Structural changes in the C-terminus of Ca^{2+} -bound rat S100B($\beta\beta$) upon binding to a peptide derived from the C-terminal regulatory domain of p53

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Abstract

S100B($\beta\beta$) is a dimeric Ca^{2+} -binding protein that interacts with p53, inhibits its phosphorylation by protein kinase C (PKC) and promotes disassembly of the p53 tetramer. Likewise, a 22 residue peptide derived from the C-terminal regulatory domain of p53 has been shown to interact with S100B($\beta\beta$) in a Ca^{2+} -dependent manner and inhibits its phosphorylation by PKC. Hence, structural studies of Ca^{2+} -loaded S100B($\beta\beta$) bound to the p53 peptide were initiated to characterize this interaction. Analysis of nuclear Overhauser effect (NOE) correlations, amide proton exchange rates, $^3J_{\text{NH-H}\alpha}$ coupling constants, and chemical shift index data show that, like apo- and Ca^{2+} -bound S100B($\beta\beta$), S100B remains a dimer in the p53 peptide complex, and each subunit has four helices (helix 1, Glu2–Arg20; helix 2, Lys29–Asn38; helix 3, Gln50–Asp61; helix 4, Phe70–Phe87), four loops (loop 1, Glu21–His25; loop 2, Glu39–Glu49; loop 3, Glu62–Gly66; loop 4, Phe88–Glu91), and two β -strands (β -strand 1, Lys26–Lys28; β -strand 2, Glu67–Asp69), which forms a short antiparallel β -sheet. However, in the presence of the p53 peptide helix 4 is longer by five residues than in apo- or Ca^{2+} -bound S100B($\beta\beta$). Furthermore, the amide proton exchange rates in helix 3 (K55, V56, E58, T59, L60, D61) are significantly slower than those of Ca^{2+} -bound S100B($\beta\beta$). Together, these observations plus intermolecular NOE correlations between the p53 peptide and S100B($\beta\beta$) support the notion that the p53 peptide binds in a region of S100B($\beta\beta$), which includes residues in helix 2, helix 3, loop 2, and the C-terminal loop, and that binding of the p53 peptide interacts with and induces the extension of helix 4.

Keywords: calcium-binding protein; NMR; p53; S100B; S100 protein

The reduced homodimeric protein, S100B($\beta\beta$), is one of the best characterized proteins of the S100 family. The solution structure of apo-S100B($\beta\beta$) shows that two subunits associate tightly ($K_d < 500$ pM) (Drohat et al., 1997) through extensive hydrophobic interactions to form a compact dimer with a highly charged surface (Amburgey et al., 1995; Drohat et al., 1996; Kilby et al., 1996; Drohat et al., 1999). Recently, the solution and crystal structures of

Ca^{2+} -bound S100B($\beta\beta$) were also solved (Drohat et al., 1998; Matsumura et al., 1998; Smith & Shaw, 1998), and a comparison of these structures to that of the apo-S100B($\beta\beta$) show that a large change in the position of helix 3 occurs in the C-terminal normal EF-hand for each S100 β subunit. However, in the N-terminal pseudo EF-hand (which consists of 14 amino acids rather than 12), no conformational change is observed probably because helix 1 remains tightly packed in the dimer interface even upon the addition of Ca^{2+} . The two EF-hand domains of S100B($\beta\beta$) are connected by a loop (loop 2) that is often referred to as the “hinge” region, and a comparison of the amino acid sequences of the S100 family reveals that the hinge and the C-terminal loop are the least conserved among the S100 family proteins. These differences in sequence were the first indication that the “hinge” and the C-terminal loop are important for specific interactions between S100 proteins and their respective targets (Kligman & Hilt, 1988).

Additional indication that the C-terminal loop and the hinge region are important for target protein binding was illustrated in a study of the interaction between S100B($\beta\beta$) and a peptide corresponding to residues 367–388 of the C-terminus of human p53

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Abbreviations: 1D, 2D, and 3D, one-, two-, and three-dimensional; PKC, protein kinase C; DTT, dithiothreitol; p53, tumor suppressor protein; S100 β , a subunit of dimeric S100B($\beta\beta$); S100B($\beta\beta$), dimeric S100B with non-covalent interaction at the dimer interface; TSP, sodium 3-(trimethylsilyl) propionate-2,2,3,3- d_4 ; TFE, trifluoroethanol; CapZ, actin capping protein; NOE, nuclear Overhauser effect; NOESY, NOE spectroscopy; TOCSY, total correlation spectroscopy; EDTA, ethylenediaminetetraacetic acid; HMQC, heteronuclear multiple quantum coherence; HPLC, high-performance liquid chromatography; HSQC, heteronuclear single quantum coherence.

