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Second Conference

"Molecular Bioscience"

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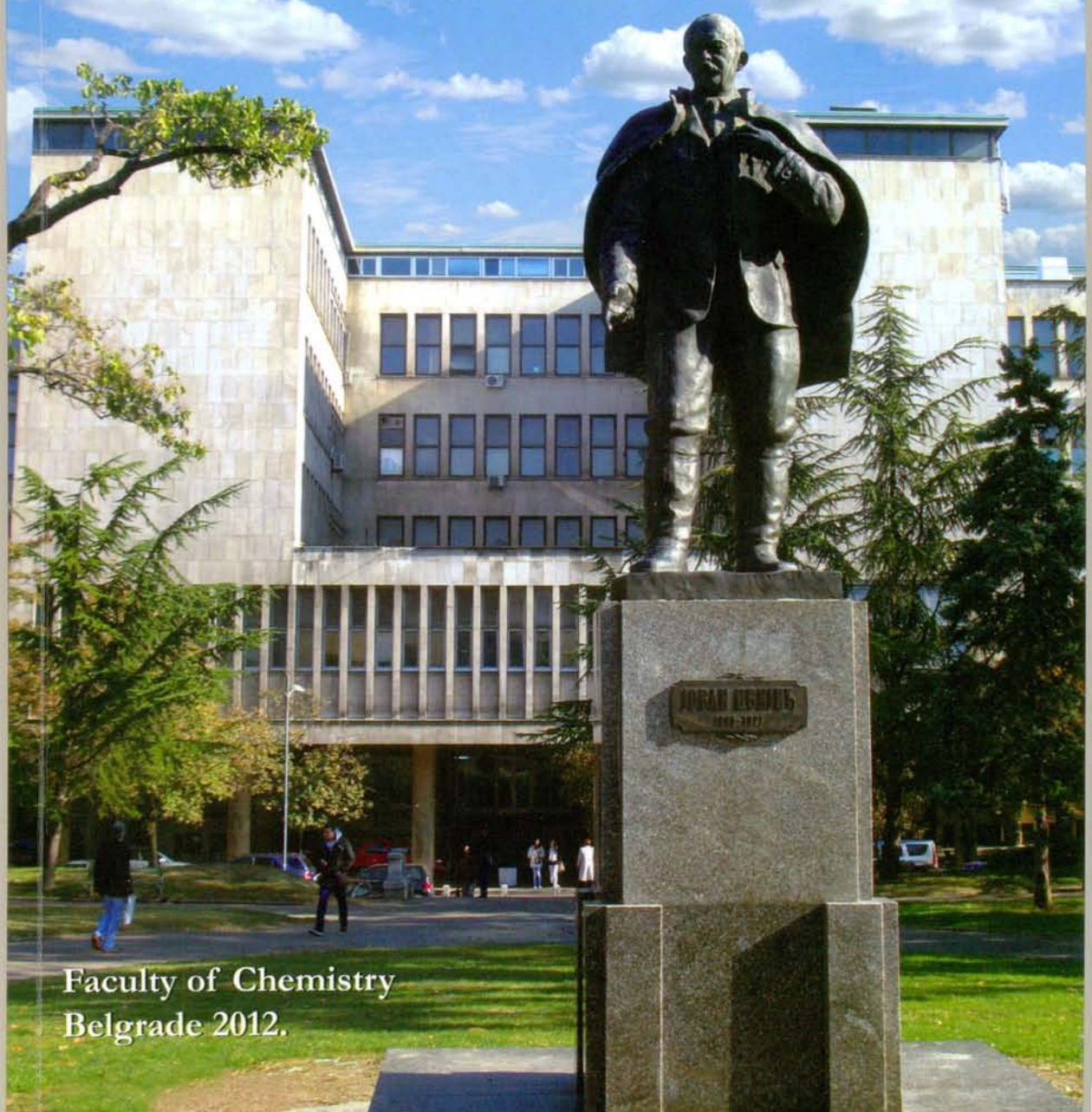

