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Impact of calcium binding and thionylation of S100A1 protein on its NMR derived structure and backbone dynamics^{†,‡}

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‡ The coordinates for the human *holo*-S100A1 and *holo*-S100A1-Hcy structures have been deposited in the Protein Data Bank (accession numbers 2LP3 and 2LP2, respectively). The ¹H, ¹³C and ¹⁵N resonance assignments and ¹⁵N magnetic relaxation data have been deposited in the BioMagResBank (accession numbers 18231 and 18230, respectively).

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3 ¹ Abbreviations: CPMG, Carr – Purcell – Meiboom – Gill pulse train; CSI – chemical shift
4 index; CSP – chemical shift perturbation; DSS-d₄, 3-trimethylsilyl-2,2,3,3-
5 tetrauteropropionic acid sodium salt; EDTA, ethylenediaminetetraacetic acid; HSQC,
6 heteronuclear single quantum coherence; HPLC, high performance liquid chromatography;
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12 TRIS-d₁₁, perdeuterated 2-amino-2-(hydroxymethyl)1,3-propanediol.
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Abstract

S100 proteins play a crucial role in multiple important biological processes in vertebrate organisms acting predominantly as calcium signal transmitters. S100A1 is a typical representative of this family of proteins. Upon binding of four Ca^{2+} ions it undergoes a dramatic conformational change, resulting in exposure, in each of its two identical subunits, a large hydrophobic cleft that binds to target proteins. It has been shown that abnormal expression of S100A1 is strongly correlated with a number of severe human diseases: cardiomyopathy and neurodegenerative disorders.

A few years ago we have found that thionylation of Cys 85 - the unique cysteine in two identical S100A1 subunits – leads to a drastic increase of the protein affinity for calcium. We postulated that the protein activated by thionylation becomes a more efficient calcium signal transmitter. Therefore, we decided to undertake, using NMR methods, a comparative study of structure and dynamics of native and thionylated human S100A1 in its *apo* and *holo* states. In this paper we present the results obtained for the both forms of this protein in its *holo* state and compare them with the previously published structure of native *apo* S100.

The main conclusion that we draw from these results is that the increased calcium binding affinity of S100A1 upon thionylation arises, most probably, from rearrangement of the hydrophobic core in its *apo* form.

Introduction

A large number of papers on structure and biological function of S100 proteins can be found in the literature and every year this number increases dramatically. There is a good reason for that: these proteins, acting predominantly as calcium signal transmitters, have been shown to play a crucial role in multiple important biological processes in vertebrate organisms [1].

S100A1 is a typical S100 protein. It is a homodimer composed of noncovalently bound, antiparallely oriented, subunits. Each of them is build of two so called “EF hand” motives bound together by a short linker. The N-terminal motives contain a 14 residue long calcium binding loop, specific for S100 proteins, flanked by two α -helices (helix I and helix II). The C-terminal ones coordinate Ca^{+2} ions by a “canonical” loop, ubiquitous in calcium binding proteins, formed by 12 amino acid residues situated in between helix III and helix IV.

Upon Ca^{2+} binding the protein undergoes a dramatic conformational change, resulting in exposure, in each of its subunits, a large hydrophobic cleft formed by residues situated in the linker, the C terminus, and helix III [2]. Numerous structural studies indicate that this region is responsible for recognition of S100 target proteins [3].

S100A1 is highly expressed in human heart muscle and brain. It is also found in skeletal muscles and kidney. It has been shown that abnormal expression of this protein is strongly correlated with a number of severe human diseases: cardiomyopathy, and neurodegenerative disorders such as Alzheimer disease [4-7].

A few years ago we have found that thionylation of Cys 85 - the strictly conserved, unique cysteine residue of S100A1 subunits by small thiol molecules such as β -mercaptoethanol, glutathione or cysteine leads to a dramatic increase of the protein affinity

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3 for calcium [8]. It prompted us to formulate the hypothesis that S100A1 can play the role of a
4
5 linker between calcium- and redox-signal pathways.
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7 Protein S-thionylation is the posttranslational modification of cysteine residues by
8
9 forming a mixed disulphide between cysteine thiol group and low molecular mass
10
11 endogenous thiols. At present, a large number of proteins undergoing thionylation has been
12
13 described [9]. Thionylation of proteins has been shown to regulate activities of enzymes,
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15 transcriptional factors, cell surface receptors, and cytoskeletal proteins. It plays an essential
16
17 part in the control of cell-signalling pathways associated with viral infections and with tumour
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19 necrosis factor [10]. The thionylation has been also suggested as a mechanism through which
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21 protein functions can be regulated by the redox status [11].
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25 To elucidate the molecular mechanism of S100A1 activation due to thionylation we
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27 decided to undertake, using NMR methods, a precise comparative study of structure and
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29 dynamics of human S100A1 protein, native and with thionylated Cys 85 residue, in its *apo*
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31 and *holo* states.
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34 Knowledge of static 3D structures of proteins, while extremely important, is not
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36 sufficient to fully understand the function of proteins and their interactions in complexes. It is
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38 believed that intramolecular motions in proteins are one of the most important factors which
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40 determine their basic physico-chemical properties, biological activity, and also interactions
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42 with ligands, receptors or nucleic acids. Magnetic relaxation of ^{15}N amide nuclei allows to
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44 monitor motions of protein backbone within the wide range of time scales. This approach of
45
46 probing dynamics of N–H groups allows characterization of motions over most of protein
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48 backbone [12-13].
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51 The results obtained by us for human *apo* S100A1 have already been published [14].
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53 In the current paper we discuss the structure and dynamics of the *holo* form of this protein and
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55 its mixed disulfide with homocysteine.
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3 The choice of homocysteine can seem rather puzzling: glutathione is by far the most
4 abundant thiol molecule in vertebrate organisms and, therefore, the most likely to form mixed
5 disulfides with proteins. On the other hand, homocysteine is situated at a critical regulatory
6 branch point in sulfur metabolism. It can be remethylated to methionine, an important amino
7 acid in protein synthesis, or converted to cysteine in the transsulfuration pathway [15]. It has
8 been shown that homocysteine has the highest tendency to create disulfide bonds with
9 proteins among such thiols as homocysteine, cysteine and glutathione in HeLa cell cultures.
10 Such S-homocysteinylation impairs the function of many enzymes, structural proteins and
11 receptors disturbing many metabolic functions in the cell [16]. It has been also shown that
12 homocysteine administered orally resulted in the increase of protein-bound homocysteine in
13 plasma with a concurred decrease in protein-bound cysteine, suggesting displacement of
14 bound cysteine [17]. Elevated level of homocysteine is associated with the increased risk of
15 cardiovascular disease [18], cerebrovascular disease, Alzheimer disease, neural tube defects,
16 osteoporosis, renal failure, and diabetes. Homocysteine can form mixed disulfides with
17 plasma proteins, such as albumin, transthyretin, fibronectin or lipoprotein(a) as well as with
18 intracellular proteins like metallothionein or glutathione peroxidase [19]. Therefore,
19 homocysteinylation of S100A1, while not reported yet, might be a biologically important
20 phenomenon: high levels of homocysteine are considered to be risk factors in the same
21 disorders which are linked with abnormal S100A1 expression [20-21].
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45 **Materials and Methods**

