The structural plasticity of the human copper chaperone for SOD1: insights from combined size-exclusion chromatographic and solution X-ray scattering studies

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The incorporation of copper into biological macromolecules such as SOD1 (Cu,Zn superoxide dismutase) is essential for the viability of most organisms. However, copper is toxic and therefore the intracellular free copper concentration is kept to an absolute minimum. Several proteins, termed metallochaperones, are charged with the responsibility of delivering copper from membrane transporters to its intracellular destination. The CCS (copper chaperone for SOD1) is the major pathway for SOD1 copper loading. We have determined the first solution structure of hCCS (human CCS) by SAXS (small-angle X-ray scattering) in conjunction with SEC (size-exclusion chromatography). The findings of the present study highlight the importance of this combined on-line chromatographic technology with SAXS, which has allowed us to unambiguously separate the hCCS dimer from other oligomeric and non-physiological aggregated states that would otherwise adversely effect measurements performed on bulk solutions. The present study exposes the dynamic molecular conformation of this multi-domain chaperone in solution. The metal-binding domains known to be responsible for the conveyance of copper to SOD1 can be found in positions that would expedite this movement. Domains I and III of a single hCCS monomer are able to interact and can also move into positions that would facilitate initial copper binding and ultimately transfer to SOD1. Conversely, the interpretation of our solution studies is not compatible with an interaction between these domains and their counterparts in an hCCS dimer. Overall, the results of the present study reveal the plasticity of this multi-domain chaperone in solution and are consistent with an indispensable flexibility necessary for executing its dual functions of metal binding and transfer.

Key words: human copper chaperone, multi-domain protein, size-exclusion chromatography, small-angle X-ray scattering, structural flexibility.

INTRODUCTION

The vast majority of species utilize copper as an essential element in aerobic respiration owing to its high redox potential [1]. Free soluble copper is a source of oxidative stress because it generates ROS (reactive oxygen species) through Fenton chemistry [2]. Consequently, the intracellular free copper concentration must be kept to a minimum. It is known to be ∼10−18 M in yeast cells, which equates to less than one free copper atom per cell [3]. Under these circumstances it is very unlikely that a newly synthesized copper-binding protein will find an amenable copper cofactor without a means of targeting copper to nascent proteins.

Copper uptake and transport into the cell begins with one of the Ctr (copper transporter family) [4]. Copper then passes along an increasing copper-affinity gradient that prevents binding by cytoplasmic low-molecular-mass metal chelators such as metallothioneine and glutathione [5]. hSOD1 [human SOD1 (Cu,Zn superoxide dismutase)] is found in the cytoplasm and the mitochondrial intermembrane space [6,7]. In each of these locations CCS (copper chaperone for SOD1) interacts directly with SOD1 and can load copper into the newly translated protein [8,9]. The hCCS (human CCS) homologue was first discovered as a homologue of a yeast protein [lyS7]yCCS (yeast CCS); indeed, hCCS is 26% identical with yCCS and displays a similar domain architecture [8] (Figure 1).

Domain I of both hCCS (residues 1–85) and yCCS shows considerable homology with the Saccharomyces cerevisiae Atx1 protein. This region contains a conserved MXCXXC motif seen at the N-terminal region of several copper transport proteins [10,11] and is known to bind Cu(I) [12,13]. However, the role of this motif in the transfer of copper to SOD1 remains enigmatic. Replacement of these cysteine residues results in less than a 15% decrease in hCCS activity in vitro [14] and one or both may be substituted in a select few species [15]. Furthermore, domain I as a whole is not necessary for yCCS function, except when copper is strictly limited [16]. This may explain the absence of an intact MXCXXC motif in some species. One could hypothesize that copper availability dictates the presence of this functional motif. Domain I is, however, essential for SOD1 copper loading by hCCS in vivo [17].

hCCS domain II (residues 86–234) exhibits 50% identity with wild-type hSOD1. Crystallographic studies on the yeast homologue indicated that this region harbours the residues involved in formation of both homodimeric yCCS [18] and heterodimeric ySOD1(yeast SOD1)–yCCS [19]. Domain III (residues 235–274) is highly conserved among CCS homologues and was proven to be essential for yCCS function under all conditions, indicating that this region loads copper into nascent SOD1 [16]. On the basis of XAS (X-ray absorption spectroscopy) data, domain III is thought to interact with the MXCXXC motif

