

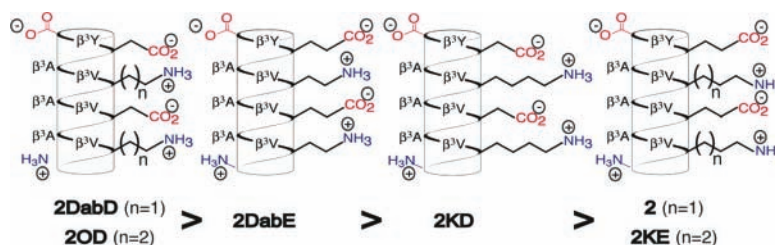
# Relationship between Salt-Bridge Identity and 14-Helix Stability of $\beta^3$ -Peptides in Aqueous Buffer

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## ABSTRACT



We report a systematic analysis of the relationship between salt bridge composition and 14-helix structure within a family of model  $\beta$ -peptides in aqueous buffer. We find an inverse relationship between side-chain length and the extent of 14-helix structure as judged by CD. Introduction of a stabilizing salt bridge pair within a previously reported  $\beta$ -peptide ligand for hDM2 led to changes in structure that were detectable by NMR.

$\beta$ -Peptides adopt a diverse array of secondary structures including a variety of helices, pleated-sheets, and tubes.<sup>1–5</sup>  $\beta$ -Peptides composed of  $\beta^3$ -amino acids often assemble into a unique helical form called the 14-helix that is characterized by a defined set of long-range hydrogen bonds and three distinct faces.<sup>6</sup> Although the majority of published work in

the  $\beta^3$ -peptide field describes molecules folded in organic solvents,<sup>5</sup> in 2001 Seebach and DeGrado reported independently that  $\beta^3$ -peptides containing an alternating pattern of oppositely charged side chains at positions  $i$  and  $i+3$  on two of three helical faces displayed moderate levels of 14-helix structure in aqueous buffer.<sup>7–9</sup> We subsequently demonstrated that the requirement for stabilizing salt bridges on two helical faces could be lifted by introducing side chains that stabilize the 14-helix macrodipole.<sup>10,11</sup> We established that  $\beta$ -peptide 14-helices stabilized in this way tolerate a vast

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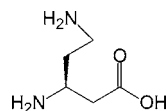
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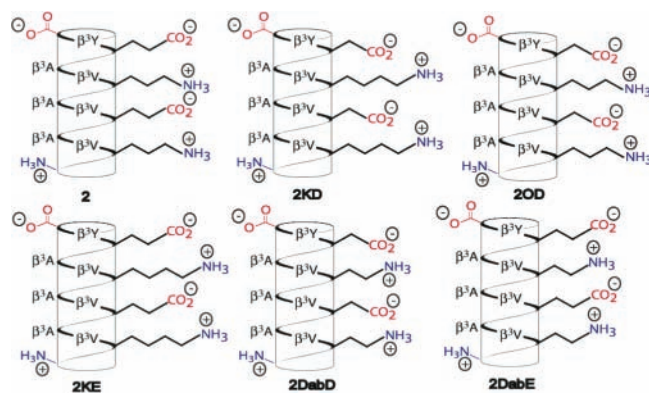
array of proteinogenic side chains<sup>10</sup> and can be modified to generate molecules that bind with moderate affinity to the proteins hDM2<sup>12</sup> and HIV gp41.<sup>13</sup> Here, we describe experiments that identify the charged side chain partners that best stabilize the 14-helix as judged by circular dichroism (CD) spectroscopy. We demonstrate that  $\beta^3$ -peptides containing  $\beta^3$ -HAspartate and either (*S*)-2,4-Homodiaminobutyric acid ( $\beta^3$ -HDab, Figure 1) or  $\beta^3$ -Hornithine along one helical face



**Figure 1.** (*S*)-2,4-Homodiaminobutyric acid ( $\beta^3$ -HDab).

provide the greatest level of 14-helix stabilization. Introduction of the 14-helix stabilizing  $\beta^3$ -Hornithine/ $\beta^3$ -HAspartate salt bridge into the previously reported hDM2 ligand,  **$\beta$ 53-1**, led to changes in 14-helix structure that could be detected by 2D-NMR spectroscopy.