46 **Sample preparation**

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49 ^{15}N -labeled and $^{13}\text{C},^{15}\text{N}$ -double labeled S100A1 protein was obtained as previously
50 described [14, 22]. Expression products were isolated using the classical method of
51 ammonium sulfate precipitation [23-24], purified by reversed-phase HPLC on a
52 semipreparative Vydac C18 column, and identified by electrospray ionization mass
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3 spectrometry using a Macromass Q-ToF spectrometer. Two forms of the protein, one with the
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5 sequence strictly corresponding to its gene sequence and another one, with the additional
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7 initiator methionine at the N-terminus, were obtained from HPLC as partly overlapping peaks.
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9 NMR measurements indicated that structural differences between both forms are small and
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11 restricted to the close proximity of the N-terminal Met residue [14, 25]. Therefore, a mixture
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13 of both forms of proteins was used in all experiments. Thionylation of S100A1 protein with
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15 homocysteine (disulfide bond formation between cysteine 85 and homocysteine, S100A1-
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17 Hcy) was performed in 0.2 M TRIS buffer containing 5 M GuHCl, 5 mM EDTA and 5 mM
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19 homocystine disulfide at pH 8.5. The reaction was carried out at room temperature for 50 min.
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21 The modified protein was purified by HPLC and identified by electrospray ionization mass
22
23 spectrometry. NMR samples of 650 μ L volume contained 0.8 - 1.0 mM protein solution
24
25 (monomer concentration) in 90%/10% H₂O/D₂O, 50 mM TRIS-d₁₁, 10 mM CaCl₂, 0.1mM
26
27 NaN₃ and 50 mM NaCl with pH adjusted to 7.2 (uncorrected value). In case of H_C detected
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29 experiments 100% deuterated buffer was used.
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34 **NMR spectroscopy**

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36 All NMR measurements were performed at the temperature carefully adjusted to 37°C
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38 checked by an ethylene glycol reference sample. Time domain data were acquired using the
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40 States-TPPI quadrature detection [26] followed by the sensitivity enhanced detection
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42 introduced by L. E. Kay [27]. 1.6 s recycling delay was used, if not stated otherwise. All
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44 chemical shifts in ¹H NMR spectra were reported with respect to external DSS-d₄. Chemical
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46 shifts of ¹³C and ¹⁵N signals were referenced indirectly using the 0.251449530 and
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48 0.101329118 frequency ratios for ¹³C/¹H and ¹⁵N/¹H, respectively [28]. Experimental data
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50 were processed using the NMRPipe software package [29]. Zero filling and 90° shifted
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52 squared sine-bell filter were applied prior to the Fourier transformation. Processed spectra
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54 were analyzed with both the CARA [30] and the SPARKY [31] software.
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3 The sequence-specific assignments were performed using uniformly ^{13}C , ^{15}N -double
4 labeled samples of *holo*-S100A1 and *holo*-S100A1-Hcy. 3D heteronuclear HNC0 [32],
5 HNCA [33], HN(CO)CA [34], HNCACB [35], CBCA(CO)NH [36], and (HCA)CO(CA)NH
6 [37] spectra were used to obtain assignments of the backbone ^1H , ^{13}C and ^{15}N resonances. The
7 assignments were additionally confirmed by analysis of sequential and medium-range NOE
8 signals in the respective 3D ^{15}N -edited NOESY-HSQC spectra [38]. The aliphatic side chain
9 ^1H and ^{13}C resonances were assigned from the analysis of the ^1H - ^{13}C HSQC, C(CO)NH [39],
10 HBHA(CBCACO)NH [40], HCCH-TOCSY [41], (H)CCH-TOCSY [41], and ^{13}C -edited
11 NOESY-HSQC spectra [42]. The aromatic side chain resonances were assigned from the
12 (HB)CBHD, (HB)CBHE [43], ^1H - ^{13}C HSQC, and ^{13}C -edited NOESY-HSQC spectra
13 recorded with the offset, spectral widths and ^{13}C - ^1H coupling constants tuned to aromatic
14 carbons. Distance constraints were obtained from the ^{15}N -edited NOESY-HSQC spectra and
15 the ^{13}C -edited NOESY-HSQC spectra separately tuned to aliphatic and aromatic carbons.
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32 Longitudinal (R_1) and transverse (R_2) relaxation rates were measured at three magnetic
33 fields: 9.4, 11.7 and 16.4 T, with ^{15}N -labeled sample using the sensitivity enhanced ^1H - ^{15}N
34 HSQC pulse sequence [27] with the option of either R_1 or R_2 measurements of ^{15}N nuclei [44].
35 The R_2 relaxation rate measurements were performed with the CPMG pulse train. A
36 refocusing time of 650 μs was used during the evolution delays. The acquisition parameters
37 for R_1 and R_2 measurements on each spectrometer were identical with the exception of the
38 delay between π (^1H) pulses used for the cross-correlation effect suppression [45]. 5 and 10
39 ms delays were used in the R_1 and R_2 measurements, respectively. Delays between the scans
40 of 1.8 s were employed in both types of experiments. $\{^1\text{H}\}$ - ^{15}N NOEs were measured with
41 the pulse sequence included in the ProteinPACK Varian Inc. (Palo Alto, CA) software at
42 16.4 T for *holo*-S100A1 and at 9.4 and 16.4 T for *holo*-S100A1-Hcy.
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3 Resonance intensities were used in calculating relaxation rates and NOE values.
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5 Experimental errors of relaxation rates were obtained from appropriate elements of the
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7 variance-covariance matrix. Experimental errors in NOE values were evaluated from signal-
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9 to-noise ratios obtained for corresponding signals in spectra with and without NOE [46].
10

11 The ^1H , ^{13}C and ^{15}N resonance assignments and ^{15}N magnetic relaxation data have
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13 been deposited in the BioMagResBank with the accession codes 18231 (*holo*-S100A1) and
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15 18230 (*holo*-S100A1-Hcy).
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18 **Structure calculations**

