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Journal:	*Journal of the American Chemical Society*
Manuscript ID:	ja-2011-06967d
Manuscript Type:	Communication
Date Submitted by the Author:	26-Jul-2011
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Solution NMR Evidence for Symmetry in Functionally or Crystallographically Asymmetric Homodimers

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Supporting Information

ABSTRACT: A recurrent theme of many crystallographic studies of homo-oligomeric protein systems is concerned with verification that the conformation observed in a crystal represents the functionally relevant structure. An asymmetric conformation adopted by two chemically identical subunits in homo-oligomers can represent an intrinsic property of a protein or be an artifact induced by crystal packing forces. Solution NMR studies can distinguish between these two possibilities. Using methyl-based NMR spectroscopy we provide convincing evidence for symmetry in the absence of ligands in several homodimeric proteins that are either asymmetric functionally and/or adopt different conformations of the two subunits in available x-ray structures.

An asymmetric conformation adopted by two chemically identical subunits in a homodimeric protein can represent its intrinsic structural property or be artifactual due to crystal packing forces.[1-3] X-ray crystallography frequently provides us with a symmetric model of a homodimer when functionally the protein is known to behave asymmetrically. Conversely, crystallographic asymmetry may be present that does not exist in solution. The structurally relevant character of crystallographically observed asymmetry may be justified when it is associated with asymmetry at the subunit interface, is persistent under several different crystal environments, and/or when a chemical basis for the asymmetry can be identified.[1,2] In many cases, however, slight conformational differences arise from individual subunits of a homo-oligomer being 'trapped' in different crystal lattice environments.[2] On the other hand, when a homodimer is predicted to be asymmetric in solution based on biochemical and functional assays (*e.g.* its subunits displaying cooperativity in ligand/substrate binding), an issue arises whether such asymmetry is 'pre-existent' or induced by external stimuli such as, for example, the binding of ligands.

Since NMR chemical shifts are exquisitely sensitive to minute changes in conformation, solution NMR can distinguish between the true and spurious structural asymmetry. Until recently, many of the homo-oligomeric protein systems have been beyond the reach of solution NMR techniques due to molecular size limitations. Isotope labeling and NMR methods developed for high-molecular-weight proteins in the past decade are now well poised to address this problem.[4,5] Here we provide compelling NMR evidence for symmetry in a number of ligand-free homodimeric proteins that are either asymmetric functionally (and are predicted to possess 'pre-existent' asymmetry of its subunits in solution), or adopt different conformations of the two identical subunits in available x-ray structures.

Recently, we have initiated NMR studies of Tyrosyl tRNA Synthetase[6,7] from the thermophilic organism *Bacillus stearothermophilus* (*Bst*-YRS) as a prototype system for understanding the relationship between structural and functional asymmetry in homodimeric proteins. YRS is a paradigmatic example of a 'half-site-reactive' homodimer (MW = 95 kDa).[6,8,9] It catalyzes the activation of Tyr via formation of Tyr-AMP and subsequent transfer of the activated Tyr to the cognate $tRNA^{Tyr}$. The truncated form of the enzyme lacking the C-terminal tRNA-binding domain (ΔYRS; MW = 72 kDa) can activate Tyr with the same efficiency as the full-length YRS but cannot charge tRNA.[10] Crystal structures of *Bst*-YRS have been solved: free, in complexes with Tyr-AMP and inhibitors.[11] The structure of the C-terminal domain of YRS (not observed in any of the available x-ray structures) has been solved separately by solution NMR.[12,13] A model of the full-length YRS assembled from the x-ray and NMR structures is shown in Figure 1. All x-ray structures including that of ligand-free YRS feature a homodimer with two-fold symmetry.[11,14,15]

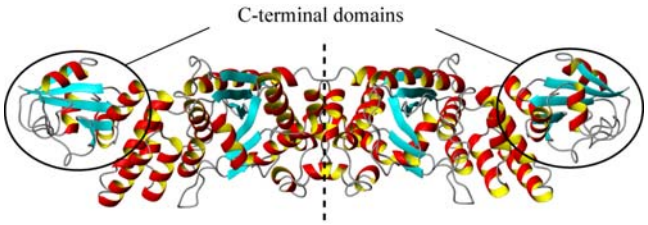

Figure 1. A structural model of *Bst*-YRS homodimer constructed from the x-ray structure of the N-terminal part (PDB ID 2TS1)[11] and the average NMR structure of the C-terminal domain (PDB ID 1JH3).[12] The molecular dyad axis is shown with a dashed vertical line.