We studied a set of six  $\beta$ -dodecamers to evaluate the effect of side-chain identity on 14-helix stability in water (Figure 2). All six molecules are derivatives of the previously



**Figure 2.** Helical net diagrams of  $\beta^3$ -peptides studied herein.

reported  $\beta$ -peptide **2**,<sup>10,11</sup> in which  $\beta^3$ -Hornithine (O) or  $\beta^3$ -HDab (Dab) replace  $\beta^3$ -HLysine (K) and  $\beta^3$ -HAspartate (D) replaces  $\beta^3$ -HGlutamate (E). All six  $\beta$ -dodecamers contain helix-promoting<sup>10,11,14</sup> aliphatic  $\beta^3$ -HValine residues at positions 2, 5, 8, and 11 along one face of the putative 14-helix and  $\beta^3$ -HALanine residues at positions 3, 6, and 9 along a second face. Each molecule also contained a  $\beta^3$ -HTyrosine

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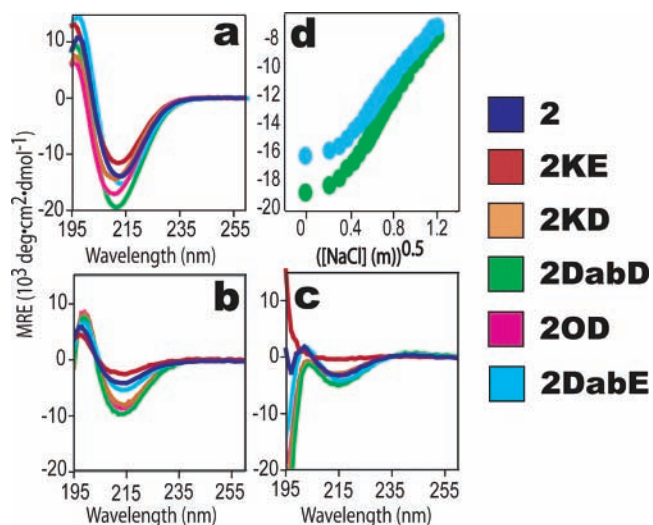
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residue to simplify spectrophotometric concentration determination. The  $\beta^3$ -peptides were synthesized using standard Fmoc solid-phase methods,<sup>15–17</sup> purified using reverse phase HPLC, and their sequences confirmed using MALDI-TOF mass spectrometry.<sup>18</sup> All six molecules are monomeric at 80  $\mu$ M as determined by analytical ultracentrifugation.

We used circular dichroism (CD) spectroscopy to monitor the extent of 14-helix structure in each  $\beta$ -peptide at 25 °C. While CD data on  $\beta$ -peptides must be interpreted carefully,<sup>19</sup> it is reasonable to assume that, for  $\beta^3$ -peptides in particular, changes in intensity of the 14-helical signature correlate to changes in 14-helical population.<sup>3,12,19,20</sup> The CD spectra of all six molecules are consistent with a 14-helix structure, with ellipticity minima between 211 and 214 nm, ellipticity maxima between 195 and 198 nm, and a crossover between negative and positive ellipticity between 200 and 202 nm (Figure 3a, Table 1).<sup>1,5,6</sup> The maximal values of negative



**Figure 3.** CD spectra of **2**, **2KE**, **2OD**, **2KD**, **2DabD**, **2DabE** at 80  $\mu$ M (PBC buffer) at: (a) pH 7 (b) pH 2 (c) pH 12. (d) MRE<sub>214</sub> of 100  $\mu$ M **2DabE** and **2DabD** vs [NaCl]<sup>0.5</sup>.

ellipticity range from  $-11500 \text{ deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}$  to  $-19500 \text{ deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}$ , representing a change of greater than 40%. The CD data suggest that the level of 14-helix structure among the six molecules is, from greatest to least: **2DabD** > **2OD** > **2DabE** > **2KD** > **2** > **2KE**.

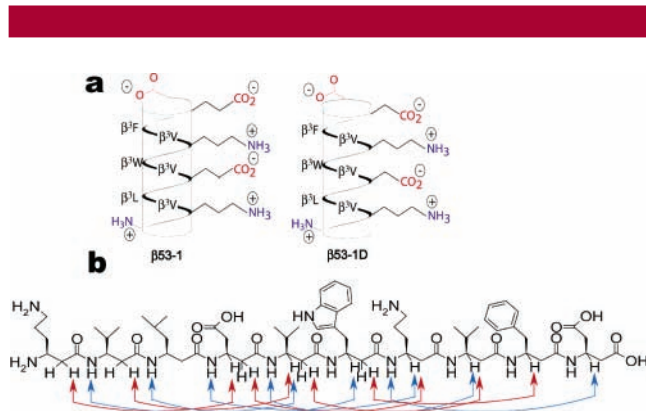
**Table 1.** Minimum MRE ( $\text{deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1}\cdot\text{residue}^{-1}$ ) for  $\beta$ -Peptides Studied Herein at 80  $\mu$ M and 25 °C

