LETTER TO THE EDITOR

Comment on "Revisiting the Ramachandran plot from a new angle"

In a recent *Protein Science* report, Zhou *et al.*¹ questioned the validity of our earlier results,² stating "...steric constraints alone are sufficient to explain the backbone dihedral angle distributions observed in proteins. Contrary to recent suggestions, no additional energetic contributions, such as hydrogen bonding, need be invoked." Specifically, we reported that the conventional Ramachandran dipeptide map^{3–5} shrinks when a previously overlooked, unsatisfied hydrogen bond is taken into account.² On the contrary, Zhou *et al.* argue that variations in τ -angles ($C'-C_\alpha-N$ angles) are sufficient to explain our results, obviating the need to consider hydrogen-bonding.¹ We reanalyzed the τ -angle distributions presented in their article and reach the opposite conclusion.

In greater detail, we reported that a large region of the sterically allowed bridge (defined as $\{\phi < 0^\circ; -20^\circ \leq \psi \leq 40^\circ\}$) is eliminated from the allowed ϕ/ψ angle distribution of an alanyl dipeptide (Ace-Ala-Nme) owing to an unsatisfied hydrogen-bonding group. To evaluate our result, Zhou *et al.* analyzed τ -angle distributions in experimentally determined protein structures (not dipeptides), focusing on residues in the bridge. However, our results are not a consequence of bridge residues *per se*. Rather, we showed that when a residue at position i adopts ϕ/ψ angles in the eliminated segment of the bridge, the amide hydrogen at position $i+1$ (NH_{i+1}) is shielded from solvent, depriving it of a hydrogen-bonding partner. Therefore, this conformation of the dipeptide would be highly disfavored in comparison with other sterically allowed, hydrogen-bond satisfied regions of the Ramachandran map. Accordingly, a valid test of our result requires inclusion of (i) both sterics and hydrogen bonding and (ii) both the bridge residue at i and the adjacent NH at $i+1$. Zhou *et al.* included neither.

What did Zhou *et al.* include in their analysis? They observed that the increase in the fraction of bridge residues "from $\tau = 105^\circ$ to 115° is consistent with the increase in area of the total allowed region of the ϕ/ψ map predicted by Ramachandran *et al.* from their hard-sphere models of dipeptides." On the basis of this observation, they tested our hydrogen-bonding result as follows. "In light of our findings concerning the t dependence of the ϕ/ψ distribution,

we chose two different amino acid types: Serine, which is capable of intrapeptide H-bonding, and Leucine, which is not, and tracked the distribution of allowed ϕ/ψ angles as a function of t for both residue types." Despite presumably opposite intrapeptide hydrogen-bonding capabilities, leucine, and serine demonstrated an equally strong correlation between increasing τ and increasing percentage of residues with ϕ/ψ angles in the bridge. From this correlation, Zhou *et al.* concluded that relaxation of steric clashes resulting from increased τ sufficiently explains the ϕ/ψ distribution of bridge residues without any "need to invoke additional interactions to explain the backbone conformations of proteins," such as hydrogen bonding.

This analysis does not address the central theme of our article. We reported that the addition of hydrogen-bonding constraints eliminates a large region of the sterically allowed bridge in the alanyl dipeptide (see Fig. 2 in Porter and Rose²). This region is disfavored because its conformers result in shielding the NH_{i+1} amide from hydrogen-bonding to solvent, and the dipeptide is too short to provide a compensating intramolecular hydrogen-bonding acceptor. However, intramolecular partners are possible in longer chains, and therefore, we hypothesized that residues in folded proteins populating the disfavored bridge region would form intrapeptide H-bonds; failure to do so would carry a substantial energetic penalty.⁶ Analysis of experimentally determined protein structures is consistent with this hypothesis: intrapeptide H-bonds to NH_{i+1} were observed in the overwhelming majority of residues for which the i th residue is situated in the disfavored bridge.

Zhou *et al.* analyzed bridge residues in isolation, and therefore the behavior of the adjacent nonbridge residue at $i+1$ is beyond the scope of their analysis. To falsify our findings, they would need to demonstrate that the adjacent NH_{i+1} amide forms significantly fewer intrapeptide H-bonds with increasing τ or, in other words, increased τ can obviate the need for intrapeptide hydrogen bonding to amide hydrogens adjacent to disfavored bridge residues. We investigated this possibility and found that, on the contrary, nearly all $N-H_{i+1}$ amides adjacent to disfavored bridge residues do form intrapeptide H-bonds, and further that the proportion of these NH_{i+1} intrapeptide H-bonds remains nearly constant with increasing τ . This result is inconsistent with the conclusion drawn by Zhou *et al.*

In particular, we extended the analysis of Zhou *et al.*, focusing as they did on serines and leucines.