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20 The NOESY interproton distance constraints were derived from the 3D heteronuclear
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22 ^{15}N - and ^{13}C -edited NOESY-HSQC experiments. The initial structure calculations were
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24 performed with the CYANA 3.0 software [47]. The dimeric interface between the protein
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26 subunits was defined basing on structural similarity to the known structure of rat S100A1
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28 protein [2] (PDB entry 1ZFS). The automatic NOESY assignment procedure [48] provided
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30 1193 intrasubunit distance constraints and 121 intersubunit distance constrains for *holo*-
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32 S100A1 protein. Appropriate numbers for *holo*-S100A1-Hcy were 1240 and 156,
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34 respectively. Stereospecific assignments for 74 (*holo*-S100A1) or 100 (*holo*-S100A1-Hcy)
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36 side chain chiral groups in each subunit were generated by the program GLOMSA [49] that is
37
38 included in the CYANA software. Additional restraints for backbone ϕ and ψ torsion angles
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40 for Ser 2–His 18, Leu 28–Glu 63, and Val 69–Thr 82 segments were predicted from the
41
42 chemical shifts using the PREDITOR server [50]. For the less defined protein regions (like
43
44 Ser 19–Lys 27 and Asn 64–Glu 68 calcium binding loop segments or the C-terminus of helix
45
46 IV starting from Val 83) the dihedral angle constraints were not applied. At first, the structure
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48 calculations were carried out using the XPLOR-NIH 2.26 program [51] with NOEs and
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50 dihedral angles as input constraints assuming the symmetry equivalence between two
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52 subunits. Before the final structure calculations, constraints defined as $r_{\text{HN-O}} = 1.5\text{--}2.5 \text{ \AA}$ and
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3 $r_{N-O} = 2.5\text{--}3.5 \text{ \AA}$ for hydrogen bonds were added based on geometric criteria. If a given
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5 hydrogen bond existed in more than 75% of structures in the ensemble, then it was selected
6
7 for the final refinement. Additional 20 constrains, defined as $r_{Ca^{2+}-O} = 2.33\text{--}2.47 \text{ \AA}$ for
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9 calcium ions coordination, were also included in final refinement. Only oxygen atoms taking
10
11 part directly in calcium coordination were selected (O of Ser 19, Glu 22, Asp 24, Lys 27 and
12
13 OE1, OE2 of Glu 32 for the first binding site, and OD1 of Asp 62, Asn 64, OD1, OD2 of
14
15 Asp 66, O of Glu-68, and OE1, OE2 of Glu 73 for the second binding site), on the basis of the
16
17 representative crystallographic structures of human S100A13 (PDB entry 2EGD) and bovine
18
19 S100B (PDB entry 1MHO). For the purpose of final refinement of *holo*-S100A1-Hcy
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21 structure, the topology of an additional amino acid, homocysteine connected to cysteine by
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23 the disulfide bond, was manually added and included in XPLOR-NIH 2.26 topology file.
24
25 Evaluation of the obtained S100A1 structures quality was done with the PROCHECK-NMR
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27 [52] and the What-If [53] programs.
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31 **Analysis of ^{15}N relaxation data**

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34 Methodology of analyzing ^{15}N relaxation data measured at multiple magnetic fields
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36 has been described in our previous paper [14]. It is based on the extended model-free
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38 approach [54] with spectral density functions combined [55-56] with anisotropic overall
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40 tumbling [57].
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43 Three Euler angles relate the directions of the principal axes of the asymmetric
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45 diffusion tensor to the molecule-fixed coordinate system. Because of the symmetry
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47 requirement one of the principal axes of the diffusion tensor of homodimeric S100A1 protein
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49 (in our case z axis) is collinear with the protein symmetry axis. This condition reduces the
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51 number of the Euler angles from three to one. The coefficients describing orientation of H–N
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53 vectors in the molecule-fixed coordinate system were calculated using the atomic coordinates
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55 of the lowest energy NMR-derived structures. The vibrationally averaged N–H distance was
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3 assumed to be 0.104 nm [58] and chemical shift anisotropy of ^{15}N chemical shift tensor equal
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5 to -170 ppm [59]. The values and ratios of the principal values of inertia tensors are given in
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7 Supporting Information, Table S1 and compared with the *apo* form of S100A1 protein.
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9 Anisotropies of the inertia moment tensors are noticeable and, therefore, anisotropic overall
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11 tumbling may be expected.
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14 The least-squares procedure used a written in-house Fortran routine optimizing the
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16 model parameters consisted of minimization through a grid-search of the target function χ
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18 given by:
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$$20 \quad \chi = \sum_{i=1}^N \sum_{j=1}^M [(P_{ij,exp} - P_{ij,calc})^2 / \sigma_{ij}^2]$$

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24 The sum was over M relaxation parameters for each of N residues, and $P_{ij,calc}$ were the
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26 appropriate relaxation parameters calculated from the assumed model. The σ_{ij} values were the
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28 corresponding standard deviations of experimentally derived $P_{ij,exp}$. The minimization
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30 procedure delivered four global parameters (three diffusion coefficients and one Euler angle)
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32 and N sets of local, residue specific, parameters comprising S_f , S_s , τ_f , τ_s , and R_{ex} . The residues
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34 of both flexible termini were also excluded from the calculation delivering the overall
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36 diffusion parameters. Those unstructured, flexible terminal segments cannot be regarded as a
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38 part of the rigid rotor and description of their motions in terms of a single overall correlation
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40 time does not seem to be appropriate [60-61]. Model parameter uncertainties derived in the
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42 minimization of target function χ were obtained as standard deviations from 200 Monte Carlo
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44 simulations [62].
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50 51 **Results**

52 53 **Sequence-specific resonance assignment**

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3 The 2D ^1H - ^{15}N HSQC spectra of both homodimeric S100A1 proteins displayed
4 relatively good dispersion of amide correlation signals (cf. Fig. 1). In *holo*-S100A1-Hcy cross
5 peaks for all residues could be assigned, except 5 residues placed, respectively, in the N-
6 terminus (Ser 2), the N-binding loop (Gly 23, Asp 24, and Lys 25) and the linker (Lys 49). In
7 *holo*-S100A1 the cross peaks of the same residues and additionally of three other ones
8 (Leu 41, Asn 86 and Asn 92) could not be identified because of line broadening caused, most
9 probably, by exchange processes. The sequence-specific backbone and side-chain
10 assignments were done using 2D and 3D NMR experiments. 91.4% of all resonances in *holo*-
11 S100A1 and 89.8% in *holo*-S100A1-Hcy were assigned. All resonances, except those of
12 histidine rings and NH_2 side chain groups of asparagines and glutamines, have been assigned
13 unequivocally. The $^{13}\text{C}_\beta$ chemical shift of Cys 85 in *holo*-S100A1-Hcy ($\delta\text{C}_\beta=40.6$), as
14 compared with that in *holo*-S100A1 ($\delta\text{C}_\beta=26.5$), clearly shows that the protein was
15 thionylated [63-64] but the resonance assignment for the unlabeled homocysteine was not
16 possible.
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33 34 **Effect of calcium binding on the ^1H - ^{15}N HSQC spectrum of S100A1**