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PROGRAM

10.00-10.10	Welcome messages from: Prof. M. B. Spasić (President of the Serbian Biochemical Society) Prof. B Jovančićević (Dean of the Faculty of Chemistry)
10.10-10.55	*FEBS lecture* Prof. Israel Pecht (FEBS Secretary-General) Department of Immunology, The Weizmann Institute of Science, Rehovot, Israel **The Type 1 Fce Receptor** **A Double -faced Immunoreceptor**
10.55-11.10	Discussion
11.10-11.30	Short break
11.30-11.50	Tanja Ćirković Veličković, PhD Department of Biochemistry, Faculty of Chemistry, University of Belgrade, Belgrade, Serbia. **Protein digestion, immunopathologies and health**
11.50-12.10	Ivanka Karadžić, PhD Department of Chemistry, School of Medicine, University of Belgrade, Belgrade, Serbia. **Topology of proteasomal core particle of *Haloferax volcanii* by chemical cross-linking, mass spectrometry and bioinformatics**
12.10-12.30	Edvard T Petri, PhD Department of Biology and Ecology, University of Novi Sad, Novi Sad, Serbia. **Application of structural biochemistry to the study of mechanisms of ion channel activation**
12.30-12.40	Discussion

Topology of proteasomal core particle of *Haloferax volcanii* by chemical cross-linking, mass spectrometry and bioinformatics

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Proteasomal 20S core particles (CPs) from the haloarchaeon *Haloferax volcanii* are cylindrial barrel-like structures of four-stacked homoheptameric rings of α- and β-type subunits organized in $\alpha_7\beta_7\beta_7\alpha_7$ stoichiometry. Chemically cross-linked peptides of the *H. volcanii* CPs were analyzed by high-performance mass spectrometry and an open modification search strategy. Distance constraints obtained by chemical cross-linking mass spectrometry (CXMS), together with the available structural data of non-halophilic CPs, facilitated the selection of accurate models of *H. volcanii* proteasomal CPs composed of α1 and β-homoheptameric rings from among several different possible PDB structures.

Introduction

Significant progress has been made toward determination of spatial and topological organization of protein and protein complexes by traditional methods such as: nuclear magnetic resonance (NMR) spectroscopy and X-ray crystallography, which provide detailed information on the structure of highly purified proteins, but have limitation when analyzing protein complexes[1,2]. In the post genomic era, large-scale analysis of protein structure by sensitive, high-throughput techniques, such as mass spectrometry (MS), play a significant role. Among the few MS techniques used for structural analysis of proteins, chemical cross-linking coupled with mass spectrometry (CXMS) has emerged as a method that can yield site-specific low resolution structure information on the distance constraints with sample quantities in two to three orders of magnitude less than required for X-ray and NMR and with rapid experimental time[1].

Chemical Cross-Linking Coupled with Mass Spectrometry (CXMS)

The general principle of CXMS is the covalent capture of juxtaposed amino acids using a variety of cross-linking reagents. CXMS can provide intra- and inter-molecular distance constraints that can be used to resolve protein folding in monomeric protein subunits and interactions at molecular interfaces in protein complexes. A CXMS analytical approach has been recently reported that is based on high performance MS to generate tandem mass spectra and the open modification, targeted de novo search strategy to interpret the data[3]. The designed method can be used to map both chemically and naturally occurring cross-links in proteins. Each peptide in the pair of cross-linked peptides is considered to be post-translationally modified by the other peptide in the pair such that each has an unknown mass at an unknown amino acid. This approach allows the use of any available open modification search engine for data analysis. To facilitate identification of cross-linked peptides and to make CXMS data analysis more generally applicable across laboratories, a database processing tool referred to as xComb has been developed[4]. xComb can be used with any standard database search engine and is publicly available.