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Figure 1. Outlines of allowed backbone dihedral angle distributions for an alanyl dipeptide (Ace-Ala-Nme) with increasing τ angles ($\tau = 111.2$, green; $\tau = 116$, yellow; $\tau = 120$, red) based on steric constraints alone (a) and steric-plus-hydrogen-bonding constraints (b). Both steric repulsion and hydrogen bonding constraints relax as τ increases, as indicated by the expanding boundaries in both plots. Despite expanding boundaries, the disfavored regions persist (b).

Their exact dataset is not available (the web link cited in their article is no longer intact), so we used a dataset with similar specifications (PISCES list with resolution ≤ 1.7 Å, $R \leq 0.25$, sequence similarity $\leq 50\%$, and side chain B-factor ≤ 30 Å²)⁷ and repeated the analysis described in Porter and Rose.² Out of the 3982/3512 leucines/serines in the disfavored bridge, almost all of the adjacent NH_{i+1} amides form intramolecular H-bonds. Specifically, 3005/2500 (75%/71%) of the adjacent NH_{i+1} amides are hydrogen bonded to a backbone acceptor and 781/750 (20%/21%) to a side chain acceptor. The remaining 196/262 (5%/7%) were shifted to the non-disfavored bridge region through minimization, with

a change in RMSD of 1.0 Å or less, as described in our article.² It should be noted that the total dataset of leucines and serines included 7595 structures, but 101 of these had missing atoms and could not be analyzed.

It should be realized that comparison of τ -angle distributions for serine versus leucine—Figure 6 in Zhou *et al.*¹—is not a discriminating test of our hypothesis. While it is true that a serine side chain can form hydrogen bonds and a leucine side chain cannot, this distinction is irrelevant. Both serine and leucine can form backbone-backbone H-bonds, which comprise the majority of those observed here. Further, the backbone $\text{C}=\text{O}_i$ cannot form an H-bond to NH_{i+1} , and the side chains of serines in the disfavored bridge rarely do. In fact, only one of the 3512 serines in our dataset forms a side chain H-bond to NH_{i+1} , demonstrating that the side chain hydrogen-bonding capacity of serines has no bearing on whether the NH_{i+1} can form an intrapeptide hydrogen bond.

Do τ -angles affect the hydrogen bonding capacities of the NH_{i+1} amide at all? To explore this question, we ran both sterics-based and sterics-plus-hydrogen-bonding-based calculations of the allowed ϕ/ψ distributions for alanyl dipeptides with three different τ -angles: 120°, 116°, and 111.2° (the τ -value used by Porter and Rose). All calculations used Ramachandran's original van der Waals radii.⁵ We found that both the allowed regions of backbone dihedral space, and, consequently, the disfavored bridge region increase with τ (Fig. 1). Experimentally determined protein crystal structures corroborate this calculation: the NH_{i+1} of both serines and leucines in

Figure 2. Fraction of NH_{i+1} hydrogen bonds (y-axis) at each τ -interval (x-axis) for both serine and leucine. For both residues, this fraction fluctuates only slightly in the first four intervals ($100^\circ \leq \tau < 116^\circ$) but decreases significantly in the largest interval ($116^\circ \leq \tau < 120^\circ$), indicating that hydrogen bonding constraints relax substantially when $\tau \geq 116^\circ$. At this point, the Ramachandran plot is distorted well beyond its conventional boundaries, as illustrated in Figure 1(a).

the disfavored bridge form intrapeptide H-bonds in nearly equal proportions for successive 4° intervals of τ in the range 100° – 116° (Fig. 2).

Ultimately, the fraction of intrapeptide H-bonds begins to diminish in the interval between 116° – 120° as τ opens wide and both steric repulsion and hydrogen-bonding constraints are relaxed (Fig. 1). At this extreme τ -value, the dipeptide map is distorted well beyond its conventional boundaries [Fig. 1(a)], yet the sharply defined disfavored regions still persist [Fig. 1(b)]. We note that this interval lies beyond the range considered by Ramachandran *et al.*,⁴ although Zhou *et al.* include it. In any event, only 6% of all disfavored bridge residues adopt τ -angles of 116° or greater, some of which may well arise from refinement errors.^{8,9} Regardless, the remaining 94% are entirely consistent with the results reported in Porter and Rose.²

We are grateful to Zhou *et al.* for further exploring our hypothesis by introducing the τ -angle as a variable. However, analysis of experimentally determined bridge residues supports our original finding that residues having ϕ/ψ angles in the disfavored bridge impose hydrogen-bonding constraints on the adjacent amides. Contrary to the conclusion of Zhou *et al.*, the distribution of τ -angles does not falsify our finding, nor could it, absent evaluation of intramolecular hydrogen bonds to these adjacent hydrogen-bonding groups. Although the dataset used by Zhou *et al.* is unavailable, we extended their analysis using a comparable dataset and, consistent with our original report, find that almost all NH_{i+1} amides adjacent to a disfavored bridge residue form an intrapeptide H-bond, and remaining outliers can be rationalized. Our findings are robust until τ exceeds 116° , at which point the dipeptide map is signifi-

cantly distorted—and yet the disfavored regions persist.

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