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36 It is well known that saturation with calcium changes significantly many of the
37 chemical shifts in S100 proteins [2]. In human S100A1 prominent examples are those of
38 Glu 63 and Gly 67 occupying the second and sixth position of “canonical” calcium-binding
39 loop. Their cross peaks in the ^1H - ^{15}N HSQC spectrum move, respectively, from
40 118.86 ppm/8.09 ppm and 109.02 ppm/8.22 ppm [14] to 129.19 ppm/8.35 ppm and
41 113.25 ppm/10.27 ppm (this paper) upon calcium binding. Coordination of Ca^{2+} ions by
42 S100A1 is also manifested by more general changes in the chemical shifts of the protein
43 backbone amide nuclei in the ^1H - ^{15}N HSQC spectra upon saturation of the *apo*-protein with
44 calcium ions. The chemical shift perturbation (CSP) upon binding of Ca^{2+} ions was calculated
45 for each residue using the equation: $\text{CSP} = [(\Delta\delta_{\text{HN}})^2 + (0.2 \Delta\delta_{\text{N}})^2]^{1/2}$ where $\Delta\delta_{\text{HN}}$ and $\Delta\delta_{\text{N}}$ are
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3 chemical shift changes in proton and nitrogen dimensions, respectively. CSP values are
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5 shown in Supporting Information, Fig. S1. The largest changes are observed for both binding
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7 loops. Pronounced changes are also visible for the linker - particularly for its first residue,
8
9 Ser 42 - and the C-terminal part of helix IV. On average, CSPs equal to 0.69 ppm for both
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11 pairs: *apo*-S100A1/*holo*-S100A1 and *apo*-S100A1/*holo*-S100A1-Hcy. Both CSP profiles
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13 mapped onto appropriate protein structures are given in Supporting Information, Fig. S2. A
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15 similar calcium-induced chemical shift perturbation profile was observed in the case of
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17 human S100A5 protein [65].
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20 21 **Structures of *holo*-S100A1 and *holo*-S100A1-Hcy**

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23 The three-dimensional solution structures of the human *holo*-S100A1 protein and its
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25 derivative modified by disulfide formation with homocysteine at Cys 85 (*holo*-S100A1-Hcy)
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27 were calculated from NMR-derived constraints (cf. Table 1). 99.2 % (99.6 %) of all residues
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29 are located in the most favored 95.1% (95.9%) or additionally allowed regions 4.1% (3.7%)
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31 of the Ramachandran plot. The statistics for the ensembles of 20 most favorable structures for
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33 both proteins are given in Table 1. In Fig. 2 the ribbon diagrams for the lowest energy
34
35 structures of *holo*-S100A1 (Fig. 2A) and *holo*-S100A1-Hcy (Fig.2B) are shown with color-
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37 encoded EF-hand helices. The atomic coordinates for all 20 structures of each protein have
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39 been deposited in the Protein Data Bank with the accession codes 2LP3 for human *holo*-
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41 S100A1 and 2LP2 for *holo*-S100A-Hcy, respectively.
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46 The structures of both proteins are almost identical, as could be expected from
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48 comparison of their ¹H-¹⁵N HSQC spectra: the average chemical shift perturbation CSP for
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50 the *holo*-S100A1/*holo*-S100A-Hcy pair equals merely 0.04 ppm (cf. Fig. S1C) and it arises
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52 predominantly from local effects of the protein thionylation. Each subunit of *holo*-S100A1
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54 (*holo*-S100A1-Hcy) contains four α -helices formed by residues Glu 3–Gly 20 (Lys 21),
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56 Lys 30–Glu 40, Ala 53–Leu 61 and Phe 71–Glu 91 (Trp 90) and one short antiparallel β -sheet
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3 formed by Lys 27, Leu 28, Val 69, and Asp 70. In the linker region joining two EF-hand
4 domains (residues 41 - 50) of *holo*-S100A1-Hcy the α -helix is formed consisting of Gly 43–
5 Asp 46. Such short helical motif does not appear in *holo*-S100A1 proteins of both, human
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8 (this work) and rat [2] species.
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11 For residues Glu 5–Cys 85 within one subunit of *holo*-S100A1 (*holo*-S100A1-Hcy),
12 the r.m.s.d. for the backbone atoms (N, C α , C') equals $0.86 \pm 0.22 \text{ \AA}$ ($0.44 \pm 0.10 \text{ \AA}$) and for
13 all heavy atoms $1.49 \pm 0.26 \text{ \AA}$ ($0.85 \pm 0.12 \text{ \AA}$), while for both subunits in the dimer the
14 corresponding values are $0.94 \pm 0.22 \text{ \AA}$ ($0.54 \pm 0.14 \text{ \AA}$) and $1.54 \pm 0.27 \text{ \AA}$ ($1.04 \pm 0.15 \text{ \AA}$),
15 respectively. Almost identical r.m.s.d. values obtained for the single subunit and the
16 homodimer in both proteins prove that the interface between subunits is correctly determined
17 and the resulting ensembles of structures represent well defined homodimeric proteins. In
18 both proteins the dimer interface is located mainly between helices I and I', I and IV' and
19 helices IV and IV'. On average 4 – 5 long-range distance restraints have been established from
20 the NOE data for residues involved in the dimer formation.
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33 **Dynamics of *holo*-S100A1 and *holo*-S100A1-Hcy**

34 For *holo*-S100A1 and *holo*-S100A1-Hcy proteins the analysis of relaxation data was
35 performed for 73 and 66 residues, respectively, allowing for the simultaneous determination
36 of four global parameters and corresponding sets of local parameters. The principal values of
37 the overall diffusion tensors are collected in Supporting Information, Table S2. As supposed
38 the overall tumbling is anisotropic with anisotropy $D_A = 2D_z/(D_x+D_y)$ equal to 0.78 ± 0.01 and
39 0.88 ± 0.04 for *holo*-S100A1 and *holo*-S100A1-Hcy, respectively. Anisotropy of the overall
40 tumbling in *apo*-S100A1 is less pronounced and equal to 0.92 ± 0.02 . The averaged isotropic
41 rotational correlation time, $\tau_R = (2D_x+2D_y+2D_z)^{-1}$, equal to $9.50 \pm 0.08 \text{ ns}$ and $9.27 \pm 0.20 \text{ ns}$ for
42 *holo*-S100A1 and *holo*-S100A1-Hcy, respectively, is typical for globular proteins of about 20
43 kDa size and corresponds well to the results obtained for other proteins from the S100 family
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3 [14, 65-68]. Comparison of the *apo* and *holo* forms reveals that overall tumbling of *holo*
4 forms is ca. 10% slower. It should not be surprising in the light of larger molecular inertia
5 moments of *holo* forms (cf. Supporting Information, Table S1).
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10 Out of five residue-specific parameters describing the local mobility of the backbone
11 amide N–H vectors within the frame of the extended model-free approach, $S^2 = S_f^2 S_s^2$ and R_{ex}
12 are of special importance. The former describes restrictions imposed on motions much faster
13 than the overall protein diffusion (the ps time scale) and the latter allows detection of much
14 slower motions, but fast enough to average chemical shifts of the exchanging sites (usually on
15 the μs – ms time scale). Site specific values of those parameters can reveal local dynamics
16 changes due to the thionylation and calcium loading for both studied proteins when compared
17 with corresponding values in *apo*-S100A1 [14]. However, differences in mobility of the
18 protein structural elements (helices, loops) on the ps time scale become much more evident
19 when the weighted means of S^2 values determined for the residues building them are
20 compared.
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25 The S^2 values averaged over structural elements presented in Fig. 3 indicate that the
26 helices are always the most rigid segments of the studied proteins (full profiles of site specific
27 S^2 values are given in Supporting Information, Fig. S3). Rigidity of both binding loops in the
28 *holo* forms is comparable to that of helices. On the other hand, the linker is the most flexible
29 part of the molecule, much more flexible than in the *apo* form. In the *holo* forms residues
30 involved in chemical exchange processes monitored by the R_{ex} parameter (Fig. 4) are
31 predominantly located in the helices I and II and the linker in contrary to the most affected by
32 exchange processes N-terminal binding loop in *apo* form.
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36 Residues of binding loops differ in their dynamic behavior between the *holo* and *apo*
37 forms. In the latter structure, the residues located in the binding loops display intense mobility
38 on the ps time scale comparable to that of the linker. Moreover, they show the exchange terms
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3 being the largest in the N-terminal loop. Therefore, calcium binding imposes restriction on the
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5 mobility of residues comprising binding loops on fast and slow time scales. On the contrary,
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7 the mobility of the linker, on both time scales, and of large parts of helices I and II on slow
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9 time scale becomes more pronounced in the *holo* forms.
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11 The obtained model parameters reproduce well input experimental data. The plot of
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13 calculated vs. experimental R_2/R_1 ratios is given in Supporting Information, Fig. S4.
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16 Discussion