(Rustandi et al., 1998b). This binding inhibits the PKC-dependent phosphorylation of the p53 peptide (Wilder et al., 1998) and increases the Ca^{2+} -binding affinity to S100B($\beta\beta$) in the C-terminal EF-hand. Furthermore, changes in ^1H and ^{15}N chemical shifts upon p53 peptide binding indicate that the hinge region, the C-terminal loop, and helix 3 likely comprise the p53 peptide binding site on S100B($\beta\beta$) (Rustandi et al., 1998b). In this study, we report that the dimer interface and the two helix-loop-helix Ca^{2+} -binding sites of S100B($\beta\beta$) remain intact upon p53 peptide binding; however, an extension of helix 4 and significant changes in exchange rates for residues in helix 3 were observed. Together these results show that structural changes in the C-terminus of Ca^{2+} -bound S100B($\beta\beta$) occur upon target protein binding. A preliminary abstract of this work has been published (Rustandi et al., 1998a).

Results and discussion

Resonance assignments

The preliminary backbone chemical shift assignments of Ca^{2+} -loaded S100B($\beta\beta$) bound to the p53 peptide reported previously (Rustandi et al., 1998b) were confirmed, and the remaining side chain and aromatic chemical shift assignments were extended to completion (>99%) by analyzing data from 2D NOESY, 2D TOCSY, 3D ^{15}N -edited HOHAHA-HSQC, 3D HCCH-TOCSY, 3D ^{15}N -edited NOESY-HSQC, 3D ^{15}N , ^{15}N -edited HMQC-NOESY-HSQC, 3D CBCA(CO)NH, 3D HNCACB, 3D HNHA, 4D ^{13}C , ^{15}N -edited NOESY-HSQC, and 4D ^{13}C , ^{13}C -edited NOESY-HMQC NMR experiments. Standard methods were used in the assignment procedure, which relied primarily on scalar connectivities, with the exception being for assignments of aromatic ring protons (δ , ϵ , ζ), which were determined using NOE correlations to numerous assigned resonances (Wuthrich, 1986). The complete listing of the chemical shift assignments (>99%) of Ca^{2+} -loaded S100B($\beta\beta$) bound to the p53 peptide are in supplementary material (Table 1S).¹

NOE assignments

Short-range $\text{H}^{\text{N}}\text{H}^{\text{N}}(i, i + 1)$, $\text{H}^{\alpha}\text{H}^{\text{N}}(i, i + 1)$, $\text{H}^{\beta}\text{H}^{\text{N}}(i, i + 1)$, and medium-range $\text{H}^{\text{N}}\text{H}^{\text{N}}(i, i + 2)$, $\text{H}^{\alpha}\text{H}^{\text{N}}(i, i + 2)$, $\text{H}^{\alpha}\text{H}^{\text{N}}(i, i + 3)$, $\text{H}^{\alpha}\text{H}^{\text{N}}(i, i + 4)$, $\text{H}^{\alpha}\text{H}^{\beta}(i, i + 3)$ NOE correlations were assigned using the 3D ^{15}N -edited NOESY-HSQC and 3D ^{13}C -edited HMQC-NOESY experiments. It was also necessary to collect 3D ^{15}N , ^{15}N -edited HMQC-NOESY-HSQC, 4D ^{13}C , ^{15}N -edited NOESY-HSQC, and 4D ^{13}C , ^{13}C -edited NOESY-HMQC NMR data to resolve ambiguities in the assignment of NOE correlations in S100B($\beta\beta$) bound to the p53 peptide.

Figures 1 and 2 show strips of the ^{15}N -edited NOESY-HSQC and ^{15}N , ^{15}N -edited HMQC-NOESY-HSQC spectra, respectively, illustrating residues in helix 4 (F70–F87). Standard methods were used to identify helical conformation such as strong $\text{H}^{\text{N}}\text{H}^{\text{N}}(i, i + 1)$, weak $\text{H}^{\alpha}\text{H}^{\text{N}}(i, i + 1)$, together with $\text{H}^{\alpha}\text{H}^{\text{N}}(i, i + 2)$, $\text{H}^{\alpha}\text{H}^{\text{N}}(i, i + 3)$, $\text{H}^{\alpha}\text{H}^{\text{N}}(i, i + 4)$, and $\text{H}^{\alpha}\text{H}^{\beta}(i, i + 3)$ NOE correlations and small $^3J_{\text{NH-H}\alpha}$ coupling constants typical of

