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In Vitro Reconstitution of the Highly Active and Natively Folded Recombinant Human Superoxide Dismutase 1 Holoenzyme

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SOD1 is an antioxidant enzyme that exists as a highly stable dimer in healthy humans. Each subunit contains an intramolecular disulfide bond and coordinates one zinc and one copper ion. The dimer is destabilized in the absence of the ions and disruption of the disulfide bond, which leads to the formation of small oligomers and subsequently larger insoluble aggregates. An acquired toxic function of destabilized SOD1 is postulated to be associated with amyotrophic lateral sclerosis (ALS), which is a neurodegenerative disease characterized by peripheral and central paralysis and by 3- to 5-year median survival after diagnosis. In this study, we present a protocol for heterologous expression of human SOD1 in *E. coli* and total reconstitution of the holoenzyme, which exhibits the highest reported specific activity (four-fold higher) of recombinant hSOD1. Biophysical characterization confirms the native state of this protein. The presented protocol provides highly active hSOD1 that will benefit *in vitro* investigations of this protein.

Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal adult-onset motor neuron disease affecting both upper and lower motor neurons. Patients suffering from ALS generally die within 5 years of diagnosis because of respiratory failure.[1] ALS predominantly occurs sporadically (sALS) with ~10% of cases appearing within families (fALS), and thus arise more than once in a family lineage and are usually inherited in an autosomal dominant manner.[2] Approximately 20% of the disease causing mutations are located in the gene coding for the enzyme superoxide dismutase 1 (SOD1).[3] Aggregates of human SOD1 have been detected in the spinal cord of mutant-SOD1 fALS patients and in non-SOD1 sALS, leading to the hypothesis that SOD1 misfolding is part of a pathomechanism that is common to all types of ALS.[4]

The native conformation of the metalloenzyme Cu,Zn-SOD1 is a stable homodimer and functions in the conversion of superoxide to peroxide. Posttranslational modification of SOD1 includes formation of an intramolecular disulfide bond, and coordination of a zinc ion in the zinc loop and a copper ion in the active site of each subunit. A consistent pattern of glutathionylation and phosphorylation occurs in close proximity to the dimer interface of endogenous SOD1 derived from human erythrocytes.[5] All posttranslational modifications affect the monomer-dimer equilibrium of SOD1. Metal binding and an intact intramolecular disulfide bond promote dimer formation, whereas glutathionylation facilitates dissociation.[5] Natively folded human SOD1 (hSOD1) is an extremely thermostable protein. Depending on its metal content, melting temperatures up to 83 °C have been reported.[6] Structural integrity and enzymatic activity of bovine SOD is maintained in 8 M urea or in the presence of 4% SDS.[7] Protein destabilization by mutations, oxidation or deprivation of metal ions renders hSOD1 prone to dissociation and oligomerization. The resulting soluble oligomers subsequently form amorphous aggregates or even fibrils.[8] In particular, the small soluble oligomers are hypothesized to exhibit neurotoxic properties in ALS.[9]

Studies have focused on the structure and function of hSOD1 in health and disease for many years. *In vitro* approaches examining the aggregation propensity of hSOD1 and success of therapeutic agents are crucial for the development of therapeutic strategies to treat ALS. Experiments critically rely on the native fold of recombinantly expressed hSOD1. Currently, *in vitro* preparation of hSOD1 produced in *E. coli* often results in incomplete metalation levels, and strategies to improve the

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metal content of recombinant hSOD1 have been proposed.[5,10] Documented specific activities of the heterologous expressed enzyme have not exceeded values of ~5,600 U/mg.[11]

Here, we report a procedure for total reconstitution of recombinant hSOD1 expressed in *E. coli* without using the human copper chaperone for SOD1 (hCCS). The applied reconstitution procedure partly follows established protocols.[10a,12] Notably, the oxidation state, incubation time and temperature during holoenzyme reconstitution were identified as crucial factors for ensuring an optimal metal load. The resulting isolated holoenzyme displayed the highest reported specific activity of recombinant hSOD1.