	$-\theta_{\min}$ (pH 7)	$-\theta_{\min}$ (pH 2)	$-\theta_{\min}$ (pH 12)	% $\Delta$ (pH 2/7)	% $\Delta$ (pH 12/7)
2DabD	19500	9690	4970	50	74
2OD	17100	8710	3370	49	80
2DabE	15200	5350	4110	65	73
2KD	14400	8040	2880	44	80
2	13900	4130	3190	70	77
2KE	11500	2570	376	78	97

Several trends emerge when the relative stabilities of the six  $\beta$ -peptides are compared. First, molecules containing  $\beta^3$ -HAsp display higher levels of 14-helix structure than otherwise identical molecules containing  $\beta^3$ -HGLu (compare **2DabD** vs **2DabE**, **2OD** vs **2**, and **2KD** vs **2KE**). Second, molecules containing  $\beta^3$ -HDab display higher levels of 14-helix structure than otherwise identical molecules containing  $\beta^3$ -HORN (compare **2DabD** vs **2OD**, and **2DabE** vs **2**). Finally, molecules containing  $\beta^3$ -HORN display higher levels of 14-helix structure than otherwise identical molecules containing  $\beta^3$ -HLys (compare **2OD** vs **2KD**, and **2** vs **2KE**). These trends suggest that the level of 14-helix structure in  $\beta$ -peptides related to **2** correlates inversely with side chain length: shorter side chains are better. Interestingly, solvent exposed salt bridges often contribute minimally to protein stability,<sup>21</sup> and glutamate, not aspartate, is the preferred partner for intra- $\alpha$ -helical salt bridges in proteins of known structure.<sup>22</sup>

The 14-helix stabilities of **2DabD** and **2DabE** were examined further by monitoring their CD spectra as a function of NaCl concentration at pH 7 in PBC buffer (Figure 3d). Both **2DabD** and **2DabE** become significantly less 14-helical as the NaCl concentration increases from 0 to 1.5 M as judged by the change in MRE<sub>214</sub>. In both cases the dependence of MRE<sub>214</sub> on NaCl concentration is approximately sigmoidal with a midpoint at 0.5 M NaCl; the plateaus observed at low salt suggest the formation of a stable conformation under these conditions. These CD data are highly reminiscent of those reported by DeGrado for a 15-residue  $\beta$ -peptide containing  $\beta^3$ -HLys/ $\beta^3$ -HGLu salt-bridges on two 14-helical faces and a C-terminal D-Asp; this molecule also showed a sigmoidal dependence of MRE<sub>214</sub> on NaCl concentration with a midpoint of 0.4 M NaCl.<sup>8</sup>

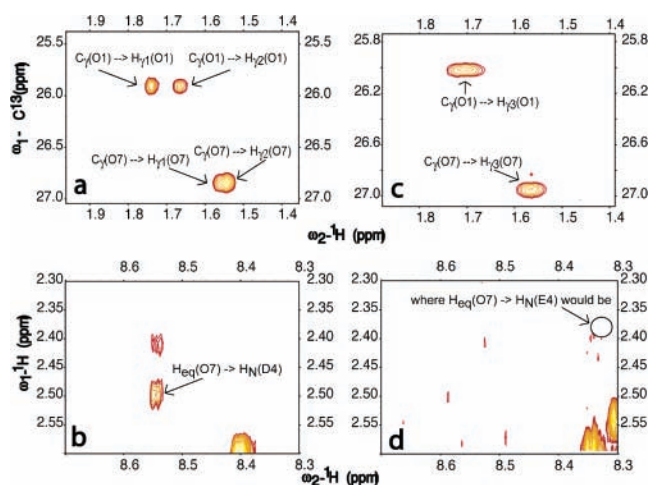
**$\beta$ 53-1**<sup>12</sup> is a structurally well-characterized<sup>23</sup>  $\beta$ -peptide that binds the oncoprotein hDM2 (Figure 4a). Based on the CD spectra of **2** and **2OD**, we hypothesized that substitution of  $\beta^3$ -HAsp for both  $\beta^3$ -HGLu residues in  **$\beta$ 53-1** would lead to differences in structure observable by NMR. As expected, the ROESY spectrum of  **$\beta$ 53-1D** at 10 °C in CD<sub>3</sub>OH showed multiple (ten of thirteen possible) long-range ROEs characteristic of the 14-helix conformation: five of seven possible C $\alpha$ H(*i*)  $\rightarrow$  C $\beta$ H(*i*+3) ROEs and five of six possible C<sub>N</sub>H(*i*)  $\rightarrow$  C $\beta$ H(*i*+3) ROEs (Figure 4b). Additional backbone ROEs



