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Cyclic Analogue of Human Heat Shock Protein 70(29–42) Fragment. Synthesis, Conformational Studies and Evaluation of Its Immunogenicity*

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The cyclic hexadecapeptide containing human heat shock protein 70(29–42) fragment cyclized by the disulfide bridge between two L-cysteine residues introduced at the N- and C-termini was synthesized by the solid phase method. It was established that the cyclic analogue, contrary to its linear counterpart, had much lower ability to generate immune response in rabbits. Conformational studies of cyclic peptide performed using 1D and 2D ^{1}H-NMR spectroscopy in conjunction with theoretical conformational analysis revealed that the cyclization constrained the 3D structure of this peptide, reflected by the observed rate of *cis/trans* isomerization of Arg9–Thr10 peptide bond and the presence of Gly7–Asn8 peptide bond in *cis* geometry. We, therefore, postulate that the conformational flexibility in the case of Human Heat Shock Protein fragments is a key element for their immunogenicity.

Key words: solid phase peptide synthesis, cyclic analogue of human heat shock protein70 fragment, immunogenicity, conformational studies, 2D NMR

Several proteins are known as heat or stress inducible proteins [1–3]. All cells in both prokaryotes and eukaryotes react to the temperature increase (heat shock) by synthesizing new proteins named heat shock proteins (hsp). A similar effect is caused by other stresses. Independently, significant amounts of hsp are present in a cell even in the absence of stress. For example, the 70-kDa heat shock proteins (hsp-70) perform a variety of functions in the maintenance of normal cellular metabolism and in the response to stress.

Recently we synthesized the human heat shock protein 70(29–42) fragment [4]. As determined by enzyme-linked immunosorbent assay (ELISA), the synthetic hsp70(29–42) peptide generated relatively strong humoral immune response in immunized rabbits. Antibody titers were comparable with anti hsp70 antibody serum level that was induced by immunization with the recombinant protein (hsp70). It was

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* Abbreviations: The symbols of amino acids and peptides are in accordance with the 1983 Recommendations of the IUPAC–IUB Joint Commission on Biochemical Nomenclature [*European J. Biochem.*, **138,** 9 (1984)] and European Peptide Society [*J. Peptide Sci.*, **5**, 465 (1999)].

established that the polyclonal antibodies directed against hsp70(29–42) peptide could be applied in ELISA for detecting hsp70 in body fluids and tissues [4].

It is frequently reported that the biological activity of cyclic analogues with constrained structure happens to be essentially different than that of the corresponding linear forms [5]. Seeking for analogues with immunogenicity different than hsp70(29–42) fragment, we used the solid-phase method to synthesize a hexadecapeptide containing tetradecapeptide of hsp70(29–42) fragment cyclized by the disulfide bridge between two L-cysteine residues additionally induced at the N- and C-termini:

Cys-Ile-Ala-Asn-Asp-Gln-Gly-Asn-Arg-Thr-Thr-Pro-Ser-Tyr-Val-Cys (**1**)

Immunogenicity of the cyclic peptide and his linear counterpart was investigated in rabbits. Since we established that the cyclic analogue of hsp70(29–42) had difficulties to generate humoral immune response in rabbits, we decided to recognize if the linear hexadecapeptide containing tetradecapeptide fragment of hsp70(29–42) with additional Cys(Acm) residues on the N- and C-termini had the same ability to generate immune response in rabbits as hsp70(29–42) fragment:

Cys(Acm)-Ile-Ala-Asn-Asp-Gln-Gly-Asn-Arg-Thr-Thr-Pro-Ser-Tyr-Val-Cys(Acm) (**2**)

We also decided to determine the solution structure of the cyclic peptide **1**. Such investigation might help in finding structural motives responsible for the immunological activity. Conformational studies were performed using 1D and 2D ^{1}H-NMR spectroscopy in conjunction with theoretical conformational analysis.