Results and Discussion

Yields and findings of the hSOD1 purification and reconstitution

Protein expression was performed in *E. coli* BL21 (DE3) (Figure 1A) and yielded about 4 g cell pellet per liter culture. The tagless hSOD1 was purified via ammonium sulfate precipitation and hydrophobic interaction chromatography. Human SOD1 was released from the Source 15PHE column at ~45% buffer B (235–224 mS/cm) (Figure 1B) and fractions containing hSOD1 with a purity of more than 98% were pooled (Figure 1C), supplemented with an equimolar amount of zinc sulfate and stored at –80 °C. The amount of purified hSOD1 obtained per gram wet cell pellet was 1.3 mg. Between 5 and 6 mg purified hSOD1 (2.5–3 mg/mL) were applied to the reconstitution protocol, which gave a final yield of 0.35–2 mg of the reconstituted holoenzyme. The SEC run (Figure 2A) resulted in a considerable loss of protein because oligomeric and monomeric species of hSOD1 are separated from the dimeric protein and could not be used in further experiments. In addition, during renaturation and copper loading a constant loss of hSOD1 was observed, which might arise from adhesion of the protein to the dialysis membrane or to the test tubes.

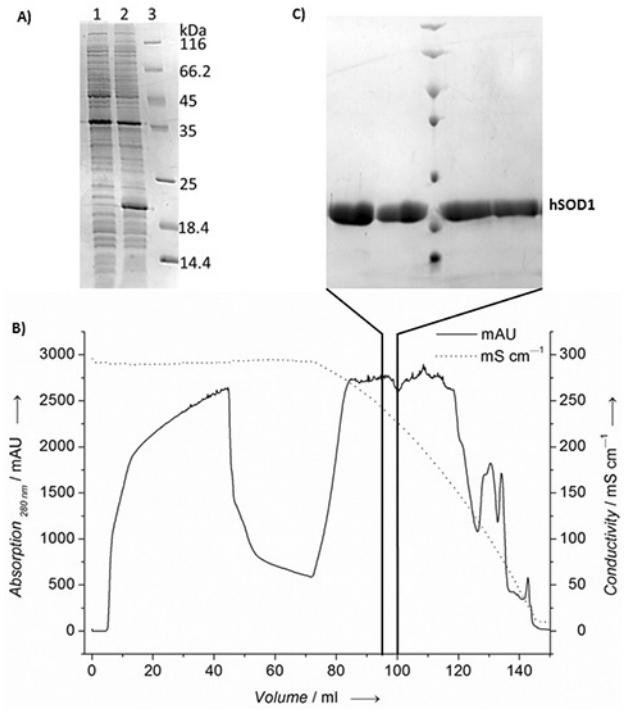

Figure 1. Expression and purification of hSOD1. A: Heterologous expression of tagless hSOD1 in *E. coli* BL21 (DE3) before (lane 1) and after induction with 1 mM IPTG and incubation overnight at 37 °C (lane 2). The molecular weight marker (26610, Thermo Fisher Scientific) is shown in lane 3. Human SOD1 migrates at about 20 kDa. B: Elution profile of the hydrophobic interaction chromatography purification of hSOD1 from the cell lysate after ammonium sulfate precipitation. Human SOD1 elutes at about 230–250 mS/cm. C: SDS PAGE of fractions containing hSOD1 with a purity of more than 98%. Marker bands correspond to Figure 1A.

Holoenzyme reconstitution partly followed established protocols. Notably, reduction of hSOD1 before copper loading was crucial for improved protein renaturation. We found the incubation temperature and time of the copper loading procedure to be important factors for increasing the copper content of hSOD1 significantly. By adjusting the incubation temperature to 37 °C and by extending the incubation time to seven days we were able to reach metal loading states of ~0.7 equivalents Cu as well as ~0.7 equivalents Zn per hSOD1 monomer, as determined by ICP-MS (Table 1).

Table 1. Properties of reconstituted hSOD1 derived from three independent reconstitution events.

	Reconstitution #1	Reconstitution #2	Reconstitution #3
Cu equivalents per hSOD1 monomer	0.70 ± 0.04	0.73 ± 0.05	0.62 ± 0.01
Zn equivalents per hSOD1 monomer	0.86 ± 0.06	0.71 ± 0.05	0.64 ± 0.01
Specific activity [U/mg]	15966 ± 1888	14949 ± 594	11952 ± 1932
Normalized activity [U/mg/Cu]	22808 ± 2697	20478 ± 814	19277 ± 3116
Melting temperature [°C]	86.01	85.20	83.62

Reconstituted recombinant Cu-Zn-hSOD1 is a dimer exhibiting long time stability without the tendency to aggregate