17 Impact of calcium binding on human S100A1 structure and its backbone dynamics

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19 The structural transition in human S100A1 protein induced by calcium binding is
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21 strictly similar to that observed in rat S100A1 [2, 69] and characteristic for the majority of
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23 S100 proteins [65, 70]: helix III changes its orientation by ca. 100° (cf. Table 2 and Fig. 5)
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25 that leads to the creation of a large hydrophobic cleft on the surface of the protein comprising
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27 the residues situated in the linker, helix III, and helix IV. To this region of *holo*-S100A1 the
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29 target proteins bind [71-72].
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34 From studies on rat S100A1 [2, 69] the authors have concluded that elongation of
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36 helix IV at its C-terminus is an additional structural change induced by calcium binding in the
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38 protein. A similar conclusion has been drawn from studies on human S100A5 [65]. In human
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40 *apo*-S100A1 [14] we have found one turn more in helix IV than reported for the rat *apo*
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42 protein [69] but the residues participating in its formation (Asn 87 - Trp 90) are in equilibrium
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44 between helical (3_{10} -helix) and non-helical conformation. In the *holo* forms of S100A1
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46 presented in this paper the equilibrium is shifted toward the α -helical conformation.
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50 As could be expected the striking difference in dynamics of *apo*-S100A1 and *holo*-
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52 S100A1 proteins is the significant restriction of motion of both calcium binding loops. S^2
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54 values averaged over structural motifs are larger for calcium binding loops in *holo*-S100A1
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56 (cf. Fig. 3). In calcium bound form those previously mobile on subnanosecond time scale
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3 structural elements become as rigid as helices. In other words, quite expectedly calcium
4 binding imposes restriction on fast motions of the loops. Additionally, the linker region in
5 *holo*-S100A1 becomes more mobile and thionylation of Cys 85 enhances this effect. The
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7 latter phenomenon can be explained by possible interactions between attached homocysteine
8 and residues within the linker (cf. Fig. 2B). Moreover, the analysis of differences in R_{ex} terms
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10 for those two proteins reveals that calcium binding restricts the mobility of S100 specific
11 calcium binding loop. There is another feature that characterizes studied *holo* protein forms,
12 namely the significant increase of exchange terms in helices I and II in comparison with *apo*
13 form. This effect has not been reported for any of S100 proteins up to now. It points out to the
14 increased mobility on the μ s–ms time scale of secondary structure elements which usually
15 remain rigid. This behavior can be a hallmark of the target binding site as recently reported
16 for the S100A6 complex with a fragment of C-terminal domain of Siah-1 protein [3]. It was
17 observed that complex formation resulted in both, chemical shift perturbation in H/N
18 correlation spectrum and broadening or disappearing of numerous cross peaks corresponding
19 to several regions of S100A6 including most of the residues within helix I. It can also
20 correlate with the increased exchange rates of several amide protons with solvent within
21 helices I and II of human [73] and rat [74] S100B proteins.

41 **Comparison of *holo*-S100A1 structures**

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43 Until now there are three available structures of *holo*-S100A1 proteins: rat (1ZFS) [2]
44 and presented in this paper human (2LP3) and human thionylated with homocysteine (2LP2).
45 All these structures, determined by NMR methods, are very similar. The r.m.s.d. values
46 calculated for backbone atoms of residues Glu 5 - Cys 85 in structure pairs 1ZFS/2LP3,
47 1ZFS/2LP2, and 2LP2/2LP3 equal to 1.92 ± 0.20 Å, 2.24 ± 0.11 Å and 1.63 ± 0.09 Å,
48 respectively. The positions of secondary structure elements as well as mutual orientations of
49 helices (see Table 2) are also very similar in those three structures. The only structural
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3 difference is visible in the linker region (residues Leu 41 - Asp 50). It does not contain any
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5 elements of secondary structure in rat and human *holo*-S100A1 proteins, while in *holo*-
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7 S100A1-Hcy an α -helix segment consisting of Gly 43–Gln 46 has been found. Nevertheless,
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9 we believe that it is simply more populated because in all these proteins the linker region is
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11 very flexible (see Fig. 3). Existence of a short α -helix in the linker region has been found in
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13 all crystallographic structures of calcium loaded S100 proteins [75-77]. Moreover, the
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15 chemical shift index (CSI) values of C α nuclei obtained for all discussed here S100A1
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17 proteins indicate that residues 42-46 (42-45 in rat S100A1) are likely to adopt the α -helical
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19 conformation (cf. Supporting Information, Fig. S5).
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23 The results presented in this paper show that the protein thionylation is not reflected in
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25 any significant changes in the structure or dynamics of its *holo* form. Therefore, the rationale
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27 of increase of the protein affinity for calcium due to the thionylation of Cys 85 should be
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29 searched in conformational changes in its *apo* form.
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33 Therefore, it seems obvious that we should to investigate this problem by thoroughly
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35 analyzing the structure of *apo*-S100A1-Hcy. Unfortunately, the signal dispersion in NMR
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37 spectra of this protein is very poor precluding spectral assignments at the level allowing for
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39 the precise structure determination. Nevertheless, at least partial assignment of a number of
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41 resonances was possible. It allowed us to identify C/H correlations for several aromatic
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43 moieties and calculate CSP values caused either by calcium binding or thionylation of the
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45 protein. The aromatic ^1H - ^{13}C HSQC spectra, which can be regarded as fingerprints of the
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47 hydrophobic core arrangement, are virtually identical for both studied *holo* proteins (S100A1
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49 and S100A1-Hcy) and the *apo* form of S100A1-Hcy, while the corresponding spectrum of
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51 native *apo* S100A1 markedly differs from them. Visual inspection of superpositions of
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53 selected pairs of 2D aromatic ^1H - ^{13}C HSQC spectra (Supporting Information, Fig. S6)
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55 confirms this statement. Selected CSP values of aromatic C/H cross peaks and N ϵ 1/H ϵ 1 side
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3 chain Trp residue are large and similar for *apo*-S100A1/*holo*-S100A1 and *apo*-S100A1/*apo*-
4 S100A1-Hcy pairs. On the other hand, they are much smaller for the *apo*-S100A1-Hcy/*holo*-
5 S100A1-Hcy pair (cf. Supporting Information, Table S3). One can conclude that the
6 rearrangement of hydrophobic side chains of aromatic residues due to the protein thionylation
7 reflected by aromatic C/H correlation spectrum of *apo*-S100A1-Hcy is similar to that
8 resulting from calcium binding to S100A1. This conclusion is in line with our previous
9 finding that Cys 85 thionylation stabilizes C-terminal part of helix IV in S100A1 protein [68]
10 as does the calcium binding.
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20 **Conclusions**