EXPERIMENTAL

Chemical part

Starting materials: N^{α}-t-Butoxycarbonyl (Boc) amino acids, trifluoacetic acid (TFA), dicyclohexylcarbodiimide (DCC), N-hydroxybenzotriazole (HOBt), dichloromethane (DCM), triethylamine (TEA), dimethylformamide (DMF), dimethylsulfide (DMS), acetic acid (AcOH), diethyl ether, p-cresol and p-thiocresol were from Fluka AG (Switzerland). Dimethylsulphoxide–d_6 (DMSO-d_6) was from Aldrich (USA). Chloromethylated resin (copolystyrene-1% divinylbenzene, 0.75 mmol of Cl/g of resin) Bio-Beads SX1 was from Bio-Rad Laboratories (USA), Sephadex G-15 was from Pharmacia (Sweden). Methanol and acetonitrile (HPLC grade) were from E. Merck (Germany).

Analytical procedures: Thin layer chromatography (TLC) was carried out on silica plates (Merck, Germany). The spots were visualized by iodine and ninhidrin. The following solvent systems were used: (A) 1-butanol:pyridine:AcOH:water (5:1:4:5, v/v), (B) 1-butanol:ethyl acetate:AcOH:water (1:1:1:1, v/v). Solutions containing 10–50 μg of the sample were applied to the plates and chromatograms were developed to a minimum length of 10 cm. In all cases, single spots were observed in the purified materials. For amino acid analysis, the peptide (approx. 0.5 mg) was hydrolyzed with the constantly boiling hydrochloric acid (400 μl) containing phenol (20 μl) in a sealed glass tube at 110°C for 24 h. The analysis was performed on a Beckman Model 121 analyser. The optical rotation was measured with a Perkin Elmer Model 141 polarimeter with an accuracy of ± 0.01°. HPLC analysis of peptides was performed on a Beckman Gold System chromatograph with the solvent system (A) 0.1% TFA in water, (B) 80% acetonitrile in (A), flow rate 1 ml min^{-1}; A_{254}. The mass spectrum (FAB MS) was measured using an AMD-604 mass spectrometer with BE geometry. The ion source was equipped with the Cs^+ gun of 12 keV energy.

Synthesis of peptides: Peptides were synthesized manually by the solid phase method [6] in the 0.5 mmole scale. Chloromethylated resin was esterified with Boc-Cys(Acm) according to Gisin [7] to a load of 0.3 mmol g^{-1}. The Boc group was used for the protection of α-amino groups throughout the synthesis with the following side chain protecting groups: Cys(Acm), Tyr(OBzl), Asp(OBzl) and Arg(Tos). The synthesis was carried out according to the standard procedures involving: (i) 2 and 30 min deprotection steps using 33% TFA in the presence of anisole (2%) in DCM; (ii) neutralization with 10% TEA/DCM twice for 5 and 10 min respectively; (iii) coupling in DCM/DMF (1:1) performed by the use of 0.5 mmole DCC/DCM in the presence of 1 mmole HOBt for 90 min. The completeness of each coupling reaction was monitored by the ninhydrin test [8]. Recoupling was performed when the test was positive. Finally, peptidyl-resin was treated according to the low-high HF procedure [9]. In the first step, 1 g of the protected peptidyl-resin was reacting with 15 ml of the mixture DMS/p-thiocresol/p-cresol/HF (6.5:0.25:0.75:2.5, v/v) for 2 h at 0°C, then HF and DMS were evaporated *in vacuo*. The reaction vessel was refilled with 15 ml anhydrous, redistilled liquid HF. The cleavage was completed at 0°C after 1 h. After the evaporation of HF *in vacuo*, the resin was washed with 10% AcOH (6 × 15 ml). The combined washings were extracted with diethyl ether (6 × 15 ml), degassed and lyophilized (twice) to obtain the crude peptide. Crude peptide was desalted on a Sephadex G-15 column (1.9 × 160 cm) eluated with aqueous AcOH (10%) with flow rate 15 ml h^{-1}. The eluate was fractionated and monitored for absorbance at 254 nm. The fractions comprising the major peaks were pooled and lyophilized. Linear Acm-hexadecapeptide (**2**) was purified on a semipreparative HPLC LiChrospher 100 RP-8, 10μm particle size column (10 × 250 mm) (gradient 20–50% B, 40 min). Pure linear product (**2**) was obtained with 53% yield. The amino acid composition of the peptide was consistent with expectations. TLC: $R_{FA} = 0.7$, $R_{FB} = 0.4$; HPLC: $R_T = 18.2$ min, $k' = 3.9$; $[\alpha]_D^{20} = -48°$ (c = 1, 10% AcOH); FAB MS (molecular ion): calc. – 1882, found – 1883 ± 1. Part of the linear Acm-peptide (94 mg, 0.05 mmol) was taken to remove acetamidomethyl groups (Acm) with mercury(II) acetate from sulfhydryl groups of L-cysteine residues according to procedure described in [10]. After removal of Acm groups, peptide was oxidized with iodine according to the procedure described in the literature [11]. Solvents were evaporated and the peptide in aqueous solution was lyophilized. Cyclic peptide was desalted on a Sephadex G-15 column (1.9 × 160 cm) eluated with aqueous AcOH (10 %) with flow rate 15 ml·h^{-1}. Fractions containing major peak were pooled, lyophilized and purified on a semipreparative HPLC LiChrospher 100 RP-8, 10 μm particle size column (10 × 250 mm) (gradient 10–55% B 55 min). Pure cyclic product (**1**) was obtained with 10% yield. The amino acid composition of the peptide was consistent with expectations. TLC: $R_{FA} = 0.5$, $R_{FB} = 0.6$; HPLC: $R_T = 17.8$ min, $k' = 3.8$; $[\alpha]_D^{20} = -60°$ (c = 1, 10% AcOH); FAB MS (molecular ion): calc. – 1738.9, found – 1739 ± 1.