SEC analysis with reconstituted hSOD1 was performed to investigate protein stability. Destabilized hSOD1 dissociates to monomers and these monomeric species are known to aggregate.[13] The elution profile and retention volume of the analytical SEC run indicated a homogenous hSOD1 dimer in the reconstituted sample without the presence of any mono- or oligomeric species (Figure 2B). Repetition of the experiment with a lower protein amount after sample storage for 14 weeks at 4 °C resulted in a very similar elution profile (Figure 3), which provides evidenced of a well-folded holoenzyme that exhibits

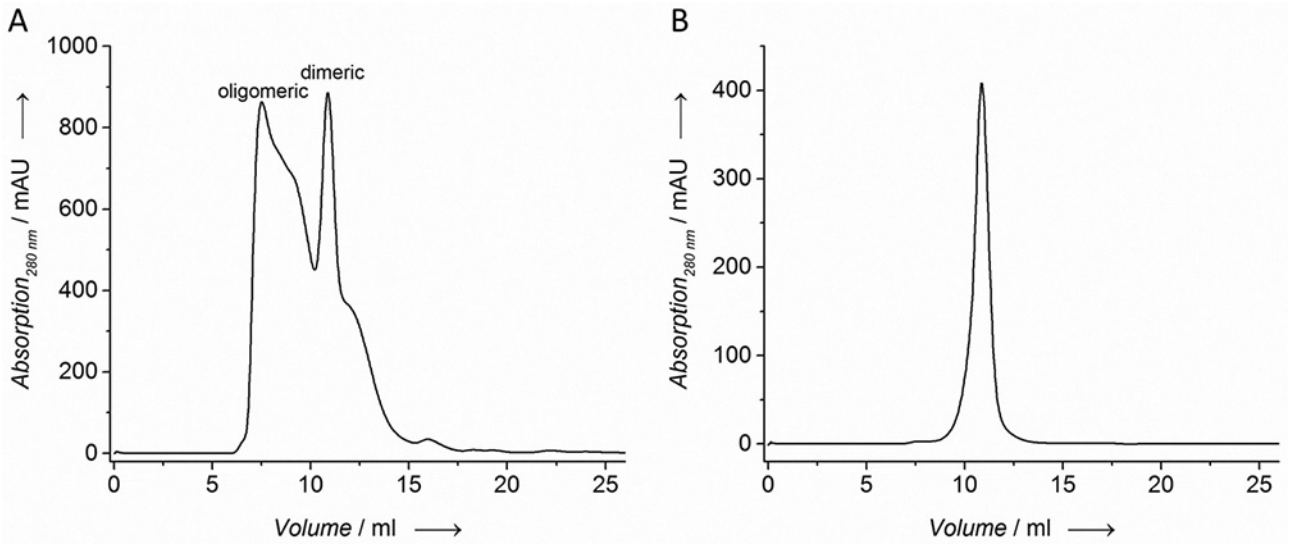

Figure 2. Isolation of the hSOD1 dimer via SEC. A: Reconstituted human SOD1 was loaded onto a superdex 75 10/30 HR column pre-equilibrated with 50 mM sodium acetate, pH 6.0, and a flow rate of 0.25 mL/min. The dimeric enzyme eluted at about 10.8 mL. Fractions containing dimeric hSOD1 (elution volume of 10.5–11.2 mL) were pooled. Oligomeric species (6–10 mL retention volume) and hSOD1 that eluted after 11.2 mL were discarded. B: 80 µL of hSOD1 (237 µM dimer) was applied to a superdex 75 10/30 HR column immediately after reconstitution. A homogenic peak with a retention volume of about 10.8 mL representing the dimeric state of hSOD1 was observed.