Cross-Linking Reagents

A large and increasing set of chemical cross-linking reagents has been investigated for use as a molecular ruler to provide information on distances between cross-linked amino acid residues that are relevant to both the tertiary and quaternary structure of proteins. Aside from the large number of described and commercially available cross-linkers that are utilized, only a few organic reactions based on nucleophilicity of the functional groups of proteins are used to attach the cross-linkers[5]. Cross-linking reagents targeting amino groups in proteins are the most common reagents. Based on the reaction of acylation of ε-amino group of lysine, a list of homobifunctional and heterobifunctional cross-linkers containing N-hydroxysuccinimide esters has been synthesized and used in CXMS protocols[5]. Homobifunctional N-hydroxysuccinimide (NHS) esters primarily target the ε-amino group of lysine residues of proteins and can differ in the length of the spacer arm from 6.4 to 11.4 Å. Although the most commonly used NHS esters are often described as reactive exclusively towards primary amines, side reactions with other amino acid residues have also been reported. In particular, NHS reactivity with serine, threonine and tyrosine residues is significant but highly dependent on the pH and on adjacent amino acids within the protein[6]. In contrast to most bifunctional reagents such as NHS, which introduce a bridge between cross-linked residues, the zero length carbodiimide cross-linkers mediate creation of a covalent bond between carboxylate and an amine group without an intervening linker, allowing a direct evaluation of contact interactions between protein surfaces[5].

CXMS and Comparative Modeling

Distance determinations obtained by the CXMS technique can lead to important advances in mapping the protein topography in low resolution structures refined by computational

methods. *Ab initio* and comparative modeling of protein complexes result in a number of possible structures. Although the number of actual proteins is vast, most proteins belong to a limited set of tertiary structural motifs[7,8]. Thus, comparative protein modeling, which uses previously solved structures/templates as starting point, can be very effective in providing a preliminary estimate of protein structure. Distance constraints generated by CXMS can then facilitate the evaluation and/or construction of the modeled protein structure. Even a small number of intermolecular cross-link constraints are sufficient to validate the topology prediction of a protein complex[9,10,11]. Thus, acquisition of cross-linking distance constraints of protein assemblies is a realistic approach to use on a routine basis to improve modeling accuracies[9]. The gap between CXMS and protein structure modeling has been addressed by a new software platform called MSX-3D, which facilitates validation of a theoretical models based on CXMS data[12].

20S Proteolytic Core Particles (CPs) from the Haloarchaeon *Haloferax volcanii*

Archaea are one of three major evolutionary lineages of life. Recent advances in the genome sequencing of halophilic archaea (or haloarchaea) have made this group of microbes ideal for providing insight into cell physiology[13]. Haloarchaea have developed into model organisms that are used to study many biological processes, including the function of the ubiquitin-proteasome system[25]. Proteasomes from archaea are highly related to those of eukaryotes. *Haloferax volcanii* is the only archaeon that has been demonstrated to synthesize three different proteasomal proteins: α1, α2 and β [encoded by *psmA* (HVO_1091), *psmC* (HVO_2923) and *psmB* (HVO_1562), respectively] that are classified in the 20S proteasome superfamily[14].

Proteasomes are self-compartmentalized nanomachines, composed of triple A-ATPases (Rpts) and 20S core particles (CPs) that are important in proteolysis and often essential for growth[13]. The proteasomal CP is a cylindrical complex of four-stacked heptameric rings of α- and β-type subunits organized in $\alpha_7\beta_7\beta_7\alpha_7$ stoichiometry. The α-type subunits form the outer rings and the β-type subunits form the two inner rings that harbor the proteolytic active sites[15,16]. As *H. volcanii* synthesizes two different α subunits (α1 and α2), this organism has the potential to make three different CPs: two symmetric (α1ββα1, α2ββα2) and one asymmetric (α1ββα2)[15]. Since the α1 and α2 proteins share only 55.5% identity, significant structural differences in the homoheptameric rings formed by α1 and α2 are predicted[15].

Structural characterization of eukaryotic proteasomes by CXMS has been recently published[16,17]. Although electron micrographs of the proteasomal CPs from *H. volcanii* reveal a four-stacked ring structure with a central channel[14], the structural details for these protein complexes are limited. PDB entries of archaeal proteasomes deposited to date in RCSB protein data bank are: 1j2q of *Archaeoglobus fulgidus*[23], 3h4p of *Methanocaldococcus jannaschii*[26], and 1pma of *Thermoplasma acidophilum*[27]. All deposited archaeal proteasomes are non-halophilic.