helices (Fig. 4) (Wuthrich, 1986). The significant chemical shift degeneracy observed in helical proteins such as S100B($\beta\beta$) made the NOE assignments with 3D data alone a difficult process. For example, A83H $^{\alpha}$ directly overlaps with M79H $^{\alpha}$, and hence, an $\text{H}^{\alpha}\text{H}^{\text{N}}(i, i + 4)$ NOE correlation remains ambiguous in the 3D ^{15}N -edited NOESY-HSQC spectrum. This degeneracy was resolved in the 4D ^{13}C , ^{15}N -edited NOESY-HSQC spectrum (Fig. 3) and demonstrates unequivocally the $\text{H}^{\alpha}\text{H}^{\text{N}}(i, i + 4)$ NOE correlation for M79 and A83. Similarly, for residue A75 in the 3D ^{15}N -edited NOESY-HSQC experiments, resonance A75H $^{\alpha}$ overlaps with M74H $^{\alpha}$, F73H $^{\alpha}$, and E72H $^{\alpha}$, and these overlapping peaks are resolved in the 4D ^{13}C , ^{15}N -edited NOESY-HSQC experiment (data not shown). Likewise, $\text{H}^{\alpha}\text{H}^{\beta}(i, i + 3)$ NOEs were difficult to assign unambiguously in the 3D ^{13}C -edited NOESY-HSQC, but they could be routinely assigned in the 4D ^{13}C , ^{13}C -edited NOESY-HMQC as illustrated for correlations from V80H $^{\alpha}$ to A83H $^{\beta}$ and for A83H $^{\alpha}$ to E86H $^{\beta}$ (Fig. 3). In summary, it was possible to unambiguously assign about 50% of the NOEs using 3D alone, whereas >98% of the resonances could be assigned with a combination of 3D and 4D NMR data.

Secondary structure and changes in the C-terminus of Ca^{2+} -loaded S100B($\beta\beta$) upon peptide binding

A diagram illustrating NOE correlations, chemical shift index, amide exchange rates, and scalar coupling constants ($^3J_{\text{NH-H}\alpha}$) used for characterizing the secondary structure of Ca^{2+} -loaded S100B($\beta\beta$) bound to the p53 peptide is shown in Figure 4 (Wuthrich, 1986; Spera & Bax, 1991; Wishart et al., 1992; Wishart & Sykes, 1994). When the p53 peptide is bound, each S100 β subunit has four helices (helix 1, Glu2–Arg20; helix 2, Lys29–Asn38; helix 3, Gln50–Asp61; helix 4, Phe70–Phe87), four loops (loop 1, Glu21–His25; loop 2, Glu39–Glu49; loop 3, Glu62–Gly66; loop 4, Phe88–Glu91), and two β -strands (β -strand 1, Lys26–Lys28; β -strand 2, Glu67–Asp69), which together form a short antiparallel β -sheet.

β -Sheet

As with apo- and Ca^{2+} -bound S100B($\beta\beta$), the two β -strands are antiparallel and bring the Ca^{2+} -binding domains of S100B($\beta\beta$) in the presence of the p53 peptide into close proximity. Residues in the two β -strands display the characteristic strong $\text{H}^{\alpha}\text{H}^{\text{N}}(i, i + 1)$ NOE correlations, upfield-shifted $^{13}\text{C}^{\alpha}$ and downfield-shifted $^1\text{H}^{\alpha}$ resonances. Furthermore, the measured $^3J_{\text{NH-H}\alpha}$ coupling constants of these residues have the largest values (6.6–9.1 Hz), and their amide proton exchange rates are among the slowest indicative of hydrogen bonding or solvent inaccessibility. In addition, long-range NOE correlations between the two β -strands are observed in 3D ^{15}N -edited NOESY-HSQC, 3D ^{15}N , ^{15}N -edited HMQC-NOESY-HSQC, 4D ^{13}C , ^{15}N -edited NOESY-HSQC experiments and confirm the antiparallel alignment of these two β -strands (data not shown).

α -Helices

Characteristic NOE correlations, downfield-shifted $^{13}\text{C}^{\alpha}$ and upfield-shifted $^1\text{H}^{\alpha}$ resonances, small $^3J_{\text{NH-H}\alpha}$ coupling constants (<5 Hz), and the slow amide proton exchange rates (>18 h) typical of α -helices are observed in each of the four helices. Interestingly, helix 4 is longer by four to five residues (A83–F87) in the peptide complex as compared to helix 4 in Ca^{2+} -bound rat S100B($\beta\beta$) (Drohat et al., 1998). While there is a possibility that these residues (A83–F87) have a tendency to form a nascent helix

¹The additional side-chain and aromatic chemical shift values were deposited into the BioMagResBank chemical shift database for Ca^{2+} -loaded S100B($\beta\beta$) bound to the p53 peptide (accession number 4099).