NMR experiment: The sample concentration of peptide **1** was approximately 4 mM in 0.5 ml DMSO-d_6. The ^{1}H NMR experiments were performed by means of a Varian Unity 500 Plus (Varian Instrument, USA) spectrometer, operating at 500 MHz. All spectra were recorded at 295 K except for the temperature coefficients of the amide protons chemical shifts, measured in the temperature range 295–318 K. For the 1D experiments, 30272 data points and a spectral width of 8000 Hz were acquired, the zero-filled to 32 K. For the 2D experiments, the time-domain matrices consisted of 2048 × 1024 complex data points with spectral width 5160 Hz for the TOCSY, NOESY and ROESY, and for the DQF-COSY the time-domain matrices consisted of 4096 × 2048 complex data points with spectral width 5160 Hz. Mixing times of 100 msec for TOCSY, 200 msec for ROESY and 300 msec for NOESY were used. All data were processed and analyzed using a VNMR 4.3 software (Varian Instruments, USA) and X-PLOR [12] implemented on a Sun Ultrasparc workstation.

Assignment of ^{1}H NMR signals: The proton resonance assignment was performed by means of TOCSY, DQF-COSY, NOESY, and ROESY experiments. The analysis of the residue spin-coupling correlation systems was straightforward, being performed by the combination of the sequential-specific assignment procedure in the TOCSY and the sequential NOE network along the peptide backbone protons (Table 1).

Temperature dependence of NH protons chemical shifts: The values $\Delta\delta/\Delta T$ were calculated from the 1D spectra recorded at 295, 298, 303, 308, 313 and 318 K.

The NOE effects are generated in both NOESY and ROESY spectra. All NOE cross-peaks for the peptide studied were picked up on the NOESY spectra. For some residues (*e.g.* Arg9, Thr10, Tyr14) double sets of cross-peaks on NOESY and ROESY spectra were observed. In further analysis only signals of major species were considered; 148 and 17 NOE effects were identified for major and minor species, respectively.

The vicinal coupling constants between H_α and NH ($^3J_{HNH\alpha}$) for the peptide were extracted from 1D spectra.