YRS exhibits 'half-of-the-sites' activity (extreme form of negative cooperativity) with respect to Tyr binding, Tyr-AMP formation and tRNA charging - it binds to, activates, and charges tRNA with only 1 mole of Tyr per mole of dimer.[16,17] Earlier, it was predicted to be asymmetrical in solution in the absence of ligands from extensive kinetic and mutagenesis

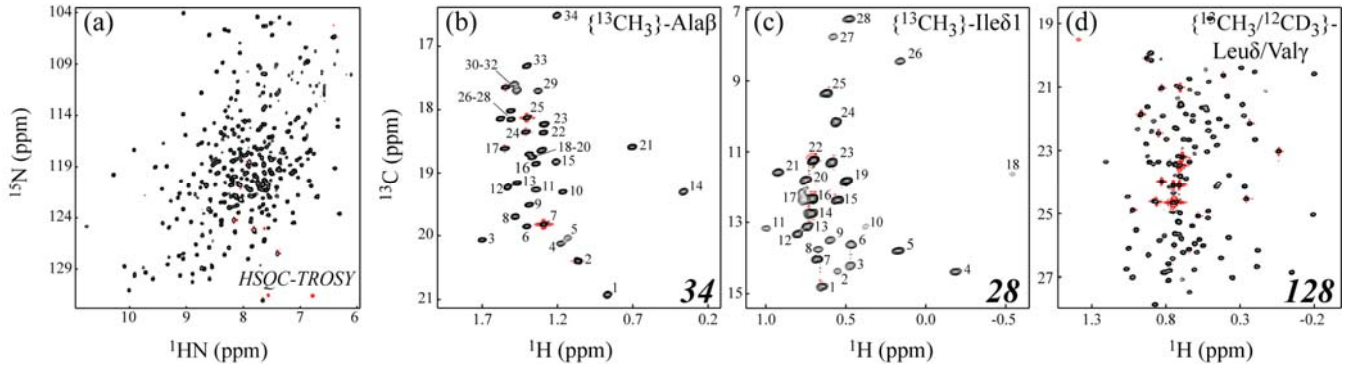

Figure 2. **(a)** ^{1}H-^{15}N HSQC-TROSY correlation map of full-length {U-^{2}H,^{15}N}-*Bst*-YRS (800 MHz, 50°C). **(b-d)** ^{1}H-^{13}C methyl-TROSY maps showing {^{13}CH$_3$}-methyl correlations of **(b)** Ala$^{\beta}$, **(c)** Ile$^{\delta1}$, and **(d)** Leu$^{\delta}$/Val$^{\gamma}$. The number of residues of each type in the amino-acid sequence of *Bst*-YRS is shown in bold italics at the right bottom corners of panels (b-d). The number of identifiable separated cross-peaks in (d) is 124 at 800 MHz. The spectra in (b-d) have been acquired in 99% D_2O (800 MHz, 50°C).