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22 Loading of S100A1 protein with calcium results in stabilization of the C-terminal part
23 of α -helix IV in addition to drastic reorientation of helix III. As could be expected, the
24 calcium binding loops are much more rigid in the *holo* form of the protein.
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29 Quite surprisingly, helices I and II and, in particular, the linker region in the *holo*
30 form, are more flexible than in the *apo* form. It can be of functional importance facilitating
31 molecular recognition of the protein target molecules.
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36 Neither the structure nor the dynamics of *holo*-S100A1 are perceptibly affected by the
37 protein thionylation. That leads to the following important conclusion: the observed increase
38 of S100A1 affinity for calcium upon its Cys 85 thionylation results generally from
39 conformational changes in the *apo* form of the protein which seem to arise from
40 rearrangement of aromatic residues constituting its hydrophobic core.
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52 spectrometers available to us.
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Supporting Information

Detailed information on structure and dynamics of studied proteins contains three tables and six figures. This material is available free of charge via the Internet at <http://pubs.acs.org>

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Table 1. NMR-derived constraints and statistics for human *holo*-S100A1 and *holo*-S100A1-Hcy proteins calculated with XPLOR-NIH 2.26

NOE distance constraints within subunit	1193	1240
Intraresidual & sequential ($ i-j \leq 1$)	713	723
Medium-range ($1 < i-j < 5$)	302	270
Long-range ($ i-j \geq 5$)	178	247
Intersubunit NOE distance constraints per subunit	121	158
Hydrogen bond constraints	29	50
Restraints per residue	14.4	15.6
Restraints for Ca^{2+} ion per subunit	10	10
Torsion angle constraints:		
Backbone (ϕ/ψ)	74/71	67/67
Side chains (χ^1/χ^2)	0/0	0/0
Mean r.m.s.d. from experimental restraints (\pm s.d.)		
NOE (\AA)	0.0126 ± 0.0012	0.0192 ± 0.0013
Dihedral angles (deg)	1.13 ± 0.20	0.59 ± 0.08
r.m.s.d. from idealized covalent geometry (region 1..93) (\pm s.d.)		
Bonds (\AA)	0.0038 ± 0.0003	0.0056 ± 0.0003
Angles (deg)	0.68 ± 0.03	0.84 ± 0.02
Impropers (deg)	0.56 ± 0.03	0.65 ± 0.02
Ramachandran plot (1..93)		
Residues in the most favored regions (%)	95.1	95.9
Residues in additional allowed regions (%)	4.1	3.7
Residues in generously allowed regions (%)	0.6	0.2
Residues in disallowed regions (%)	0.2	0.2
Ramachandran plot (5..85)		
Residues in the most favored regions (%)	94.4	95.7
Residues in additional allowed regions (%)	4.6	4.0

Residues in generously allowed regions (%)	0.7	0.1
Residues in disallowed regions (%)	0.3	0.2
r.m.s.d. to the mean subunit structure		
Ordered backbone atoms (1..93) (Å)	1.06 ± 0.25	0.84 ± 0.20
Ordered heavy atoms (1..93) (Å)	1.70 ± 0.26	1.19 ± 0.17
Ordered backbone atoms (5..85) (Å)	0.86 ± 0.22	0.44 ± 0.10
Ordered heavy atoms (5..85) (Å)	1.49 ± 0.26	0.85 ± 0.12
r.m.s.d. to the mean structure of the whole dimer (both subunits)		
Ordered backbone atoms (1..93) (Å)	1.17 ± 0.29	0.89 ± 0.20
Ordered heavy atoms (1..93) (Å)	1.78 ± 0.29	1.13 ± 0.17
Ordered backbone atoms (5..85) (Å)	0.94 ± 0.22	0.54 ± 0.14
Ordered heavy atoms (5..85) (Å)	1.54 ± 0.27	1.04 ± 0.15
Structure Z-scores		
1st generation packing quality	2.081 ± 0.525	2.088 ± 0.451
2nd generation packing quality	4.573 ± 1.451	3.885 ± 1.278
Ramachandran plot appearance	1.687 ± 0.364	1.954 ± 0.319
χ^1 and χ^2 rotamer normality	2.010 ± 0.438	0.369 ± 0.412
Backbone conformation	0.647 ± 0.504	-0.181 ± 0.200
Equivalent X-ray resolution of Ramachandran plot (1..93) (Å)	1.0	1.0
Equivalent X-ray resolution of Ramachandran plot (5..85) (Å)	1.0	1.0
Equivalent X-ray resolution of χ^1 and χ^2 (1..93) (Å)	1.0/1.0	1.1/1.0

Table 2. Angles (in degrees) between helices I, II, III and IV in human and rat S100A1 structures^a.

Helices	human	rat	human	human
	<i>apo</i> -S100A1 ^b	<i>holo</i> -S100A1 ^c	<i>holo</i> -S100A1 ^d	<i>holo</i> -S100A1-Hcy ^e
I → II	133 ± 1	132 ± 1	132 ± 2	139 ± 1
I → IV	117 ± 1	131 ± 2	119 ± 2	130 ± 1
II → IV	-34 ± 1	-29 ± 1	-26 ± 3	-35 ± 1
I → I'	-144 ± 2	-157 ± 3	-158 ± 2	-154 ± 1
IV → IV'	151 ± 1	152 ± 3	144 ± 6	149 ± 1
III → IV	-167 ± 2	121 ± 2	130 ± 4	112 ± 2

^a Interhelical angles were calculated using interh1x (K. Yap, University of Toronto).

^b Taken from NMR structure (PDB entry 2L0P).

^c Taken from NMR structure (PDB entry 1ZFS).

^d Taken from NMR structure (PDB entry 2LP3), this work.

^e Taken from NMR structure (PDB entry 2LP2), this work.

Sign of the interhelical angle was chosen according to convention proposed in [78].