Fig. 1. Strips extracted from the ^{15}N planes of a 3D ^{15}N -edited NOESY-HSQC spectrum to illustrate NOE correlations in helix 4 (residue F70 to F87). Strip plots of 3D ^{15}N -edited NOESY-HSQC spectra of Ca^{2+} -loaded S100B($\beta\beta$) bound to p53 peptide for helix 4. Lines trace the connectivities of sequential strong $\text{H}^{\text{N}}\text{H}^{\text{N}}(i, i + 1)$ and weak $\text{H}^{\alpha}\text{H}^{\text{N}}(i, i + 1)$, while the $\text{H}^{\beta}\text{H}^{\text{N}}(i, i + 1)$ correlations have been omitted for clarity. Whenever visible, the $\text{H}^{\alpha}\text{H}^{\text{N}}(i, i + 3)$ and $\text{H}^{\alpha}\text{H}^{\text{N}}(i, i + 4)$ connectivities are depicted by enclosed solid and dashed boxes, respectively.

in the Ca^{2+} -bound state based on chemical shift indexes (Drohat et al., 1998), NOE correlations typical of stable helix formation were not detected. This could perhaps be due to conformational exchange on the chemical shift time scale that is observed for residues T81 to F87 (Fig. 5A). Figure 5B illustrates that any exchange broadening effects in helix 4 are virtually eliminated upon the addition of the p53 peptide to Ca^{2+} -bound S100B($\beta\beta$). The narrowing of the HSQC correlations for residues in the C-terminus of helix 4 upon peptide binding is accompanied by the appearance of strong NOE correlations consistent with their inclusion in helix 4 (Fig. 4). Thus, helix 4 is extended to include residues A83–

F87, and there are no longer any intermediate or slow conformational exchange processes for residues of helix 4 in the peptide complex (Fig. 5).

The extension at helix 4 to include A83–F87 can also occur for Ca^{2+} -loaded human S100B($\beta\beta$) in the absence of target protein (Smith & Shaw, 1997); however, the lack of exchange broadening for the C-terminal residues of helix 4 in this study is likely induced by the 10% TFE included in the NMR samples. Furthermore, the crystal structure of Ca^{2+} -bound bovine S100B($\beta\beta$) shows a longer helix 4 than that of Ca^{2+} -bound rat S100B($\beta\beta$) (Matsumura et al., 1998), but this difference in structure could be the result of inter-

Fig. 2. Strips extracted from the ^{15}N planes of a 3D ^{15}N , ^{15}N -edited HMQC-NOESY-HSQC spectrum to illustrate NOE correlations in the extension of helix 4 (residue A83 to F87). Strip plots of 3D ^{15}N , ^{15}N -edited HMQC-NOESY-HSQC spectra of Ca^{2+} -loaded S100B($\beta\beta$) bound to p53 peptide illustrating the $\text{H}^{\text{N}}\text{-H}^{\text{N}}$ connectivities for residues A83 through F87. The lines trace the connectivities of sequential strong $\text{H}^{\text{N}}\text{H}^{\text{N}}(i, i+1)$ correlations.

molecular crystal lattice packing, which could stabilize the longer helix in the crystalline state. Our data support the notion that the C-terminus of Ca^{2+} -loaded S100B($\beta\beta$) is in a slow conformational averaging regime in a totally aqueous environment. In solution, these residues (A83–F87) form a stable helix upon target protein binding or by the addition of organic solvents such as TFE; crystallization can also stabilize the extra turn of helix in the absence of a bound protein target.