studies of Fersht and co-workers.[6,17] Specifically, heterodimers between the full-length YRS and its truncated form (ΔYRS) were constructed. Such heterodimers have two sites for formation of Tyr-AMP intermediate but only one site for tRNA binding.[10] A mutation which drastically reduces the rate of Tyr-AMP formation, has been introduced into the binding site of a pre-determined subunit.[18] It was found that heterodimers containing mutation on either subunit form ½ mole of Tyr-AMP/mole of dimer rapidly at the wild-type rate, while the remaining ½ mole are formed 10^4-fold slower - at the rate consistent with the mutant. It was concluded from these and other experiments that the dimer is 'frozen' in two states: ½ is active on one subunit and ½ on the other.[6,17] The YRS dimer has been for many years the most compelling case of 'pre-existent' asymmetry in a homodimeric protein despite an utter contradiction with available symmetric crystal structures[11,15] (Figure 1). Although very slight differences between the conformations of the two subunits have been observed in the x-ray structure of Tyr-bound ΔYRS, it cannot apparently be related to 'half-site' activity as both subunits in the crystal structure have Tyr molecules bound.[15]

Surprisingly, the asymmetry in the ligand-free YRS cannot be confirmed by solution NMR data. Clearly, any degree of structural differences between the two subunits should be reflected in the number of correlations observed in NMR spectra. It may be difficult to estimate the exact number of ^{1}H-^{15}N correlations from the HSQC-TROSY[19] map of ligand-free *Bst*-YRS (Figure 2a) because of (i) resonance overlap, (ii) missing correlations due to fast exchange with the solvent or (iii) incomplete back-protonation of amides after protein production in D_2O. It is much more straightforward to obtain the exact number of correlations in selectively methyl-labeled samples. Figures 2b-d show that the number of methyl ^{1}H-^{13}C correlations observed in the methyl-TROSY[20] spectra of selectively {^{13}CH$_3$}-methyl-labeled full-length *Bst*-YRS closely matches the number of corresponding residues in the amino-acid sequence of the protein, indicating the lack of structural asymmetry of the ligand-free enzyme.

To ensure that the observed symmetry is not a temperature-related artifact, NMR spectra have been collected at several temperatures in the 30-to-50°C range. Figure S1a in the Supporting Information shows that the changes in chemical shifts of Ala$^{\beta}$ ^{13}CH$_3$ methyls are small, indicating that no major changes in the conformations of both YRS subunits occur in this temperature range. In fact, YRS is known to form an exceptionally stable dimer (K_D = 85 pM);[21] it is therefore inconceivable that it dissociates under NMR conditions. At room temperature, the molecular weights of YRS and ΔYRS correspond to dimeric species based on size-exclusion chromatography elution times and PAGE electrophoresis under native conditions, while at higher temperatures, ^{15}N(^{13}C) relaxation

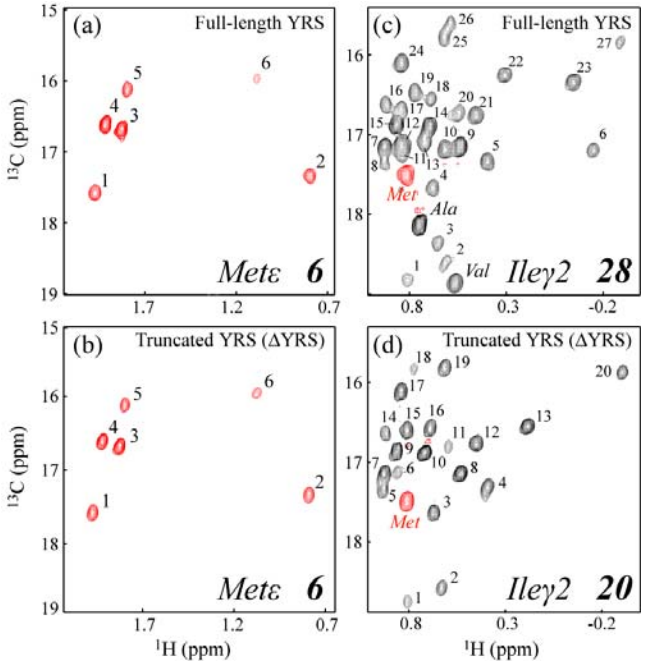

Figure 3. Selected regions of the ^{1}H-^{13}C constant-time HSQC spectra showing {^{13}CHD$_2$}-methyl correlations of **(a)** Met$^{\varepsilon}$ in full-length YRS; **(b)** Met$^{\varepsilon}$ in ΔYRS; **(c)** Ile$^{\gamma2}$ in full-length YRS, and **(d)** Ile$^{\gamma2}$ in ΔYRS. The number of residues of each type in the amino-acid sequence of YRS is shown in bold italics at the right bottom corner. Negative contours (Met) are shown in red. Met residues are absent from the C-terminal domain of YRS. All NMR data were collected at 40°C in 99% D_2O (600 MHz).