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8 Figure 1. ^1H - ^{15}N HSQC spectrum of human *holo*-S100A1 protein. The terminal NH_2 groups
9 of Asn and Gln residues are not labeled. One of two cross peaks labeled W90 at 9.621/128.53
10 corresponds to side chain NH group of indol moiety.
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14
15 Figure 2. Ribbon representations of the *holo*-S100A1 (A) and *holo*-S100A1-Hcy - (B)
16 structures. Subsequent helices of EF-hand motifs are colored: helix I - blue, helix II - cyan,
17 helix III - red and helix IV - yellow. Calcium ions are shown as spheres and homocysteine in
18 stick representation.
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25 Figure 3. Weighted means of generalized order parameter values $\langle S^2 \rangle$ with corresponding
26 error bars for amino acid residues in various structural elements of human *holo*-S100A1 and
27 *holo*-S100A1-Hcy proteins compared with corresponding values obtained for *apo*-S100A1
28 [14]. Relaxation data were analyzed assuming fully anisotropic overall tumbling [57] and
29 extended model-free spectral density functions [54].
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37 Figure 4. Data (vertical bars) for exchange terms R_{ex} at 9.4 T with corresponding error bars
38 for human *apo*-S100A1 (A) *holo*-S100A1 (B) and *holo*-S100A1-Hcy (C) proteins vs. residue
39 number. Insignificant R_{ex} values smaller than 0.5 s^{-1} and, therefore, close to their accuracy are
40 shown as hatched bars. The horizontal lines indicate positions of four helices in EF-hand
41 motifs.
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49 Figure 5. Comparison of most representative structures for human *holo*-S100A1 (red) and
50 *apo*-S100A1 (blue) proteins. Calcium ions are represented as yellow spheres.
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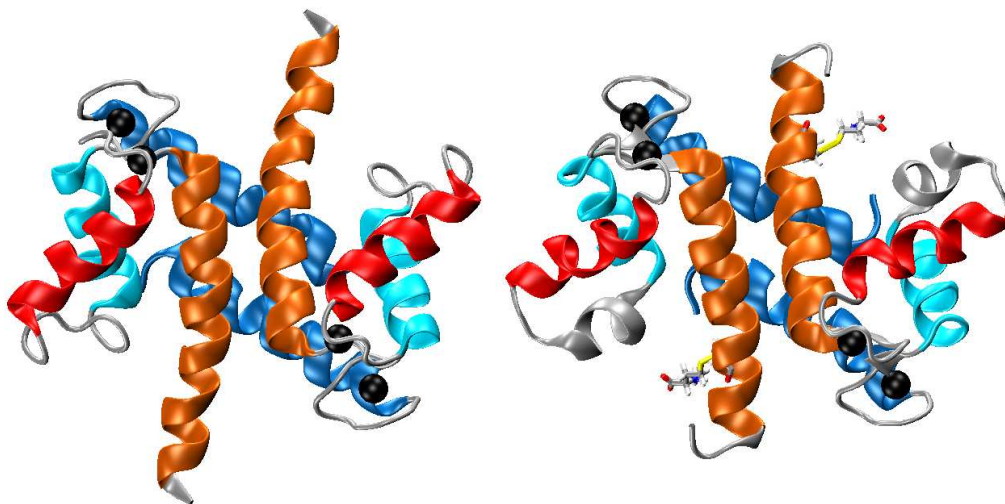