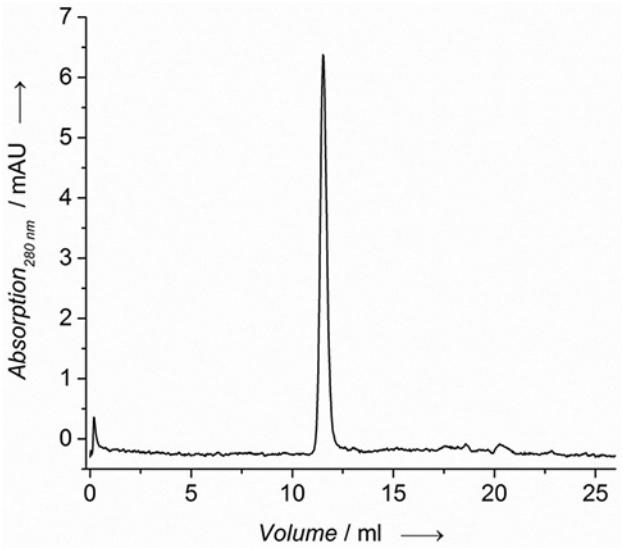

Figure 3. SEC analysis of reconstituted hSOD1. After sample storage for 14 weeks at 4 °C, 70 µL of hSOD1 (3 µM) was applied to a superdex 75 increase 10/300 GL column. A homogenous peak with a retention volume of about 11.5 mL was observed, indicating that the dimeric stoichiometry of hSOD1 showed no tendency to form monomers or oligomeric species.

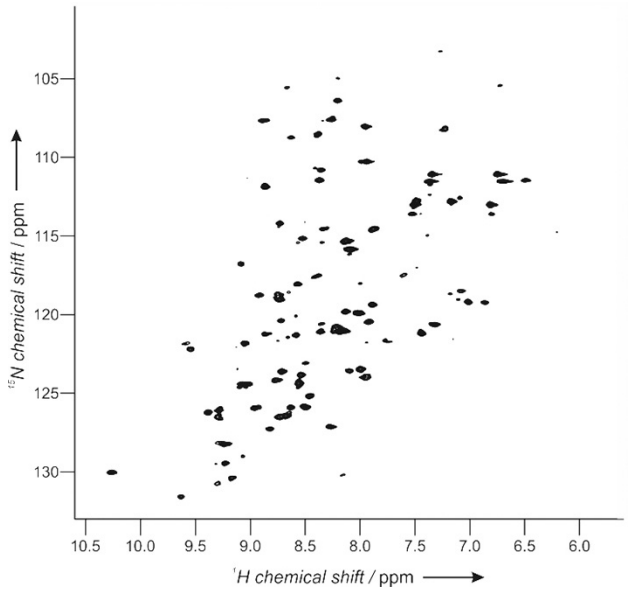

Figure 4. NMR spectrum of hSOD1. 2D ^{1}H-^{15}N-HSQC spectrum of 16 µM [*U*-^{15}N]-hSOD1 recorded at 25 °C.

longtime stability without any tendency to aggregate Structural information about the reconstituted hSOD1 holoenzyme was collected via NMR spectroscopy. The 2D ^{1}H-^{15}N HSQC spectrum of hSOD1 showed a well-dispersed set of signals, indicating a well-folded protein (Figure 4). Several resonance signals showed low signal intensity, possibly due to the presence of the paramagnetic Cu^{2+} in close proximity.

Cu-Zn-hSOD1 displays high dimer stability

Human SOD1 retains its homodimeric integrity, enzymatic activity and metal binding ability in the presence of 1% SDS.[14] We used semi-native SDS-PAGE (0.4% SDS) to investigate the dimer stability of fully reconstituted Cu–Zn-hSOD1^{S-S}. For this purpose we incubated the protein for 30 min with the above mentioned loading buffers at 37 °C. The samples were subjected to a conventional SDS gel and run for 2 h at 80 V. After

staining and destaining, the hSOD1 dimer was detected at about 45 kDa while the monomeric protein was detected at about 20 kDa.[14]

In the absence of DTT and EDTA in the loading buffer, hSOD1 migrated as a single band at about 45 kDa (Figure 5, lane 1), thus indicating the purely dimeric assembly. Addition of 2 mM DTT in the loading buffer did not facilitate dimer dissociation (Figure 5, lane 2), whereas buffer supplement with 20 mM DTT caused considerable dissociation of hSOD1 (Figure 5, lane 3). The dimer destabilizing effect of DTT was further strengthened by addition of 20 mM EDTA (Figure 5, lanes 4–7). This is in accordance to data published for endogenous hSOD1 isolated from human erythrocytes[14] and demonstrated high dimer stability of the recombinantly expressed and reconstituted hSOD1 holoenzyme.