times of amides(methyls) agree with those predicted for dimeric species. Likewise, temperature dependent isothermal titration calorimetry (ITC) measurements indicate that the 'half-of-the-sites' activity is retained at higher temperatures (Figure S1b; Supporting Information).

Recently, we developed a series of NMR experiments for detection of $^{13}CHD_2$ methyl isotopomers in residually protonated large proteins produced in D_2O using {U-^{13}C}-glucose as a carbon source.[22,23] Albeit at the ~6-fold expense in sensitivity, correlations of *all* protein methyls can be detected in these experiments, with Ala$^{\beta}$ and Thr$^{\gamma 2}$ positions usually targeted for selective observation.[22] We have applied this methodology to {U-^{13}C;^{2}H}-labeled ΔYRS and YRS with the same result - no evidence of differences between subunit conformations on the chemical shift time-scale. Met$^{\varepsilon}$ and Ile$^{\gamma 2}$ regions of methyl correlation maps of full-length YRS and ΔYRS are shown in Figure 3 (not labeled with $^{13}CH_3$ methyls in Figure 2). The number of observed {$^{13}CHD_2$}-methyl correlations closely corresponds to the number of Met and Ile residues in each subunit of the homodimers. Figure S2 (Supporting Information) shows the remaining $^{13}CHD_2$ methyl correlations observed in ΔYRS, lending even more confidence in the absence of any detectable asymmetry of the YRS structure in solution. Note that it is especially straightforward to confirm the identification of Met methyls (Figures 3a-b) as Met$^{\varepsilon}$ carbons are not ^{13}C-coupled to adjacent carbon positions in non-constant-time ^{1}H-^{13}C HSQC correlation maps.

The homodimeric 'half-site-reactive' enzyme Tryptophanyl-tRNA Synthetase (*Bst*-WRS; dimer MW = 74 kDa) is structurally homologous to YRS and catalyzes similar reactions of Trp activation and tRNA charging.[24] Several crystal structures of *Bst*-WRS free and in complexes with Trp/ATP and Trp-AMP have been solved.[24-27] WRS crystal structures provide substantial insight into domain rearrangements during the catalytic cycle. Based on ATP/Trp-dependent kinetics, SAXS measurements, and x-ray structures a mechanistic model for WRS was proposed in which ATP acts as an allosteric effector.[26] The 2.9Å crystal structure of ligand-free *Bst*-WRS (PDB ID 1D2R) shows asymmetry although the two subunits are found in several different crystal packing environments, and the interface between the two subunits is symmetrical.[25] We have used NMR techniques to assess the asymmetry of the unliganded homodimer in solution. Figure S3 (Supporting Information) shows the ^{1}H-^{15}N correlation map and selected regions of {$^{13}CHD_2$}-methyl HSQC spectra recorded on ligand-free {U-^{15}N,^{13}C,^{2}H}-*Bst*-WRS. As with YRS above, the number of observed methyl correlations closely corresponds to the number of methyl-bearing side-chains of each type, indicating that under the conditions used for NMR measurements the two subunits of the WRS homodimer adopt the same conformation.