Amide exchange

The exchange rates of many amide protons of Ca^{2+} -loaded S100B($\beta\beta$) bound to p53 peptide are slower than in the absence of bound peptide (Fig. 4). In particular, the amide proton of residues in helix 1 (L10), helix 2 (K33 and N37), the hinge region (F43 and I47), helix 3 (K55, V56, E58, T59, L60, and D61), loop 3 (G66), and helix 4 (Q71, T82, A83, and C84) are present after 0.25 h in D_2O for S100B($\beta\beta$) bound to the p53 peptide, but they are absent after 0.25 h in the peptides absence (Drohat et al., 1998). Interestingly, 12 out of the 16 residues that showed a distinct slowing in amide exchange rates occur for amide protons in helix 3, the hinge, and helix 4. The fact that these same regions of S100B($\beta\beta$)

Fig. 3. NOE spectra illustrating sequential and medium-range NOE correlations. **A:** A plane from the 4D ^{13}C , ^{15}N -edited NOESY-HSQC spectrum shows NOE correlations of $\text{H}^{\beta}\text{H}^{\text{N}}(i, i+1)$, $\text{H}^{\alpha}\text{H}^{\text{N}}(i, i+2)$, $\text{H}^{\alpha}\text{H}^{\text{N}}(i, i+3)$, $\text{H}^{\alpha}\text{H}^{\text{N}}(i, i+4)$. In particular, NOE correlation of $\text{H}^{\alpha}\text{H}^{\text{N}}(i, i+4)$ is clearly observed. This is ambiguous in 3D ^{15}N -edited NOESY-HSQC due to chemical shift degeneracy of A83H^{α} and M79H^{α} . **B:** A plane from 4D ^{13}C , ^{13}C -edited NOESY spectrum shows the medium NOE correlations of $\text{H}^{\alpha}\text{H}^{\beta}(i, i+3)$ between A83H^{α} and E86H^{β} . **C:** Similarly, A plane from 4D ^{13}C , ^{13}C -edited NOESY spectrum shows the medium NOE correlations of $\text{H}^{\alpha}\text{H}^{\beta}(i, i+3)$ between V80H^{α} and A83H^{β} . Note that several peaks are folded in the ^{13}C dimension; the actual chemical shift values for the folded peaks can be calculated using a sweep width of 24 ppm.

Fig. 4. Diagram of NMR data used to determine the secondary structure of Ca^{2+} -loaded S100B($\beta\beta$) bound to p53 peptide. The amide proton exchange rates are categorized as fast ($T < 0.25$ h), medium ($0.25 \text{ h} < T < 18 \text{ h}$), and slow ($T > 18 \text{ h}$) with no circle, half-shaded, and solid, respectively. The $^3J_{\text{NH-H}\alpha}$ couplings are labeled as small ($J < 5 \text{ Hz}$), medium ($5 < J < 8 \text{ Hz}$), and large ($J > 8 \text{ Hz}$) with solid, half-shaded, and no square, respectively. The asterisks denote residues which lack H^α or H^N cross peaks in the HNHA and/or in HSQC. The sequential and medium NOE correlations are represented by bar height indicating the strength of the NOE (strong, medium, weak, and very weak) and by horizontal lines connecting the two participating residues, respectively. The deviation in the $^{13}\text{C}^\alpha$ and $^1\text{H}^\alpha$ chemical shifts from those of a random coil are indicated by the positive and negative bars as described (Spera & Bax, 1991; Wishart & Sykes, 1994). The overall secondary structure of Ca^{2+} -loaded S100B($\beta\beta$) bound to p53 peptide is depicted as spirals for helices and arrows for β -strands.

give rise to large chemical shift perturbations and intermolecular NOE correlations (Table 1) to the p53 peptide upon binding (Rustandi et al., 1998b) further supports the notion that residues in this region of the protein comprise a site that interacts with the p53 peptide.

Model for target protein binding

A large conformational change in S100B($\beta\beta$), in which helix 3 reorients by $\sim 90^\circ$, is required for its binding to protein targets such as a peptide derived from the negative regulatory domain of p53 (Drohat et al., 1998, 1999). This conformational change exposes several residues in the hinge region, helix 3, helix 4, and the C-terminal loop of S100B($\beta\beta$). Residues in these regions of S100B($\beta\beta$) likely define the target protein binding site since the addition of the p53 peptide causes chemical shift perturbations

(Rustandi et al., 1998b), slows amide exchange rates (Fig. 4), extends helix 4 by one turn, and give rise to intermolecular NOE correlations between the peptide and S100B($\beta\beta$) (Table 1; Figs. 6, 7).