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Abbreviations used: CCS, copper chaperone for Cu,Zn superoxide dismutase; Ctr, copper transporter family; DTT, dithiothreitol; hCCS, human CCS; SAXS, small-angle X-ray scattering; SEC, size-exclusion chromatography; SOD1, Cu,Zn superoxide dismutase; hSOD1, human SOD1; TEV, tobacco etch virus; yCCS, yeast CCS; ySOD1, yeast SOD1.
of domain I and this facilitates copper transfer between these two distal and largely independent regions [20].

Although full-length yCCS has been structurally characterized, hCCS is still relatively unknown, other than a 2.75 Å (1 Å = 0.1 nm) resolution crystal structure of the central SOD1-like domain [21] and a solution structure of domain I. Full-length hCCS is described as ‘difficult’ and ‘recalcitrant’ when analysed by crystallization propensity predictors [22,23]. The 60 kDa molecular mass of hCCS is beyond the current upper size limit for structural characterization by solution-state NMR.

SAXS (small-angle X-ray scattering) is the method of choice for studying large flexible biological macromolecules and their complexes in solution. In 2004, Mathew et al. [24] documented a combination of the standard SAXS technology with a HPLC apparatus. This synthesis facilitates the separation of impurities, different oligomeric states and aberrant high-molecular-mass aggregates from the species of interest, by SEC (size-exclusion chromatography), directly before exposure to the X-ray beam. It eliminates any averaging contribution from such species and prevents buffer mismatch between blank and sample through the buffer-exchange effect of SEC [25]. Although this combination of two powerful techniques was demonstrated in 2004, its wider application has not been achieved. The recent installation of a combined SEC–SAXS instrument on the SWING beamline at the French synchrotron SOLEIL [26] has made this technique available to its scientific user community. In the present paper we report the solution structure of hCCS as determined by SEC–SAXS and discuss our findings in the light of the currently received mechanism of copper ion transfer to nascent SOD1.

EXPERIMENTAL

Recombinant hCCS cloning, expression and purification

hCCS-coding DNA was amplified by PCR from an IMAGE clone (gi:85567354). PCR products were ligated into the pETM11 expression vector. hCCS expression was performed in BL21 (DE3) Escherichia coli cells. Cultures were grown at 37°C with agitation until $D_{600} \sim 0.5$, whereupon expression was induced with 0.4 mM IPTG (isopropyl β-D-thiogalactopyranoside). Cultures were then incubated at 30°C overnight with agitation. Harvested cells were resuspended in lysis buffer [20 mM NaH$_2$PO$_4$ (pH 7.4), 500 mM NaCl, 10 mM imidazole, 5 mM DTT (dithiothreitol), 0.2 mg/ml lysozyme, 10 μg/ml DNaseI and 1:1000 protease inhibitor cocktail set III (Calbiochem)] and lysed by sonication on ice. Insoluble cell debris was separated from the soluble fraction by centrifugation at 30000 g for 2 h at 4°C.

His-tagged hCCS was purified using a His-Trap column (GE Healthcare) and then digested with TEV (tobacco etch virus) protease, produced at the University of Liverpool, added to a final concentration of 110 μg/ml and incubated at 4°C overnight. Pure protein was dialysed against storage buffer [50 mM Tris/HCl (pH 8.0), 150 mM NaCl and 5 mM DTT] and concentrated. Protein was subsequently analysed by SEC on a Superdex 200
SAXS data collection, processing and interpretation