Figure 2

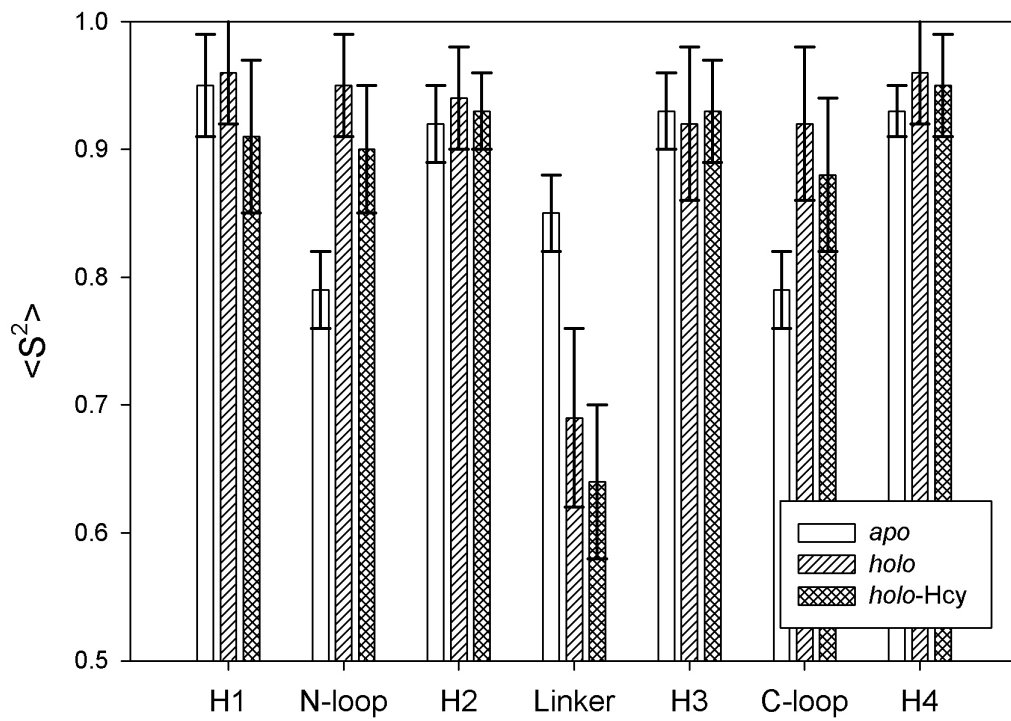


Figure 3

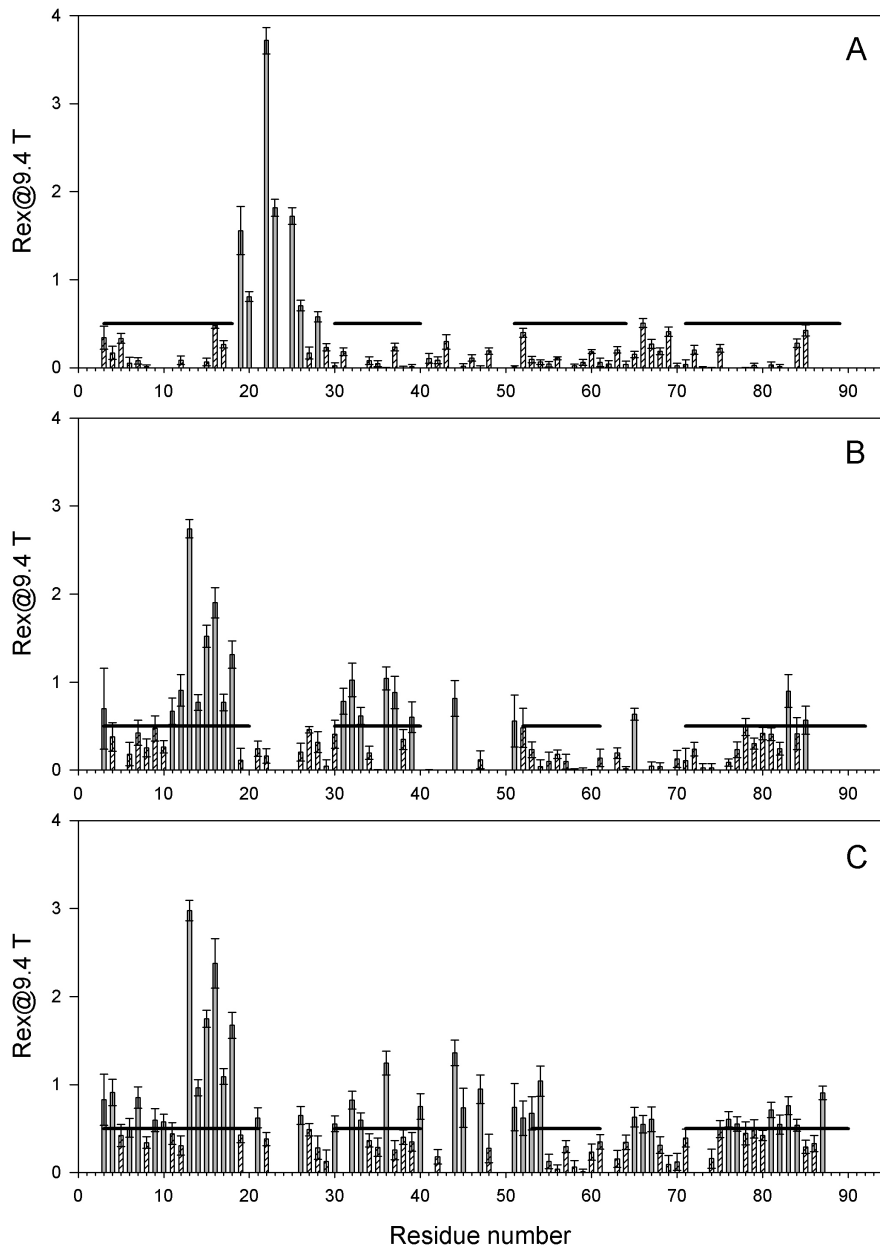


Figure 4

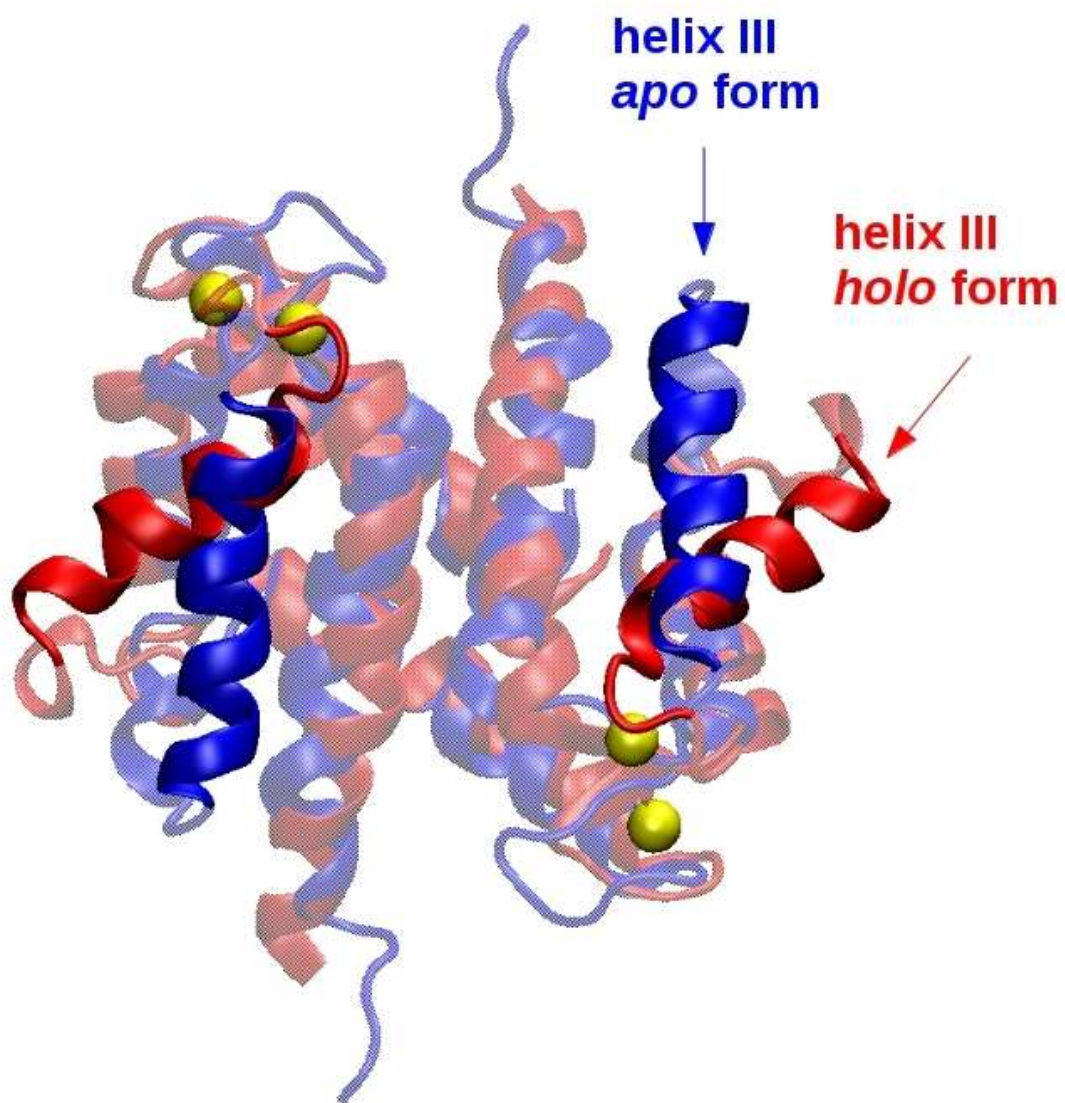
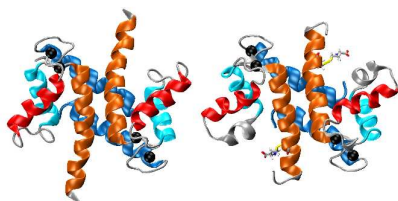


Figure 5

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12 Impact of calcium binding and thionylation of S100A1 protein on its NMR derived
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14 structure and backbone dynamics
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16 Michał Nowakowski, Katarzyna Ruszczyńska-Bartnik, Monika Budzińska, Łukasz
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18 Jaremko, Mariusz Jaremko, Konrad Zdanowski, Andrzej Bierzyński, and Andrzej Ejchart
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