. 1991, 1995). Many of these same residues displayed relatively strong sequential $d\alpha\text{N}$ ($i, i+1$) and weaker $d\alpha\text{N}$ (i, i) nuclear Overhauser enhancements (NOEs) in the 3D ^1H - ^{15}N NOESY spectrum, leading to the positive identification of the eight strands of the β -barrel. Expected upfield- and downfield-shifted $^1\text{H}\alpha$ and $^{13}\text{C}\alpha$ chemical shifts are observed for most of residues in the α -helical regions (Fig. 3), and the same regions showed the $d\text{NN}$ ($i, i+1$) NOEs expected for helices. The β -strands and α -helices thus identified by using the combined information from NOEs, CSI, and H-D exchange compare well with the location of their counterparts in the αTS component of the $\alpha_2\beta_2$ complex from *S. typhimurium* (Fig. 3).

Figure 1. ^1H - ^{15}N heteronuclear single quantum coherence (HSQC) spectrum of the α subunit of tryptophan synthase from *Escherichia coli* (αTS), at 25°C (pH 7.8), in 50 mM phosphate, 0.2 mM K_2EDTA , and 2 mM dithioerythritol. The assignments are indicated by the single-letter amino acid code and the residue number. The horizontal lines connect the side-chain NH_2 resonances of asparagine and glutamine. The protein concentration was ~ 0.6 mM.

The absence of assignments for most of helix $\alpha 2'$ and the preceding loop may reflect exchange broadening. This region, located at the interface between the two subunits, is dynamic even in the $\alpha_2\beta_2$ complex and only becomes ordered in the presence of ligands (Hyde et al. 1988). The dynamic behavior is thought to play a role in catalysis and in allosteric communication between the α and β subunits (Miles et al. 1999). The region between $\beta 6$ and $\alpha 6$ (residues 178–189) is highly disordered in the native enzyme complex, as evidenced by the poor electron density. The NMR

data imply that these regions remain flexible in the isolated αTS in solution.

Tertiary interactions in E. coli αTS

Abnormal chemical shifts often imply unique features in the tertiary structure, that is, the global fold. For example, the NH proton of Ala103 in $\alpha 3$, shows an abnormal downfield shift (Fig. 1) that is consistent with the hydrogen bond interaction with the O $\delta 1$ of Asp130 observed in the crystal

Figure 2. ^1H - ^{15}N heteronuclear single quantum coherence (HSQC) spectra of the subunit of tryptophan synthase from *Escherichia coli* (αTS), selectively ^{15}N -labeled at phenylalanine (A), valine (B), isoleucine (C), and leucine (D) residues at 25°C (pH 7.8). The unassigned resonances are indicated by an asterisk. The protein concentration was ~ 0.6 mM, and the buffer used is described in the caption for Figure 1.

structure. Similarly, the downfield shifts for Phe19 and Ile97 NHs of $\beta 2$ and $\beta 3$, respectively, are consistent with hydrogen bonds to the O $\delta 2$ s of Asp46 and Asp124, respectively. The observed upfield $^1\text{H}\alpha$ chemical shift of 2.85 ppm for Val 106 of $\alpha 3$ is consistent with its proximity to the aromatic ring of Phe114 and in close agreement with the value predicted (Williamson and Asakura 1993) from the crystal structure coordinates. These abnormal chemical shifts imply that the tertiary structures of the isolated αTS from *E. coli* and αTS in the $\alpha_2\beta_2$ complex from *S. typhimurium* are similar, consistent with the presumption of a conserved global fold.

Secondary structure in a stable folding intermediate

Thermodynamic analysis of the urea-induced unfolding of αTS from *E. coli* revealed the presence of two stable intermediates, *I1* and *I2*, maximally populated at ~ 3.0 and 5.0 M urea, respectively, at pH 7.8 and 25°C (Gualfetti et al.

1999). On the basis of a far-UV CD spectrum reconstructed from singular value decomposition analysis, it has been proposed that the β -barrel remains folded and is largely responsible for stabilizing the *I1* intermediate. The *I2* species lacks secondary and tertiary structure and is thought to be stabilized by long-range hydrophobic interactions between strings of nonpolar side-chains found in β -strands (Saab-Rincon et al. 1996). Stable folding intermediates similar to *I1* have also been observed for other TIM barrel proteins (Jasanoff et al. 1994; Andreotti et al. 1997; Sanchez del Pino and Fersht 1997; Forsyth and Matthews 2002), indicating that the β -barrel motif may play a significant role in defining the equilibrium and, perhaps, the kinetic properties of the folding reactions.

This conjecture is supported by the observation that the majority of the ~ 50 cross-peaks observed 22 h after dissolving the protein in D_2O at pH 7.8 and 25°C correspond to almost all the amide protons of the eight strands that stabilize the β -barrel; very few of the amide protons in helices

Figure 3. Summary of the chemical shift index data of the $^1\text{H}\alpha$ and $^{13}\text{C}\alpha$ nuclei and the hydrogen-deuterium (H-D) exchange data for slowly exchanging amide protons. The amino acid sequences of the α subunits of tryptophan synthase from *Salmonella typhimurium* (α TSSt) and *Escherichia coli* (α TSEc) are shown. The secondary structure for the α TSSt derived from the crystal structure (Protein Data Bank code 1BKS) by using the program DSSP (Kabsh and Sander 1983) is indicated; helices and strands are shown in red and blue, respectively. The filled circles indicate amide protons observed in the ^1H - ^{15}N heteronuclear single quantum coherence (HSQC) spectrum recorded 22 h after dissolving the protein in $^2\text{H}_2\text{O}$ at 25°C and pH 7.8. Residues of the α subunit involved in hydrogen bond interactions with the β subunit residues in the tryptophan synthase complex are indicated by open circles, and residues 178–189 (boxed) do not show electron density in the crystal structure complex. The gaps in chemical shift information are owing to either lack of assignments or cross-peaks in the ^1H - ^{15}N HSQC spectrum.