SAXS data were collected on the SWING beamline at SOLEIL synchrotron, St Aubin, France, using the HPLC integrated SAXS set-up [26]. Protein (400 μg in 40 μl of storage buffer) was loaded on to a pre-equilibrated Shodex KW402.5-4F 150 kDa SEC column at a flow rate of 300 μl/min at 25°C before passing through the beam which was set to take 250 frames over the course of protein elution. Scattering was recorded on an AVIEX170×170 CCD (charge-coupled device) detector over an angular range $q_{\text{min}} = 0.01$ Å$^{-1}$ to $q_{\text{max}} = 0.5$ Å$^{-1}$. Data averaging and reduction, including preliminary $R_g$ and $I(0)$ calculations, were carried out using the Foxrot suite developed at SOLEIL for the SWING beamline. Further analysis was performed with PRIMUS [27], followed by particle distance distribution function $p(r)$ analysis using GNOM [28]. Ab initio three-dimensional shape reconstruction was then performed without the imposition of symmetry restraints using the GASBOR web server (EMBL, Hamburg) [29]. In total, 30 models were averaged with DAMAVER [30] and the resulting structures were compared with the crystal structure of hCCS domain II in PyMOL (http://www.pymol.org).

The published crystal structure of hCCS domain II [21] was used as the structural model for the hCCS amino acid region 88–232. The secondary structure of region 12–69 was inferred using models of the Atx1-like domain I proposed by the I-TASSER web server [32]. Similarly, the secondary structure of the short $\alpha$-helix found in domain III, amino acids 250–258, was inferred using I-TASSER. Terminal and linker regions 1–11 (with the addition of two extra amino acids remaining from TEV cleavage of the His$_6$ tag), 77–87, 233–249 and 259–274 are predicted to be unstructured. The rigid body modelling software BUNCH [33] was then used to generate 25 hCCS models constrained by the position of the SOD1-like domain II that forms the interface between two CCS monomers. Unrestrained movement of domain I and III together with the flexible linker regions was permitted. BUNCH only generates $\alpha$-atom positions for flexible structural segments such as linkers and peptide terminal ends. The protein backbone and side-chain positions of these flexible regions were reconstructed from their $\alpha$-traces using the SABBAC web server [34]. The scattering profile of each reconstructed and complete model was then computed with CRYSOl [35].

The molecular co-ordinate files generated during the course of the present study are available upon request from the authors.

RESULTS AND DISCUSSION

Characterization of recombinant hCCS and static SAXS measurements

hCCS produced as described has a molecular mass of 29.2 kDa and contains a stoichiometric amount of zinc, but negligible copper, as determined by electrospray ionization MS and inductively coupled ionization MS respectively. When examined by SEC a dominant peak at ~60 kDa is observed, indicating that hCCS is predominantly dimeric. Tetrameric protein that elutes prior to the dimeric form is observed, as previously described by Winkler et al. [36]. Aggregated protein that elutes from the column in the void volume was also observed (Figure 2A). When dimeric protein is separated and stored, these higher molecular-mass species reform and eventually lead to extensive aggregation of the protein (Figure 2A, inset). Standard SAXS experiments on recombinant hCCS at synchrotron beamlines at both SSRL (BL4-2, Stanford, U.S.A. [37]) and SPring-8 (BL45XU, Harima, Japan [38]) yielded incongruence in scattering profiles and deduced structural parameters (Table 1 and Figure 3A), such as substantially increased values for the radius of gyration ($R_g$), indicating a problem with protein monodispersity.

Measurement of hCCS in solution by combined SEC–SAXS

In order to overcome oligomerization in bulk solutions, it was crucial to isolate dimeric hCCS immediately prior to X-ray analysis. Scattering experiments were therefore performed on 16/60 or 10/300 column (GE Healthcare) calibrated with aldolase (158 kDa), BSA (67 kDa) and ovalbumin (43 kDa) (Sigma).

Table 1 Structural parameters with errors derived from SAXS experiments of hCCS collected at three different synchrotron radiation facilities

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SSRL</th>
<th>SPring-8</th>
<th>SOLEIL</th>
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<tbody>
<tr>
<td>$R_g$ (Å)</td>
<td>38.3 ± 0.3</td>
<td>31.4 ± 0.1</td>
<td>38.3 ± 0.3</td>
</tr>
<tr>
<td>$I(0)$</td>
<td>160 ± 7</td>
<td>118 ± 5</td>
<td>160 ± 7</td>
</tr>
</tbody>
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Figure 2 SEC and SAXS measurements of recombinant hCCS