Conformational calculations: To generate the 3D solution structure of the peptide studied, the NMR data (NOE interproton distance constrains and dihedral angles) were used in the structure determination. The standard modules of the X-PLOR programme [12] were used applying *Distance Geometry* (DG) and *Simulating Annealing* (SA) algorithm. The calculations were carried out using the CHARM force field [13] *in vacuo* starting from a random structure. According to the NMR data, the interproton distances were calculated by CALIBA using the distance 1.78 Å for geminal protons for calibration, torsion angles were computed by HABAS, and geometry of the peptide groups (all, except Gly7–Asn8, were found to be in *trans* geometry) were kept fixed. Also the chirality of all C_α atoms was fixed to L and the distance between sulfur atoms was fixed to 2.3 Å. The starting conformation was set to a random form. For the peptide studied, 300 cycles of SA were carried out. Each cycle included 27000 interactions of 80 ps with the 3 ft steps. In the first 50 interactions (1 ps), as well in the last 200 interactions (1 ps), energy minimization with the use of Powell's algorithm [14] was performed. The molecule was heated and annealed at 1000 K for 50 ps. During SA refinement of structure the molecule was cooling slow from 1000 K to 100 K for 30 ps. Finally, 200 energy-minimized starting conformations were obtained.

The sets of the final conformations were clustered (using the minimal-tree algorithm). The root mean square deviation (RMSD) between C_α atoms at optimum superposition was taken as a measure of the dis-

Table 1. ^{1}H chemical shifts, the NH temperature coefficients $\Delta\delta/\Delta T$ and vicinal coupling constants $^3J_{HNH\alpha}$ of peptide **1** in DMSO-d_6 at 295 K.

Residue	Chemical shifts [ppm]						$^3J_{HNH\alpha}$	$\Delta\delta/\Delta T$
	NH	α-CH	β-CH	γ-CH	δ-CH	others	[Hz]	[ppb/K]
Cys	7.91	4.54	2.91 2.67				6.84	–4.02
Ile	7.92	4.16	2.09	1.78 1.95	0.84		7.81	–5.22
Ala	8.37	4.35	1.70				6.84	–3.81
Asn	7.78	4.38	3.83				5.86	–5.18
Asp	8.28	4.77	3.12 2.99				–	–3.69
Gln	8.32	4.50	2.55 2.52	2.72 2.68			7.81	–4.10
Gly	8.01	3.71 3.67					–	–3.86
Asn	8.19	4.52	2.71 2.67				6.84	–3.59
Arg	8.93	4.23	1.76	1.55 1.47	3.07	HN_ε:7.43	7.81	–4.32
Thr	7.64	4.42	3.85	1.08			7.81	–5.10
Thr	7.87	4.90	3.99	1.00			7.81	–2.90
Pro	–	4.35	1.98	1.84 1.88	3.69 3.59		–	–
Ser	7.95	4.25	3.50				–	–2.37
Tyr	8.04	4.54	2.57 2.43				–	–3.67
Val	8.63	4.31	1.70	0.86 0.82			8.79	–1.96
Cys	8.56	4.51	3.21 2.95				8.79	–5.31

tance between conformations, and a cut-off value of 2.1 Å was used to separate the families. Ten low-energy conformations were selected for further analysis.

The β-turns detected in the conformations calculated were defined according to Lewis *et al.* [15].

Immunological part

Immunization of rabbits with derivatives of the synthetic hsp70(29–42) fragment: Sera from two animals were taken before and after immunization with two derivatives of the synthetic hsp70(29–42) fragment, the cyclic analogue of hsp70(29–42) (peptide **1**) and the linear peptide with Cys(Acm) residues on the N- and C-termini (peptide **2**). Each peptide was injected into two rabbits in a following way: one of the animals was immunized with Freund's complete and incomplete adjuvant supplementation, and the second one, without any adjuvants. The number and routes of injections, as well as time intervals between the antigen applications were the same as previously described [4]. A dose of 100 μg of the peptide was applied in each injection. Two weeks after the final antigen application, the rabbits were bled by marginal ear vein incision.