Halophilic CP nanomachines by CXMS

Halophilic proteins are quite specific with regard to amino acid composition often having an extremely high content of acidic amino acids and unusual abundance of surface exposed acidic residues[18]. With regard to CXMS investigation of *H. volcanii* CPs, several points must be emphasized. First, haloarchaeal proteins are typically halophilic (salt-loving) comprising a high content of acidic residues that are often surface exposed and a low number of basic amino acid residues (*e.g.*, lysine, a target of many cross-linkers). In addition, the *H. volcanii* CPs do not contain any cysteine residues. All of these factors limit and complicate the investigation of the halophilic CPs by CXMS, since lysine and cysteine residues are often targets of commercial cross-linkers. Furthermore, only a few crystal structures of haloarchaeal proteins and proteasomal CPs (with the latter derived only from non-halophilic archaea or eukarya) have been resolved to date. In spite of these limitations regarding halophilic proteasomes, CXMS coupled with protein modeling, could emerge as a powerful approach to predict the structure of these unusual, acidic proteins.

Taking into account advantages of CXMS to elucidate tertiary and quaternary structure of proteins, a symmetric $\alpha 1\beta\beta\alpha 1$ core particle of *H. volcanii* proteasomes was studied using two commercial chemical cross-linkers. Zero length EDC and homobifunctional amine-specific BS2G were used as cross-linkers to determine the juxtaposed amino acids in the CP and to validate the theoretical 3D protein structures predicted by comparative modeling[24].

Identification of cross-linked peptide

Tandem mass spectra (MS/MS) were analyzed to confirm the identity and to evaluate the type of cross-linked peptides. To identify cross-linked peptides, MS data were acquired and analyzed using the open modification search strategy reported by Singh *et al.*[3]. Briefly, all data were generated at high mass accuracy on an LTQ-Orbitrap mass spectrometer (Thermo Fisher, USA) using data dependent acquisition of tandem mass spectra by collision induced dissociation (CID) of ions $\geq (M+4H)^{4+}$. The resulting database was searched and refined with respect to the used cross-linkers by xComb and uploaded in Phenyx. Tandem mass spectra of cross-linked peptides were annotated using MS2Assign[19]. Strict criterions were applied on MS2 analysis: 1. Fragment ions from both cross-linked peptides had to be assigned; 2. Cross-linked amino acid blocks peptide cleavage by proteases, so peptides that contain one missed cleavage were considered; 3. Fragment ions containing cross-linked sequences of both peptides had to be assigned; 4. High mass accuracy was prioritized. According to the classification of cross-linking products three types of single modification were considered: type 0 was defined as dead-end, type 1 was defined as intrapeptide and type 2 was defined as interpeptide with the two peptides denoted as α and β chains, where α is the longer of the two peptides[19].

Protein structure validation

Theoretically calculated models of α1 and β protein subunits were obtained from the publicly available MODBASE (http://modbase.compbio.ucsf.edu/modbase-cgi/index.cgi. Structures designated as α1 (Model ID: 4de8eea6b134a909401c34d144ce816c) and β (Model ID: b0e4c2ed79c3a4a1971f2bb8e71bd170) were used. To analyze 3D protein models using mass spectrometry data, MSX-3D, version 3.4.23 (http://proteomics-pbil.ibcp.fr/cgi-bin/msXsetup.pl) was used[12]. Pairwise comparison of protein structures was established using DaliLite workbench software (http://www.ebi.ac.uk/Tools/dalilite/index.htmL)[20]. The coordinates of heptameric complexes of α1 and β were obtained by DaliLite software using PDB entries 1j2p, 1ryp, and 1fnt (for α1) and 1ryp, 1j2q, and 1fnt (for β). Coordinates of the PDB structures with high Z scores and a strong match to α1 and β subunits were used to assemble proteins into heptameric ring structures of $\alpha 1_7$ and β_7 and double heptameric rings of $\beta_7\beta_7$ by Vega ZZ molecular modeling software[21].