(A) hCCS (17 mg) was loaded on to a calibrated Superdex 200 16/60 gel-filtration column in a 1 ml volume. Inset, aggregated hCCS after extended incubation at 4°C on a Superdex 200 10/300 column. (B) hCCS $R_g$ and $I(0)$ acquired on the SEC–SAXS setup at SWING. Values were determined from scattering data, plotted over a time course of elution of hCCS from the SEC column represented here as frame number of exposure to the X-ray beam. Frames 168–175, used for subsequent analysis, are highlighted in grey. au, arbitrary units.
the SWING beamline at SOLEIL using the SEC–SAXS set-up [26]. This system combines an optimized synchrotron radiation SAXS instrument with HPLC sample delivery. hCCS samples at a concentration of 10 mg/ml were passed along a Shodex KW402.5-4F 150 kDa SEC column before exposure to the X-ray beam.

Scattering data were collected over the course of protein elution with approximately 20 frames taken before protein reached the beam for background subtraction purposes. Figure 2(B) shows information extracted over the course of the SAXS measurements for hCCS with approximate $R_g$ and $I(0)$ values indicating the heterogeneity of the sample. The $I(0)$ value is directly related to protein concentration and reflects the SEC UV-absorption profile. The region with the highest protein concentration and steady $R_g$ values was identified and averaged. Prior to this peak significantly higher $R_g$ values were observed, pointing to the presence of larger molecular-mass species. Subsequent examination yields a $R_g$ value of $31.3 \pm 0.81$ Å and a maximum particle dimension ($D_{max}$) of $118 \pm 5$ Å (Table 1 and Figure 3B, inset), supporting the notion that the hCCS dimer is significantly larger than the crystal structure of the equivalent dimer from yeast [18] that yields $R_g = 25.9$ Å and $D_{max} = 85$ Å. Each monomer in this structure has a predicted molecular mass of 25.8 kDa as opposed to the 29.1 kDa recombinant hCCS used in the present study. This molecular mass difference is a contributor to the $R_g$ and $D_{max}$ value disparity.

Quaternary structure of hCCS and a mechanism of copper transfer to SOD1

Low-resolution three-dimensional models of the hCCS dimer were restored using the ab initio shape reconstruction program GASBOR [29]. These models were used to examine the overall shape and possible domain arrangements of hCCS (Supplementary Figure S1 at http://www.BiochemJ.org/bj/439/bj4390039add.htm). Given the flexible nature of full-length hCCS, however, it is not expected to exist as a static globular macromolecule in solution. hCCS is more likely to exhibit...
conformational plasticity with domains I and III able to move freely with respect to domain II. In order to explore the conformational space occupied by hCCS and the biologically relevant interactions between these domains, we modelled their arrangement and positions using the program BUNCH [33] in conjunction with models of the three hCCS domains (Figure 1A–1C). This provides a more plausible dynamic structural depiction of the hCCS dimer in solution (Figure 4A).

Each different rigid body model generated by this process is consistent with the SAXS data, as indicated by a very good scattering profile fit to the experimental results (Figure 3B). These models collectively, therefore, represent a pool of potential domain arrangements that hCCS could adopt in solution. Figure 4(B) indicates that domain I forms a hemispherical halo around the central SOD1-like domain II and appears to be constrained by the length of the interdomain linker. In contrast, however, domain III does not appear to be constrained as such and forms an extension into the solvent that is free to move. These models highlight the possibility of an interaction between the Cu(I)-binding regions of domains I and III from each monomer as described by Eisses et al. [20]. Conversely, there is no obvious indication of a domain swap between the two hCCS monomers. The possibility of a domain I–I or domain III–III interaction is also unlikely owing to their spatial separation. Figure 4(B) illustrates how the various hCCS conformations, directly observed in the present study for the first time, fit into the presently accepted mechanism of copper transfer from the membrane-bound copper transporter Ctrl through hCCS, and the eventual delivery to SOD1 via an increasing Cu(I) affinity gradient [5].