Detection of specific antibodies by enzyme-linked immunosorbent assay (ELISA): The ELISA was performed in the same manner as previously described [4]. Briefly, microtiter plates were coated with the appropriate antigen: recombinant human hsp70 protein {(rhsp70) – StressGen, Canada}, hsp70(29–42) fragment [4], peptide **1** or peptide **2** and kept at 4°C overnight. After washing with phosphate buffered saline (PBS) pH 7.2, blocking with 3% bovine serum albumine (POCh, Poland), and a next wash step, rabbit sera were added in dilutions from 1:10 to 1:20 000. In the present experiment, apart from sera of rabbits immunized with the cyclic peptide **1** and linear peptide **2**, polyclonal antibodies to hsp70 [4] and to linear hsp 70(29–42) fragment [4] were tested. After an overnight incubation at 4°C and further wash with PBS, the plates were incubated with peroxidase conjugated goat anti-rabbit immunoglobulins (DAKO, Denmark). As a substrate for the colourimetric reaction ortho-phenylenediamine (Sigma-Aldich, Germany) was applied and absorbance was measured at 492 nm with a Microelisa Reader (Organon Teknika, Finland). After correction for the background absorbance, the serum dilution was considered positive for specific antibodies if the optical density exceeded 0.15. Results are presented in Table 2.

Table 2. The reactivity of rabbit postimmunization sera with the synthetic hsp 70 (29–42) fragment, its both derivatives (peptide **1** and peptide **2**) and recombinant human hsp 70 protein in enzyme-linked immunosorbent assay (ELISA).

	Antigen used for immunization	Rabbit No.	Antibody titer in ELISA *			
			hsp 70 (29–42) peptide **	Cyclic peptide **1** **	Peptide **2** **	Human hsp 70 recombinant protein**
RABBIT SERA	hsp 70 (29–42) peptide	1	1:2560 [4]	1:160	1:2560	1:640 [4]
	hsp 70 (29–42) peptide with Freund's adjuvant	2	1:1280 [4]	1:240	1:1280	1:640 [4]
	Cyclic peptide **1**	3	neg.	neg.	1:80	neg.
	Cyclic peptide **1** with Freund's adjuvant	4	neg.	neg.	1:360	neg.
	Peptide **2**	5	neg.	neg.	1:5000	neg.
	Peptide **2** with Freund's adjuvant	6	neg.	neg.	1:1280	neg.
	Human hsp 70 recombinant protein with Freund's adjuvant	7	1:640 [4]	1:80	1:480	1:5000 [4]

* mean value of the highest serum dilution with positive reaction calculated from two independet experiments; ** antigen used for detection; neg. – negative reaction; [4] – results from reference [4].

RESULTS AND DISCUSSION

The cyclic hexadecapeptide (**1**) containing human heat shock protein 70(29–42) fragment cyclized by the disulfide bridge between two L-cysteine residues introduced at the N- and C-termini was synthesized by the solid-phase method. Boc/Bzl chemistry was used. Also its linear counterpart, peptide (**2**) containing tetradecapeptide fragment of hsp 70(29–42) with additional Cys(Acm) residues on the N- and C-termini, was obtained. Both peptides were homogeneous as judged by the HPLC analysis and revealed the expected amino acid composition and molecular weights.

The conformation of cyclic peptide (**1**) was studied by 1D and 2D ^{1}H-NMR. The proton shifts, NH temperature coefficients and values of $^{3}J_{HNH\alpha}$ of peptide **1** are summarized in Table 1. In the ROESY spectra several chemical exchange signals were observed. As can be seen in Fig. 1, for the Arg9–Thr10 peptide bond exchange cross-peak of $m\alpha H_i$-$M\alpha H_i$ is present indicating that the Arg9–Thr10 peptide bond is involved in *cis/trans* isomerization. The presence of strong cross-peaks of αH_i–NH_{i+1} in the NOESY and ROESY spectra (figure not shown) for major species and αH_i–αH_{i+1} for minor species for this peptide bond indicates that for major species the geometry of this peptide bond is *trans* and for minor species is *cis*. In the case of the