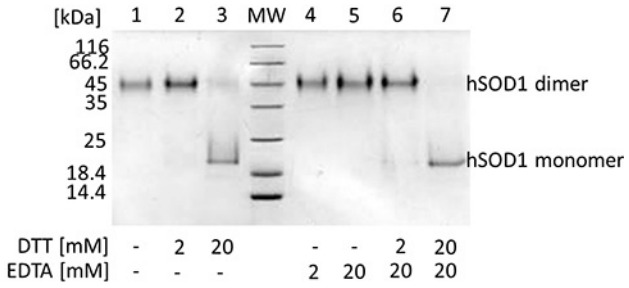

Figure 5. Dimer stability of human SOD1 investigated by partially denaturing SDS-PAGE. Reconstituted hSOD1 was pre-incubated for 30 min at 37 °C in loading buffer consisting of 62 mM Tris, pH 6.8, 10% glycerol, 0.05% bromphenol blue and different amounts of DTT and EDTA as indicated. The protein molecular weight marker was boiled before loading. 2 µg hSOD1 was applied per lane and the 15% gel was run at 80 V for 2 h with the standard gel running buffer. The gel was stained with pre-warmed Coomassie staining solution at RT. After destaining, migration of hSOD1 was documented using a ChemiDoc MP Imaging System (Bio-Rad). Dimeric hSOD1 migrates at ~45 kDa, whereas the monomeric hSOD1 can be detected at ~20 kDa.

Rodrigues et al. determined the melting point of purified hSOD1 by differential scanning calorimetry. Dependent on the protein metal content three melting points of about 60 °C (E_2ZnE SOD1), 72 to 76 °C (E_2Zn_2 SOD1) and 83 °C ($CuEZn_2$ SOD1) were reported.[6a] We performed thermal melting point analysis of reconstituted hSOD1 via circular dichroism (CD) spectroscopy. Melting curves were monitored at 260–190 nm and increasing temperatures (Figure 6A). The resulting T_m of about 86 °C (Figure 6B) indicated a well-folded, metal coordinated protein. Measurements of hSOD1 derived from two independent reconstitution procedures resulted in similar melting temperatures (84 to 85 °C) and are provided in Table 1. Thus, reconstitution of human SOD1 that has been recombinantly expressed in *E. coli* yields protein with high thermal stability, as specified for the hSOD1 holoenzyme.

E. coli produced and reconstituted Cu-Zn-hSOD1 displays the highest specific activity reported for recombinantly produced hSOD1

Specific activity of hSOD1 critically relies on coordination of copper in the active site of the enzyme; however, the specific activity of hSOD1 is commonly reported as units per mg protein without regard to its copper loading state. Specific activities of 3000 to 6000 U/mg have been documented for recombinant hSOD1[11,15] and normalization of specific activity to the copper loading state of hSOD1 has rarely been reported. The maximal theoretical specific activity for the purified protein has been estimated to be between 7000 and 8000 U/mg protein/Cu for wild-type hSOD1.[11] There is a sole publication that determined the specific activity of endogenous hSOD1 assayed in red blood cell lysates to be as high as 23,700 U/mg.[16]

The SOD determination kit (Sigma) was used according to manufacturer's instructions. Human SOD1 from three inde-