Irrespective of the degree of crystallographically observed asymmetry in aminoacyl tRNA synthetases, the inter-subunit interfaces are symmetric in all available x-ray structures of ligand-free (and ligated) YRS, ΔYRS, and WRS. The recently solved 2.0Å crystal structure of the ligand-free homodimeric cAMP receptor protein, CRP (also known as catabolite gene activator protein, CAP; dimer MW = 50 kDa) from *Mycobacterium Tuberculosis* features "profound" asymmetry that encompasses the interface between the two subunits and extends far beyond,[28] with the r.m.s.d. between C$^{\alpha}$ atoms of the two subunits of 3.5Å. Figure 4a shows the ribbon diagram of *Mtb*-CRP crystal structure dissected by the molecular dyad axis.[28] Upon binding of the effector cAMP in the N-terminal domain, the protein undergoes a conformational transition that enables specific binding of DNA to the C-terminal domain and transcription initiation. Thus, the functional role of the crystallographically observed asymmetry in the apo-*Mtb*-CRP might be the blockage of transcription in the absence of the ligand (cAMP), even though the NMR structure[29] and the crystal structure[30] of its close homolog from *E.coli*, *Ec*-CRP, both feature symmetric homodimers (PDB IDs 2WC2, 3HIF).

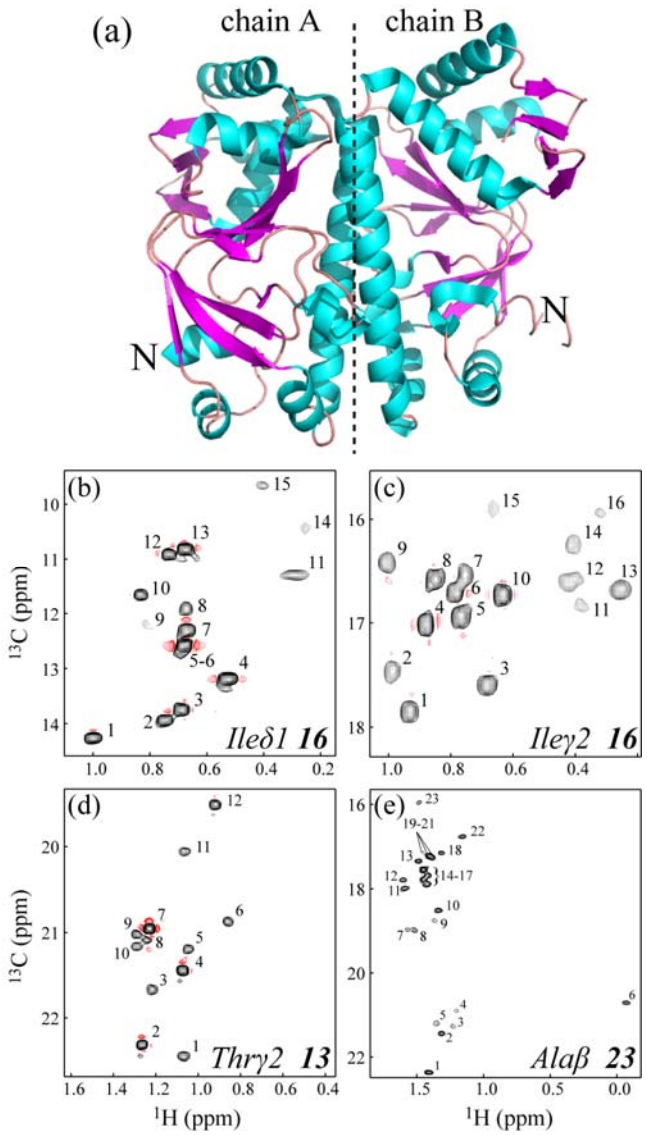

Figure 4. **(a)** A ribbon diagram of the crystal structure of the apo-*Mbt*-CRP homodimer (PDB ID 3D0S).[28] The axis of the molecular dyad is shown with a dashed vertical line. **(b-e)** Selected regions of the ^{1}H-^{13}C correlation map of CRP showing {$^{13}CHD_2$}-methyl correlations: **(b)** Ile$^{\delta 1}$, **(c)** Ile$^{\gamma 2}$, **(d)** Thr$^{\gamma 2}$ and **(e)** Ala$^{\beta}$. The number of residues of each type in the amino-acid sequence of CRP is shown in bold italics at the right bottom corners in (b-d). Selective detection of Thr$^{\gamma 2}$ and Ala$^{\beta}$ correlations with suppression of the rest of methyl signals has been employed.[22] All NMR spectra were recorded at 37°C in 99% D_2O (600 MHz).