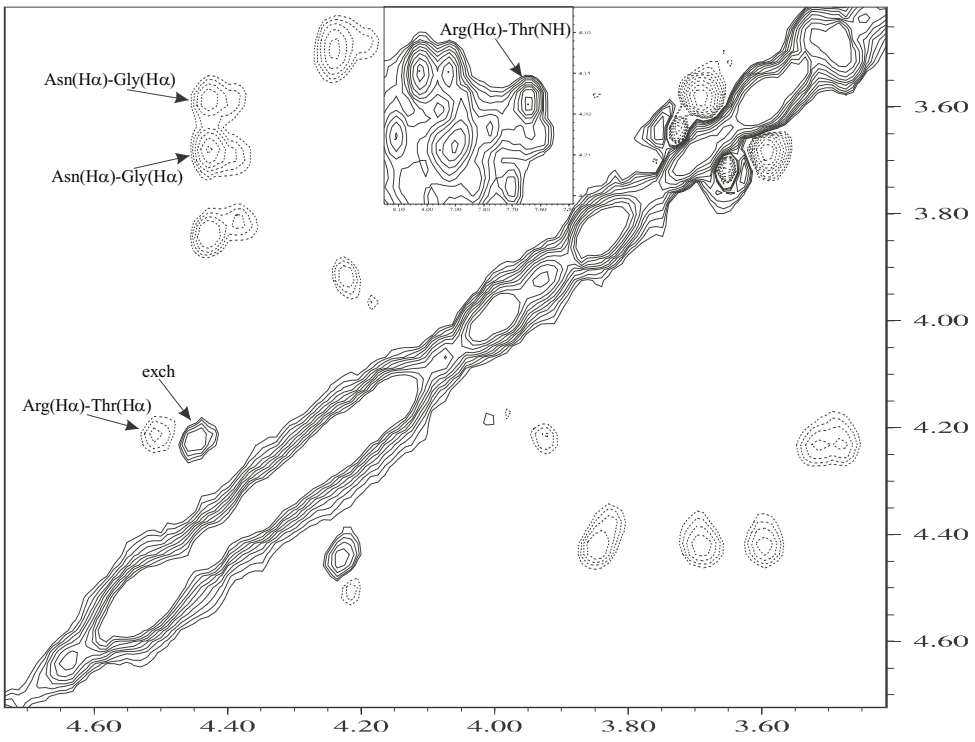

Figure 1. The α–α proton and NH-α regions of the ROESY spectrum of peptide **1** in DMSO-d_6 (exch. – chemical exchange cross-peaks).

Gly7–Asn8 peptide bond only a cross-peak αH_i–αH_{i+1} was found suggesting that this peptide bond adopted *cis* geometry. Fig. 2 presents NOE pattern of peptide **1**. The most pronounced effects were observed for $d_{\alpha N}$ (i, i+1), $d_{N\beta}$ (i, i+1) and $d_{N\beta}$ (i, i) mainly in the region 8–16. This finding suggests that the extended structure is dominant in the peptide. Nevertheless, the final conclusion about the solution structure of peptide **1** is drown based on the calculations using interproton distances and torsion angles as constraints. Fig. 3 shows 10 lowest-energy conformations selected for the peptide studied. They superimpose well in the 6–12 fragment (the calculated RMSD of α-carbon atoms is 0.72 Å). These conformations are stabilized by several β-turns (not shown); the presence of some of them in the C-terminal fragment is additionally confirmed by the values of $\Delta\delta/\Delta T$ obtained for amide protons (*e.g.* Thr 11, Ser13, Val 15).