On the other hand, these data are not consistent with a model for target protein binding put forward by Matsumura et al. (1998). Matsumura's model predicts that a single target protein binds per S100B dimer, and that it interacts solely with residues in helices 4 and 4' at the dimer interface. This original model for binding is unlikely because thermodynamic titrations indicate that one p53 peptide binds each S100 β subunit (Rustandi et al., 1998b). In addition, the Matsumura model does not adequately explain the slower amide exchange rates for residues in helix 3, many of the chemical shift perturbations observed previously (Rustandi et al., 1998b), or several of the intermolecular NOE correlations between the p53 peptide and S100B($\beta\beta$) (Table 1; Fig. 7). For example, while the intermolecular NOE correlations from helix 1 (L3 and M7) and

Fig. 5. A comparison of HSQC peaks between (A) Ca²⁺-loaded S100B(ββ) and (B) Ca²⁺-loaded S100B(ββ) bound to the p53 peptide. The experiments were performed under identical conditions. Note that peaks of residue T81 through F87 in Ca²⁺-bound S100B(ββ) are much weaker due to intermediate and/or slow conformational exchange broadening when compared to Ca²⁺-loaded S100B(ββ) bound to the p53 peptide.

helix 4 (M74, V77, M79, V80, T82, and A83) could be explained by the Matsumura model, the intermolecular NOE correlations from loop 2 (L44, I47, K48), helix 2 (L35, I36), and helix 3 (E51, V52, V53, K55, V56, M57) can only be explained by a model for the target protein binding that also includes residues in helix 2, helix 3, and loop 2. Since these residues are distant from the dimer interface, a revised model is required that includes all of the res-

Table 1. Intermolecular NOEs from S100B(ββ) residues to the p53 peptide in the S100B(ββ)-Ca²⁺-p53 peptide complex

Residue in S100B(ββ)	δ (ppm)	Protons δ (ppm) observed in p53 peptide
L3δ	1.11	0.95, 0.85
M7ε	1.77	0.86, 0.77
L35δ	0.75	1.00, 0.59
I36γ ₂	1.04	0.92, 0.77
L44δ	0.77	1.83, 1.68, 1.61, 1.31
I47δ	0.71	1.01, 0.59
K48γ	1.34	1.71
E51γ	2.34	2.08
V52γ ₂	1.08	1.12, 0.86, 0.82
V53γ ₁	0.91	3.20, 1.65, 0.77
V53γ ₂	0.91	1.12, 0.77
K55γ	1.53	2.05, 1.97, 1.70, 1.12
V56γ ₁	0.52	2.27, 1.64, 0.90, 0.77
M57ε	1.91	2.26
M74ε	1.64	0.86
V77γ ₁	0.26	1.64, 0.86
M79ε	2.11	3.16, 2.60, 2.46, 1.63, 0.86, 0.77
V80γ ₁	0.34	1.63, 0.92, 0.88, 0.77
V80γ ₂	0.70	0.90, 0.77
T82γ ₂	1.32	2.47, 2.43, 1.63, 1.41, 0.77
A83β	1.24	1.60, 0.86, 0.77

Fig. 6. Plane of the 3D isotope filtered NOESY spectra illustrating intermolecular NOE correlations (labeled with asterisks) between M79 (of S100B(ββ)) and several resonances of the p53 peptide. This plane also contains the methyl group of A9, which is not in proximity to the peptide based on its lack of intermolecular NOE correlations.

idues that give rise to intermolecular NOE correlations (Table 1; Fig. 7). As expected, this group of residues forms a surface that is distinctly present on each S100β subunit of dimeric S100B(ββ), which is consistent with the thermodynamic binding studies.

A general role for the hinge and the C-terminal loop in the binding of S100 protein targets

It has been known for a long time that the hinge region (loop 2) and the C-terminal loop are the least conserved regions of the S100 protein family (Kligman & Hilt, 1988). A number of studies are reported that demonstrate a role for these regions in various S100-target protein complexes. For example, a peptide based on the

Fig. 7. Ribbon diagram of Ca²⁺-bound S100B(ββ). Residues in one of the two symmetrical subunits of S100B(ββ) that show intermolecular NOE correlations to the p53 peptide (Table 1) are shown in yellow. While the same intermolecular NOE correlations exist for residues in the other target protein binding site of dimeric S100B(ββ), these residues are not highlighted in yellow.