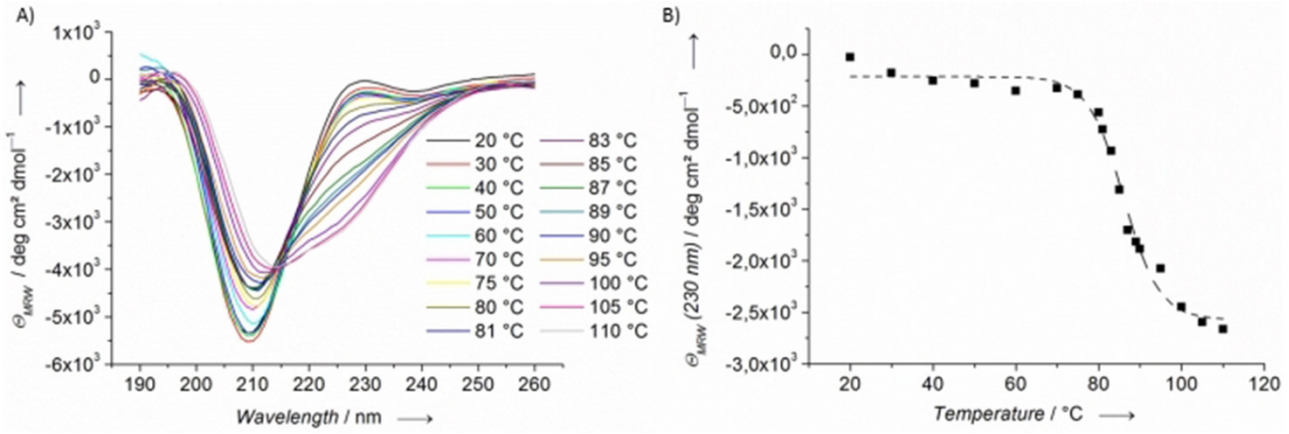

Figure 6. Thermal stability of reconstituted hSOD1 determined via CD spectroscopy. A: Spectra with 0.26 to 0.28 mg/mL hSOD1 in 50 mM sodium acetate, pH 6.0, were recorded in a temperature interval measurement (20 to 110 °C) at 260 to 190 nm with a scan speed of 20 nm/min. Five accumulations per measurement were performed and a buffer sample was subtracted from all protein containing spectra. Data were processed to mean molar ellipticity. B: The apparent melting temperature of about 86 °C was determined from data recorded at 230 nm via a Boltzmann sigmoidal fit (Origin 8).

pendent reconstitution events with slightly varying copper and zinc contents were compared with hSOD1 from a previous preparation that mainly followed published protocols.[10a,17] The latter protein preparation (containing 0.68 equivalents Zn and 0.04 equivalents Cu per monomer) displayed a specific activity of ~500–1000 U/mg. Fully reconstituted hSOD1 derived by the protocol described herein contained about 0.6 to 0.7 equivalents Cu and 0.64 to 0.86 equivalents Zn per monomer and exhibited a specific activity of ~14,300 U/mg, which is the highest specific activity reported for recombinant and purified hSOD1 (Table 1). Normalization to the copper loading state of hSOD1 resulted in a maximal theoretical specific activity of about 20,900 U/mg/Cu, which is in very good agreement to the data published for endogenous hSOD1 assayed in red blood cell lysates.

Repetition of activity determination after eight months of storage at 4 °C yielded 12,300 ± 260 U/mg for reconstitution #2, which demonstrates the high stability of reconstituted hSOD1.

Conclusions

The production and reconstitution of recombinantly expressed hSOD1 is crucial to many studies that aim to elucidate the molecular pathomechanism of ALS, and to create therapeutic strategies for efficient disease prevention and cure. The present work describes a reproducible procedure for total reconstitution of hSOD1 expressed in *E. coli* without requiring hCCS. In brief, the protein was purified via ammonium sulfate precipitation and hydrophobic interaction chromatography and supplemented with zinc. SOD1 was reduced and subsequently denatured in guanidine hydrochloride. Renaturation was performed in the presence of zinc via dialysis against buffers containing decreasing concentrations of guanidine hydrochloride. The protein was supplemented with Cu^{2+} and incubated at 37 °C for 7 days. The dimeric hSOD1 holoenzyme was finally isolated via SEC. Extensive analysis indicated that the natively folded holoenzyme was produced, which exhibits high thermal stability and the highest reported specific activity of recombinantly expressed and purified hSOD1. In particular, reduction of hSOD1 before copper loading and increasing the incubation temperature and time during copper loading lead to elevated copper levels of the reconstituted protein. The final dimer purification step using SEC increased the yield of pure hSOD1 dimer. Together with the high copper loading a drastically increased specific activity of hSOD1 was reached.

Published protocols document the importance of the copper chaperone for hSOD1 integrity. Many enzymes, especially chaperones, increase the efficiency of the reaction. Here, the coordination of a copper ion into the respective copper ion-binding site is mediated. In organisms, hCCS activity might be essential because the free copper ion concentration is far below the concentrations used in our reconstitution.

In preliminary experiments we did include hCCS in the assay. Comparison of the results that included this protein versus the protocol herein showed no clear differences concerning the metal coordination level. Consequently, and to simplify the protocol, the purification and ion loading procedure in the absence of hCCS was used.

The present reconstitution procedure will be a valuable tool for prospective investigations, which critically rely on the structural integrity of the hSOD1 holoenzyme.

Supporting Information Summary

The supporting information describes hSOD1 expression and purification, the reconstitution procedure and all analytical methods (e.g., ICP-MS, NMR, SEC runs, enzyme activity assay, semi-native PAGE and melting point determination via CD) in detail.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: enzyme activity · metalloenzyme · reconstitution · SOD1 · thermostability

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