Identification of juxtaposed amino acids by CXMS and protein structure validation

To analyze the symmetric α1ββα1 CP subtypes of *H. volcanii* by CXMS the following strategy was used: 1. CP complex was treated with the homobifunctional lysine-reactive cross-linker BS2G and the zero length EDC cross-linkers; 2. Tandem mass spectra of the cross-linked CPs were analyzed using the open modification search strategy, which is essentially a targeted de novo search, to confirm the identity and to evaluate the type of cross-linkages[3]; 3. distance constraints obtained by CXMS were used for validation of theoretical models of α1 and β subunits by MSX-3D[12]. In addition to MSX-3D, VEGA ZZ program was used to display, analyze, and manage the three dimensional (3D) structure of the protein complexes[21] and to validate 3D structures of protein complexes of α1, and β subunits obtained by homology modeling according to the PDB templates: 1j2p (α-ring of the proteasome from *Archaeoglobus fulgidus*), 1ryp (20S proteasome from the yeast *Saccharomyces cerevisiae*), and 1fnt (20S proteasome from the yeast *Saccharomyces cerevisiae* in complex with the proteasomes activator PA26 from *Trypanosome brucei*). A stoichiometry of seven α1 and β subunits assembled in homoheptameric rings was considered, and the coordinates of this α- and β- ring were obtained by DaliLite software[20].

Several α1-specific linkages were detected by tandem MS after treatment of the α1ββα1 CP subtype with the homobifunctional lysine-reactive BS2G (α1 K54-K68 and K44-K47 cross-linkage) and zero-length EDC (α1 K54-E67 and K163-E62 cross-linkages). Of all the lysine residues within α1, only the intramolecular distance between K54 and K68 (9.4 Å) was found (Fig. 1d) to accomodate the length of the BS2G cross-linker spacer arm (7.7 Å) and generate an α1-interpeptide linkage. Consistent with this result, the span between reactive side chain atoms is often slightly greater than the length of the cross-linker used to connect them[22].

In accordance with the close proximity of the α1 lysine residues K54 and K68 in space, the α1 peptides in the region cross-linked by BS2G were also cross-linked with zero-length EDC (K54 and E67 were linked within α1-peptides 44-TPEGVVLAADKR-55 and 58-SPLMEPTSVEK-68), with intramolecular distance of 6.9 Å between K54 and E67. The α1 peptide 58-SPLMEPTSVEK-69 was also found connected to a peptide in the central region of α1, 150-LYETDPSGTPYEWKAVSIG-171, with fragment ions consistent with a linkage between K163 and either E62 or E67. While an intramolecular distance of greater than 30 Å between K163 and E62/67 was found, intermolecular cross-links between two α1 subunits were measured to be: 4.8 Å, 6.7 Å, and 9.7 Å, for models based on PDB coordinates of 1j2p, 1fnt, and 1ryp, respectively. The EDC cross-link identified in the heptameric α1-ring between K163 and E62 was optimally modeled using 1j2p (Fig. 1a) as a template and was estimated to have a length of 4.8 Å (Fig. 1b).