hinge region of CP-10 was found to exhibit chemotactic activity similar to that of full-length protein (Lackmann et al., 1993), mutation of residues in the C-terminus of p11 was found to abolish annexin I binding (Kube et al., 1992), chemical modification and mutagenesis of C84 in S100B($\beta\beta$) were found to decrease binding to τ -protein (Baudier & Cole, 1988) and aldolase (Landar et al., 1997), a peptide derived from the hinge region (L32–L40) of S100B($\beta\beta$) has been shown to block the S100B($\beta\beta$) activation of guanylate cyclase and inhibition of p80 phosphorylation, while a peptide derived from the C-terminal region of S100B($\beta\beta$) blocks guanylate cyclase and stimulates S100B($\beta\beta$) in inhibiting p80 phosphorylation (Pozdnyakov et al., 1998). Recently, binding studies of point and deletion mutants of another S100 protein, S100A1, to phenyl-Sepharose and CapZ peptide indicate that S100A1 lacking several C-terminal residues (C85–S93) fails to bind phenyl-Sepharose and exhibits a significantly reduced Ca^{2+} -dependent interaction with the CapZ peptide (Landar et al., 1998). Furthermore, another truncated S100A1 protein lacking several residues in the C-terminus (F88–S93) is not able to inhibit GFAP assembly; also, this truncated S100A1 mutant is less able to complex with GFAP and does not complex to tubulin (Garbuglia et al., 1999). Thus, our conclusion regarding the Ca^{2+} -dependent interaction of the p53 peptide with residues in the hinge and C-terminus of S100B($\beta\beta$) is consistent with previous studies of S100B and other S100 proteins.

Materials and methods

Materials

All chemical reagents were ACS grade or higher and purchased from Sigma (St. Louis, Missouri) unless otherwise indicated. All buffers were passed through Chelex-100 resin (Bio-Rad, Hercules, California) to remove trace metals. Perdeuterated Tris, d_{11} -Tris (1 M solution in D_2O , >98.7 atom percent deuterium) was purchased from C/D/N Isotopes, Inc. (Vandreuil, Quebec), D_2O (100.0 atom percent deuterium, low in paramagnetic impurities) was purchased from Aldrich Chemical Company (Milwaukee, Wisconsin). $^{15}\text{NH}_4\text{Cl}$ (>99%) and ^{13}C -labeled glucose were purchased from Cambridge Isotope Laboratories (Woburn, Massachusetts).

Preparation of S100B($\beta\beta$) and p53-derived peptide

Recombinant S100B($\beta\beta$) was prepared as previously described (Amburgey et al., 1995). Briefly, rat S100B($\beta\beta$) was overexpressed in *Escherichia coli* strain HMS174(DE3) transformed with an expression plasmid containing a cDNA encoding S100B($\beta\beta$) gene. Unlabeled, ^{15}N -labeled, and ^{13}C , ^{15}N -labeled S100B($\beta\beta$) were purified (>99%) under reducing condition using procedures similar to those described previously (Amburgey et al., 1995; Drohat et al., 1996), except that 0.5 mM DTT (Gibco BRL, Paisley, United Kingdom) was used as a reducing agent throughout the preparation instead of β -mercaptoethanol. The S100 β subunit concentration was determined using the Bradford assay with a known S100B($\beta\beta$) concentration sample as a standard. The concentration for the S100B($\beta\beta$) standard used in the Bradford assay was determined by amino acid analysis (Analytical Biotechnology Services, Boston, Massachusetts).

A peptide, acetyl-SHLKSKKGQSTSRHKLMFKTE-am, derived from human p53 (residues 367–388) was synthesized using

solid-phase peptide synthesis (Biopolymer Laboratory, University of Maryland School of Medicine at Baltimore, Maryland) with its N- and C-termini acetylated (acetyl-) and amidated (-am), respectively. The p53 peptide was stored as a lyophilized powder and dissolved in 1 mM Tris- d_{11} -HCl, pH 7.6 prior to use. The purity (>99%) of the p53 peptide was determined using HPLC, and its concentration and composition were confirmed by amino acid analysis (Analytical Biotechnology Services). Subsequently, the p53 peptide concentration was determined spectrophotometrically using an extinction coefficient $\epsilon_{258} = 427 \text{ cm}^{-1} \text{ M}^{-1}$ at neutral pH.

NMR spectroscopy

NMR samples contained 3.0–6.2 mM S100 β (subunit concentration), 0.1 mM EDTA, 0.34 mM NaN_3 , 5 mM DTT, 17 mM NaCl, 10 mM d_{11} -Tris-HCl, 10% D_2O , 6.3–13 mM CaCl_2 , pH 6.5. The p53 peptide was lyophilized and redissolved as a stock solution (60.5 mM) in 1 mM d_{11} -Tris-HCl. The pH of the stock solution was adjusted to 6.5 with cold dilute HCl prior to adding it to the NMR sample. The p53 peptide was titrated to a final concentration of 4.8–10 mM into Ca^{2+} -loaded S100B($\beta\beta$) samples.