Using the same isotope labeling/NMR methodology for detection of $^{13}CHD_2$ methyls as described above, we have conducted a brief solution study of apo-*Mtb*-CRP. Figures 4b-e show selected regions of $^{13}CHD_2$ methyl correlation maps recorded on {U-^{15}N,^{13}C,^{2}H}-labeled CRP (37°C). Although in this case the number of methyl correlations is in some instances lower than expected from the protein amino-acid content presumably due to conformational exchange broadening, no signs of profound asymmetry (that could be expected to lead to 'doubling' of NMR signals) can be observed in the spectra. We note that the dimer interface of CRP is rich in methyl-containing side-chains, especially those of Thr and Ala residues. Figure S4 of the Supporting Information shows additional regions of the $^{13}CHD_2$ methyl correlation map collected on {U-^{15}N,^{13}C,^{2}H}-labeled apo-*Mtb*-CRP.

In view of the presented data, it is likely that the two subunits in a homodimeric protein can sample a set of different conformational sub-states. Some of these sub-states are apparently preferred in crystals, while they may be averaged in solution leading to the observation of a single set of NMR resonances even when extensive differences exist in crystals between the conformations of the two monomers at the subunit interface. On the other hand, as is apparently the case in 'half-site-reactive' aminoacyl-tRNA synthetases, functional asymmetry does not necessarily translate to 'pre-existent' structural differences between the two subunits. The lack of detectable structural asymmetry of YRS in solution is particularly puzzling. It is possible that 'half-of-the-sites' activity in this case results from the equilibrium between various conformational sub-states of the two subunits in solution.[31,32] For example, the inter-conversion between catalytically active/inactive and inactive/active subunit conformations of the homodimer may occur fast on the chemical shift time-scale resulting in a single set of peaks in the NMR spectra. At each point in time, the enzyme would thus maintain 'transient' structural asymmetry, with energy for catalysis stored intermittently on one of the two subunits. However, so far we have not been able to confirm this hypothesis experimentally via NMR relaxation measurements that sample protein dynamics on fast (pico-to-nanosecond) or slow (milli-to-microsecond) time-scales. Alternatively, the asymmetry may be induced upon initial binding of substrates (Tyr/Trp or ATP in the case of aminoacyl tRNA synthetases).[26] We are currently investigating how the symmetry of all the ligand-free protein homodimers described here is affected in response to binding of ligands/substrates in solution.

ASSOCIATED CONTENT

Supporting Information. One figure showing {$^{13}CH_3$}-Ala$^{\beta}$ methyl correlation maps and ITC measurements acquired on full-length *Bst*-YRS as a function of temperature. One figure showing selected regions of the $^{13}CHD_2$ methyl correlation map of *Bst*-ΔYRS. One figure showing ^{1}H-^{15}N HSQC-TROSY spectrum and selected regions of the $^{13}CHD_2$ methyl spectrum of *Bst*-WRS. One figure showing selected regions of the $^{13}CHD_2$ methyl correlation map of *Mbt*-CRP. This material is available free of charge via the Internet at http://pubs.acs.org.

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ACKNOWLEDGMENT

The authors thank Dr. Paul Paukstelis (University of Maryland) for the gift of the plasmid encoding *Bst*-YRS, Dr. Prasad Reddy (NIST) for providing the plasmid of *Mbt*-CRP, Dr. Dorothy Beckett (University of Maryland) for allowing us to perform ITC measurements in her laboratory, Dr. David Weber (University of Maryland-Baltimore) for access to an 800 MHz spectrometer, and Dr. Eric First (Louisiana State University Health Center) for stimulating discussions. Identification of specific instruments, procedures, and products in this paper does not imply recommendation or endorsement by the National Institute of Standards and Technology.

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