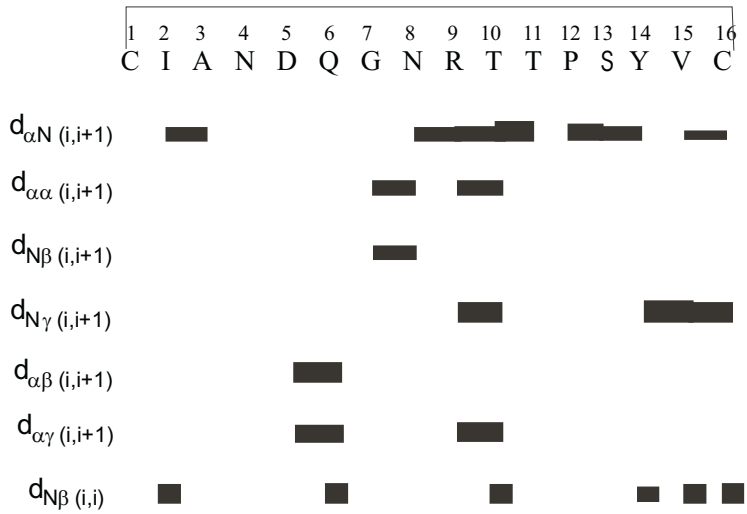

Figure 2. The NOE effects corresponding to the interproton distances observed for peptide **1**.

In the previous work [4], the immunogenicity of the hsp70(29–42) fragment was studied. It was found that the peptide generated relatively strong immune response in immunized rabbits, and that anti-peptide antibodies crossreacted with recombinant human heat shock protein 70. In the current study, the cross-reactivity of the previously obtained anti-hsp70(29–42) and anti-recombinant hsp70 antibodies [4] was estimated with derivatives of the hsp70(29–42): peptide **1** and peptide **2**. While cyclic peptide **1** showed only slight antigenic activity towards the both types of antibodies, peptide **2** was as strongly active as hsp70(29–42) peptide. The sera of rabbits immunized with peptides **1** and **2** were also tested. They did not recognize either human recombinant hsp70 protein or the hsp70(29–42) fragment. All results of immunological investigations are summarized in Table 2.

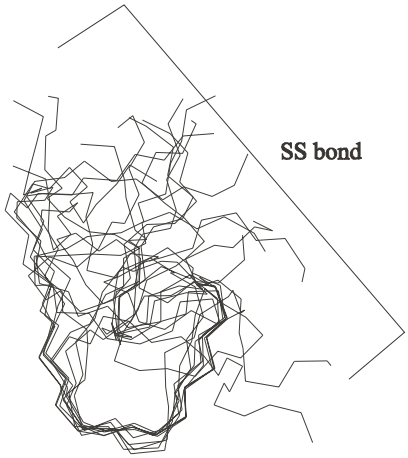

Figure 3. Superposition of low-energy conformations of peptide **1** in fragment 6–12 in DMSO-d_6 (RMSD of Cα atoms is 0.72 Å).

CONCLUSIONS

Despite of our expectation, the global constraint introduced into the hsp 70(29–42) fragment dramatically lowered its immunogenicity. As determined with the ELISA, cyclic peptide **1** was, as opposed to peptide **2**, unable to generate humoral immune response to itself. But antibodies from rabbits immunized with cyclic peptide **1** recognized peptide **2,** but to a much lower extent than did the respective antibodies. This finding suggests the existence of an epitope that is not on the surface of cyclic peptide **1** but becomes accessible to an antibody after decyclization of the peptide *in vivo*. In spite of our expectations arising from our earlier experience [4] the immunogenicity of peptide **1** was significantly lower compared to that of peptide **2** and that of the 29–42 fragment of human heat shock protein 70. The solution conformational studies of cyclic peptide **1** performed in parallel to the biological tests revealed that the disulfide bridge present in peptide **1** influenced the conformational flexibility of the peptide backbone. It is reflected by the observed *cis/trans* isomerization of Arg9–Thr10 peptide bond, and the presence of Gly7–Asn8 peptide bond in *cis* geometry. The conformational studies were not performed on peptide **2**. Based on our experience we postulate that such a liner peptide would not adopt any preferential conformation in solution. Taking into consideration the above, we would like to suggest that in this case conformational freedom of the peptide is the key element for the production of specific antibodies by B cells. It might be a more general statement, as similar conclusion was recently drawn for MHC proteins [16].

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