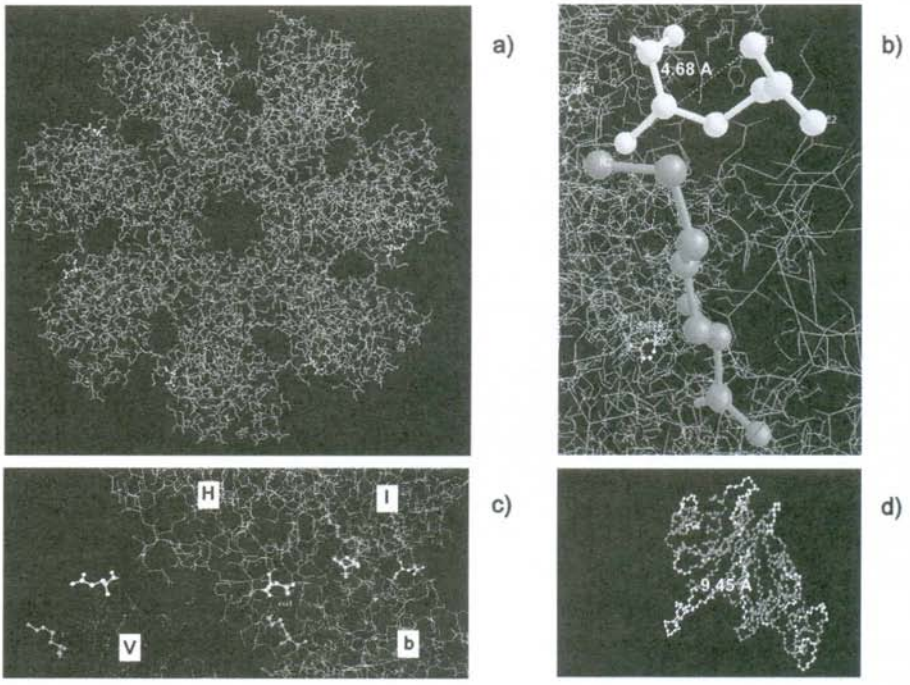

Figure 1. Structural models of the $\alpha 1_7$-ring and $\beta_7\beta_7$-rings of *H. volcanii* 20S CPs. a) Heptameric $\alpha 1_7$-ring modeled according 1j2p PDB template, b) distances between the NZ of K163 and OE1 of E-62 from the two α1 subunits estimated at 4.7 Å, c) the model of the $\beta_7\beta_7$-rings was assigned according to the PDB template 1fnt, as H, I, V, and b. The measured distance between K-78 from the b chain and E-212 from the H chain was 4.64 Å, d) MSX-3D predicts that lysine K54 from TPEGVVLAADKRSR cross-linked with lysine K68 from SPLMEPTSVEKIHK is at a distance of 9.45 Å in the model structure of the α1 subunit.

With BS2G as a cross-linker, four dead-end modifications were detected in α1 tryptic peptides, suggesting that the highly-reactive lysine residues (K68, K71, K54 and K163) are on the surface of α1.

An intrapeptide BS2G cross-linkage was detected between residues K44 and K47 of the β peptide 38-ADELGDKETKTGTTTVGIKTEEGVVLATDMRASMGYMVSSK-78. The distance between β K44 and K47 using BS2G, was measured to be a short intramolecular distance of 6.1 Å by MSX-3D. Between β K78 from 69-ASMGYMVSSKDVQK-82 and β E212 from 209-SAVER-213, cross-linked with EDC the large intramolecular distance of >30 Å was measured. However, the intermolecular distance between the β K78-E212 residues within *two* rings β7β7, was measured to be 4.6 Å in 1fnt (Fig. 1c) and 22.1 Å for 1ryp-based structure. In contrast, distances of 29.4 Å, 16.4 Å, and 26.5 Å were measured between the same residues within *one* ring for 1fnt-, 1j2q-, and 1ryp- based structures, respectively. Thus, the model based on 1fnt appears more accurate for the packing of the $\beta_7\beta_7$-rings than the models based on 1ryp and 1j2q.

Conclusion

The α1ββα1 CP from *H. volcanii* are presumed to be associated as four-stacked heptameric rings in an $\alpha_7\beta_7\beta_7\alpha_7$ symmetry based on analogy to non-halophilic archaeal CPs for which detailed X-ray crystal structures are known. It is demonstrated that CXMS (previously performed only for non-halophilic proteins of relatively neutral pI) coupled with atomic structural data (determined experimentally for non-halophilic protein homologs) provides useful distance constraints for extremely acidic halophilic protein complexes (proteasomal CP from the archaeon *H. volcanii*). Several observed cross-links were used to validate the predicted 3D-structures of the *H. volcanii* proteasomal α- and β -type subunits, and intermolecular distance constraints were used to assemble these proteins (α1, β) into the quaternary structure of the CP complexes. Distance constraints obtained by this CXMS study facilitated selection of an accurate model from several possible PDB proteasomal models and assisted in the determination of the arrangement of the α1- and β- homoheptameric rings in the complex protein structures.

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