**Figure 4.** (a) Helical net diagrams depicting  **$\beta$ 53-1** and  **$\beta$ 53-1D**. (b) Backbone ROEs observed in the ROESY spectrum of  **$\beta$ 53-1D**; C $\alpha$ H(*i*)  $\rightarrow$  C $\beta$ H(*i*+3) ROEs are in red, C<sub>N</sub>H(*i*)  $\rightarrow$  C $\beta$ H(*i*+3) ROEs are in blue.

may have been present but were obscured by resonance overlap, as was true for  **$\beta$ 53-1**.<sup>12</sup> No backbone ROEs inconsistent with the 14-helix were observed. Overall, the ROESY spectrum of  **$\beta$ 53-1D** closely matched that of  **$\beta$ 53-1**, further supporting the conclusion that  **$\beta$ 53-1D** assembles into a 14-helix.

Interestingly, aliphatic <sup>13</sup>C-HSQC<sup>18</sup> and TOCSY<sup>24,25</sup> spectra revealed that the vicinal protons in the  $\gamma$  position of  $\beta^3$ -HORN at position 1 were clearly resolved in the NMR spectrum of  **$\beta$ 53-1D** but not  **$\beta$ 53-1** (Figure 5a,c). The portion



**Figure 5.** Differences in the 2D-NMR spectra of  **$\beta$ 53-1D** (a, b) and  **$\beta$ 53-1** (c, d). Regions of the <sup>13</sup>C-HSQC spectra are shown in a and c; differences in the ROESY spectra are shown in b and d.

of the aliphatic <sup>13</sup>C-HSQC NMR spectrum shown in Figure 5 identifies the interactions between the  $\gamma$  protons on  $\beta^3$ -HORN1 and 7 and the corresponding  $\gamma$  carbon within  **$\beta$ 53-**

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**1D** (Figure 5a) and  **$\beta$ 53-1** (Figure 5c). In the case of  **$\beta$ 53-1**, the  $\gamma$  protons of both  $\beta^3$ -HOrn1 and 7 were broadened due to exchange and the exact positions of the two peaks could not be fully defined. In the case of  **$\beta$ 53-1D**, however, the  $\gamma$  protons of  $\beta^3$ -HOrn1 were narrower and resolved. The differences between  **$\beta$ 53-1D** and  **$\beta$ 53-1** were also seen in the ROESY spectra (Figure 5b,d). In the case of  **$\beta$ 53-1D**, we observed six long-range ROEs between protons on  $\beta^3$ -HOrn and those on proximal  $\beta^3$ -HAsp residue(s). These ROEs include three—those between protons of  $\beta^3$ -HAsp4 and  $\beta^3$ -HOrn7—that were not observed in the ROESY spectrum of  **$\beta$ 53-1** (Figure 5b,d). The ROESY spectra of  **$\beta$ 53-1D** and  **$\beta$ 53-1** also differed in terms of the distribution of long-range ROEs throughout the sequence: the spectrum of  **$\beta$ 53-1D** showed comparably fewer unambiguous ROEs between  $\beta^3$ -HOrn1 or  $\beta^3$ -HOrn7 and  $\beta^3$ -HAsp4 and 10 but comparably greater ROEs between  $\beta^3$ -HOrn7 and  $\beta^3$ -HAsp4. Overall, although the CD spectra of  **$\beta$ 53-1** and  **$\beta$ 53-1D** are nearly identical, the NMR data implies a subtle increase in the order of the salt-bridge side-chains in  **$\beta$ 53-1D** when compared with  **$\beta$ 53-1**.

In summary, here we provide evidence that salt bridge identity exerts an influence on 14-helix stability and identify the  $\beta^3$ -HDab/ $\beta^3$ -HAsp pair as the most stabilizing of those salt bridges studied. With this information in place, we can now apply the structure-stabilizing salt-bridge effects to the design of other biologically active  $\beta$ -peptides, thereby further assessing the delicate connection between  $\beta$ -peptide structure and function.

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**Supporting Information Available:**  $\beta^3$ -Peptide synthesis, purification, CD spectroscopy, and NMR spectroscopy. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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