Domain I is essential for hCCS-mediated incorporation of copper into SOD1 [17]. Reversible copper transfer has been observed between Ctrl and Atx1 [39] and is likely to proceed in a similar fashion between Ctrl and hCCS domain I. The initial stage of copper binding by domain I is illustrated by the model represented in Figure 4(B), where domain I is found to be free, solvent-exposed and receptive. This would permit copper transfer to the Atx1-like domain from the C-terminus of Ctrl (human Ctrl) while hCCS is in the dimer state. As with the preceding steps in this process, initial acquisition seems to occur independently for each hCCS monomer owing to the lack of interaction between opposing domains.

C-terminal domain III is then able to acquire copper from domain I. This is facilitated by a conformation in which the two domains are physically adjacent (Figure 4Bi) [40], that facilitates hCCS homodimer dissociation, enabling realization of the SOD1–CCS heterodimer.

Ultimately copper-loaded domain III is then able to move towards the empty copper site of SOD1 (in Figure 4Biii this is represented by replacement of the SOD1-like domain of the opposing hCCS monomer with a wild-type SOD1 monomer; PDB code 2VOA [41]). It is important to note that these models represent hCCS in solution and were generated directly from scattering data without the need to artificially constrain model building in order to demonstrate domain interactions, as has been documented previously using in silico methods [42].

Although the models described in the present study lend themselves to a demonstration of the feasibility of copper movement between domains, they do not represent the complete spectrum of possible hCCS conformations. It is possible that a mixture of conformations exist that satisfy the experimental data in totality but individually do not. Thus hyper-extended and compact structures may co-exist in solution. Currently there is no substantiated and non-circuitous means to determine the composition of such a conformationally heterogeneous solution for oligomeric proteins; however, this limitation does not detract from the message foregrounded in the present study.

The main pathway for SOD1 activation is by its cognate chaperone CCS [8,9]. The hCCS models delineated by the X-ray solution scattering analysis indicate that hCCS exists in a range of conformations with highly independent domains. The models propose a system whereby domains I and III of one hCCS monomer are able to interact with each other, but not with their dimer counterparts.

The size and flexibility of hCCS has thus far prohibited its structural characterization. The analysis described in the present paper is the first structural characterization of full-length hCCS in solution and would not have been possible without the combination of two complementary methods. It highlights the efficacy of the SAXS-HPLC technique for the analysis of biological macromolecules that exhibit variable oligomeric states or a propensity to aggregation. Having measured recombinant hCCS at several SAXS beamlines we find that those observations obtained by the use of this combined technique remove many of the pitfalls associated with static SAXS experiments.

AUTHOR CONTRIBUTION

Gareth Wright planned the experiments, produced the protein, performed the experiments at SOLEIL, analysed the results and wrote the paper; Günter Grossmann carried out the experiments at SOLEIL, SRL and SPring-8 and performed data analysis, contributed to the writing of the paper and supervised the project; and Samar Hasnain directed the research and contributed to the writing of the paper.

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SUPPLEMENTARY ONLINE DATA

The structural plasticity of the human copper chaperone for SOD1: insights from combined size-exclusion chromatographic and solution X-ray scattering studies

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Figure S1  Ab initio three-dimensional shape restoration of hCCS

(A) Representative ab initio shape model resulting from a single GASBOR calculation without symmetry constraints shown in three perpendicular orientations. This model was used as the reference for averaging all 30 independently restored shapes. (B) Consensus shape of all ab initio non-symmetrical SAXS models of hCCS. For comparison a ribbon model of the crystal structure of hCCS domain II (blue), which closely resembles the SOD1 dimer, is superimposed on the average three-dimensional shape model (red). The model is shown moving through an 180° rotation along the long axis with approximate dimensions added. Domain II forms the interface between hCCS monomers and this model adequately provides volume to accommodate this interaction. In every model used to generate this average there are two regions of mass that are not accounted for by the central domain II. These areas must therefore contain domains I and III. The elongated three-dimensional shape reflects the observation that the characteristic p(r) function has an asymmetric curve shape (i.e. D_{max}/2 is significantly larger than the r-value at peak maximum, see inset to Figure 3B of the main text). Moreover, the ab initio shape reconstruction confirms that the overall conformation of hCCS in solution reveals no apparent symmetry.

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