are protected (Fig. 3). Hydrogen exchange experiments (Mayo and Baldwin 1993; Bai et al. 1995; Bhuyan and Udgaonkar 1998; Fuentes and Wand 1998; Li and Woodward 1999 [and references cited therein]; Parker and Marqusee 2001) are required to identify the cooperative networks of hydrogen bonds that stabilize the partially folded forms.

Perspective

By using a combination of 3D double- and triple-resonance NMR experiments at pH 7.8 and 25°C on uniformly ^{15}N - and $^{13}\text{C}/^{15}\text{N}$ -labeled and ^{15}N -selective labeled proteins, we have assigned most of the backbone cross-peaks in a 29-kD TIM barrel protein and identified probes for monitoring the secondary structure in a stable folding intermediate. This approach can readily be extended to other TIM barrel proteins and, thereby, enable a test of the role played by the β -barrel motif in stabilizing what appear to be similar folding intermediates for one of the most common folds in biology.

Materials and methods

Uniformly ^{15}N - and $^{13}\text{C}/^{15}\text{N}$ -labeled αTS was expressed in *E. coli* BL21/DE3 cells transformed with the plasmid pT7.WS1 grown on M9 minimal medium with 1 g/L $^{15}\text{NH}_4\text{Cl}$ and 2 g/L $^{13}\text{C}_6$ glucose. Cells were grown at 37°C to an OD of 1.0 at 600 nm, induced with IPTG, and harvested after 3 h. After sonication, unfractionated cell extracts were incubated for a few minutes in 100 mM potassium phosphate (pH 7.8), 5 mM K_2EDTA , 2 mM dithioerythritol (DTE), 10 mM MgCl_2 , 0.1 M NaCl, and 0.01% Triton X-100, containing 10 $\mu\text{g}/\text{mL}$ RNase and DNase.

The protein was found in both the soluble and insoluble fractions of the cell lysate after sonication. The protein from the soluble fraction was purified by using a modified protocol of Kirschner et al. (1975). After dialysis in 10 mM potassium phosphate (pH 7.8), 2 mM K_2EDTA , and 2 mM DTE, the protein was loaded on a preequilibrated DE52 resin column and eluted by increasing the K_2EDTA concentration to 4 mM (Gualfetti et al. 1999). The insoluble fraction of the cell lysate was washed three times; solubilized in 6 M urea, 10 mM potassium phosphate, 1 mM K_2EDTA , and 1 mM DTE; and subsequently dialyzed to remove the urea. The refolded protein was then loaded onto a DE52 resin column preequilibrated with 10 mM potassium phosphate (pH 7.8), 1 mM K_2EDTA , and 1 mM DTE. After washing with 10 mM potassium phosphate, the protein was eluted using a nonlinear 10 to 300 mM potassium phosphate (pH 7.8) gradient (Yee et al. 1996). The structural integrity of the protein obtained from both the fractions was verified by the comparison of the ^1H - ^{15}N HSQC spectra. Identical spectra ensured the complete recovery of the secondary and tertiary structure in the protein recovered from the insoluble fraction of the lysate.

αTS selectively labeled with ^{15}N at the amide nitrogen of phenylalanine, valine, isoleucine, and leucine residues, respectively, was obtained using the auxotrophic strain DL39avtA/DE3 and purified following the protocol applied to the soluble fraction of the uniformly labeled protein as described above. The cells were grown on M9 minimal medium supplemented with nucleic acid

bases and all the amino acids in unlabeled form except those at which ^{15}N -labeling was desired. Protein expression was induced with IPTG.

NMR spectroscopy

The protein samples were prepared by dialyzing the protein in 50 mM potassium phosphate (pH 7.8), 0.2 mM K_2EDTA , and 2 mM DTE. The protein concentration was ~0.6 to 0.8 mM in 95% $^1\text{H}_2\text{O}$ and 5% $^2\text{H}_2\text{O}$. All the NMR experiments were conducted at 25°C by using a Bruker 600 MHz spectrometer equipped with a triple-resonance probe including Z-axis pulse field gradients. ^1H - ^{15}N HSQC, ^{15}N TOCSY-HSQC, and ^{15}N NOESY-HSQC experiments were performed on uniformly labeled αTS . ^1H - ^{15}N HSQC and ^{15}N NOESY-HSQC experiments were performed on selective ^{15}N -labeled proteins. 3D triple-resonance HNCA, HN(CO)CA, HNCO, CBCA(CO)NH, HNCACB, HCCH COSY, and HCCH TOCSY experiments were performed on uniformly $^{13}\text{C}/^{15}\text{N}$ -labeled protein. Slowly exchanging amides were identified by recording a series of ^1H - ^{15}N HSQC experiments on the lyophilized protein dissolved in $^2\text{H}_2\text{O}$. All the data were processed and analyzed using FELIX 2000 (Accelrys).

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