All NMR spectra were acquired at 37 °C with a Bruker (Karlsruhe, Germany) DMX600 NMR spectrometer (600.13 MHz for protons) equipped with four frequency channels and a triple resonance three-axis gradient probe. Unless otherwise stated, a 1 s relaxation delay was used, and quadrature detection in the indirect dimensions was obtained with States-TPPI phase cycling. For most experiments, initial delays in the indirect dimensions were set to give zero- and first-order phase corrections of 90° and –180°, respectively (Marion et al., 1989b; Bax et al., 1991). Data were processed on Silicon Graphics workstations using the processing program nmrPipe (Delaglio et al., 1995). Time-domain data in the indirect dimensions were extended by no more than one-third using standard linear prediction routines (Zhu & Bax, 1992), except for data in constant time domains that were extended twofold using mirror-image linear prediction (Zhu & Bax, 1990). All proton chemical shifts are reported with respect to the H_2O or HDO signal taken as 4.658 ppm relative to external TSP (0.0 ppm) at 37 °C. The ^{13}C and ^{15}N chemical shifts were indirectly referenced using the following ratios of the zero-point frequencies at 37 °C: 0.10132905 for ^{15}N to ^1H and 0.25144953 for ^{13}C to ^1H (Live et al., 1984; Spera & Bax, 1991; Edison et al., 1994).

NMR experiments needed to assign the backbone chemical shifts of Ca^{2+} -loaded S100B($\beta\beta$) bound to p53 peptide have been described previously (Rustandi et al., 1998b). These include 2D ^1H - ^{15}N fast HSQC (Mori et al., 1995), 3D ^{15}N -edited HOHAHA-HSQC (Bax & Davis, 1985; Marion et al., 1989a; Cavanagh & Rance, 1992), 3D HCCH-TOCSY (Kay et al., 1993), 3D CBCA(CO)NH (Grzesiek & Bax, 1992; Muhandiram & Kay, 1994), and 3D HNCACB (Wittekind & Mueller, 1993). Additional 3D experiments include a 3D ^{15}N -edited NOESY-HSQC (Kay et al., 1989) collected using a 100 ms mixing time and an acquired data matrix of $512^* \times 106^* \times 28^*$ (* indicates complex points). A 3D ^{15}N , ^{15}N -edited HMQC-NOESY-HSQC (Ikura et al., 1990) experiment with a $512^* \times 21^* \times 21^*$ matrix was acquired with a 150 ms mixing time. A sensitivity-enhanced 4D ^{13}C , ^{15}N -edited NOESY (Muhandiram et al., 1993) experiment was collected using a 100 ms mixing time and an acquired matrix of $256^* \times 13^* \times 64^* \times 19^*$ data points. A 4D ^{13}C , ^{13}C -edited NOESY (Vuister et al., 1993) spectrum was obtained with a 100 ms mixing time and a raw matrix of $256^* \times 64^* \times 18^* \times 18^*$ data points. Intermolec-

ular NOE correlations from the peptide to S100B($\beta\beta$) were recorded using 2D and 3D isotope filtered NOESY experiments (Vuister et al., 1994; Starich, 1995) with a 200 ms and 150 ms mixing time and data matrices of $512^* \times 316^*$ and $512^* \times 70^* \times 35^*$, respectively. The amide proton exchange experiments were done by lyophilizing the S100B($\beta\beta$)-Ca²⁺-p53 peptide complex from H₂O and redissolving it in D₂O. Immediately following solvation, a series of fast ¹H, ¹⁵N-HSQC experiments with intervals of 0.25, 0.33, 0.67, 1.4, 2.7, 4.7, 8.7, 12.7, 18.7, 24.7 h were collected (Marion et al., 1989a). The measurements of ³J_{NH-H α} coupling constants were obtained from the HNHA experiment as previously described (Vuister & Bax, 1993; Kuboniwa et al., 1994). Uniformly ¹⁵N-labeled S100B($\beta\beta$) in the S100B($\beta\beta$)-Ca²⁺-p53 peptide was used to collect 3D-HNHA spectrum with $512^* \times 60^* \times 80^*$ data points. The ³J_{NH-H α} coupling constants were calculated from

$$I_{\text{cross}}/I_{\text{diagonal}} = -\tan^2(2\pi J\xi)$$

where $\xi = 13.4$ ms and $I_{\text{cross}}/I_{\text{diagonal}}$ is the intensity ratio of cross-peak to diagonal peak. The relaxation effects were taken into account as described in Vuister and Bax (1993).

Supplementary material in Electronic Appendix

Included in supplemental material is a table reporting the chemical shift values for Ca-loaded S100B($\beta\beta$) bound to the p53 peptide.

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