A novel mechanism of protein thermostability: a unique N-terminal domain confers heat resistance to Fe/Mn-SODs

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Superoxide dismutases (SODs, EC 1.15.1.1) are metalloenzymes that are widely expressed in prokaryotic and eukaryotic cells. SODs catalyse the conversion of superoxide radicals (O$_2$$^-\cdot$) to H$_2$O$_2$ and O$_2$, and play a crucial role in the primary cellular defence system against oxidative stress1. Four types of SODs have been reported according to their metal cofactors: manganese SOD (Mn-SOD), iron SOD (Fe-SOD), copper/zinc SOD (Cu/Zn-SOD), and nickel SOD (Ni-SOD)2–4. Of these, Mn-SOD and Fe-SOD are closely related as their protein structures differ from the other two groups and were recently assigned into one family5. Mn-SOD and Fe-SOD are prevalent in bacteria, mitochondria and chloroplasts, and likely evolved from a common ancestor3,5. Mn-SOD and Fe-SOD are typically homodimers or homotetramers and generally require either Mn$^{2+}$ or Fe$^{2+}$ to perform their biological activities; however, some SODs, referred to as cambialistic SODs, can accommodate both ions and are often found in anaerobic organisms5,6.

Over the past 40 years, SODs have attracted tremendous attention and are widely used in the pharmaceutical, cosmetic, food, agriculture, and environmental protection industries due to their excellent antioxidant properties7–12. At one time, SODs were isolated from animal or plant sources, but the focus has recently shifted to microbial sources since these organisms can be easily induced and cultivated on a large scale1. For industrial applications, thermostability is a major requirement for commercial SODs, because thermal denaturation is a common cause of enzyme inactivation. Fortunately, the isolation of thermostable SODs from thermotolerant or thermophilic microorganisms offers a rapid and effective means to address this issue. To date, many thermostable SODs have been identified from thermophiles and hyperthermophiles, such as Aquifex pyrophilus13, Sulfolobus solfataricus14, Aeropyrum pernix15, Pyrobaculum aerophilum16, Bacillus stearothermophilus17, Chloroflexus aur-
**Methods**

**Materials and reagents.** The primers were synthesised by the AusGCT Biotechnology Corporation (Beijing, China). Tag DNA Polymerase, dNTP, and restriction enzymes were purchased from TaKaRa (Dalian China). T4 DNA ligase was obtained from Promega (Mannheim, Germany), and the DNAs I was procured from Roche (Basel, Switzerland). The Chelating Sepharose Fast Flow column and High Molecular Weight Standards were purchased from Amersham Biosciences (Hammersmith, UK). Phenylmethane-sulfonyl fluoride (PMSF) was purchased from Sigma (St. Louis, MO, USA). All other chemicals and reagents were from Shanghai Sangon, China.

**Bacterial strains and culture conditions.** *G. thermodenitrificans* NG80-2 (CGMCC 1228) was grown in Luria-Bertani (LB) medium at 60 °C with shaking. *Geobacillus* BSn5 and *Escherichia coli* BL21 (DE3) (Novagen) were grown in LB medium at 37 °C with shaking. When necessary, 50 mg kanamycin/L was added to the medium.

**Bioinformatics methods.** The alignment of SODs from *G. thermodenitrificans* NG80-2 (YP._001126309), *B. subtilis* BSn5 (ADY92836), and their closely related homologs was performed using the ClustalW program. Phylogenetic trees were generated with Weblogo. Structural modelling. The structure of SODA was obtained using 0.1% nitric acid and Mn2+ concentrations of 1.0, 2.0, and 4.0 μg/L. Similarly, an iron standard curve was obtained using 0.1% nitric acid and Fe2+ concentrations of 1.0, 2.0, and 4.0 μg/L. The absorbencies of manganese and iron were measured at 297.5 ± 248.3 nm, respectively. The SOD samples were diluted in nanopure water with 0.1% nitric acid until the absorbance was within the range of the standard curves. The samples were thoroughly dialysed in 20 mM Tris (pH 8.0) prior to the assay.

**Effects of temperature and pH on SOD activity.** To determine the optimum temperature, SOD activity was measured in the standard reaction mixture at pH 7.8 at temperatures ranging from 20 to 80 °C. To determine the optimum pH, SOD activity was measured at the optimum temperature in the pH range of 3.0 to 10.0 using sodium citrate (pH 3.0–8.0), Tris-HCl (pH 8.0 and 9.0), or glycine-NaOH (pH 9.0 and 10.0) buffers. Activity was measured as a percentage of the maximum.

**Thermostability test.** The thermal stability of SOD was tested after incubating samples at 20, 30, 40, 50, 60, 70, and 80 °C for 10, 20, 30, 40, 50 and 60 min without substrate. The remaining activities of enzymes were detected at their optimum temperature and pH and calculated as the percentage of the maximum SOD activity.

**Analytical ultracentrifugation.** Sedimentation velocity experiments were performed in a ProteomeLab XL-1 Protein Characterization System (Beckman Coulter). All interference data were collected at a speed of 42,000 rpm in an An-60 Ti rotor at 4 °C. A set of 200 scans was collected at 6-min intervals. The proteins were pre pared in 50 mM potassium phosphate buffer plus 150 mM NaCl at pH 6.8 for SODA and SODA(Tris), SODA(2225), and SOD, and at pH 7.8 for SODA(Na2) and SODA(Tris). The data were analysed using the program SEDFIT (version 11.8) in terms of a continuous c(s) distribution.

**Efects of inhibitors, denaturants, and denaturants on SOD activity.** The effects of inhibitors (ethyleneediaminetetraacetic acid (EDTA) and β-mercaptoethanol (β-ME)), denaturants (sodium dodecyl sulphate (SDS)), and denaturants (urea and guanidine guanidine hydrochloride) on SOD activity were investigated according to the method described by Zhu.

**Circumstantial dichroism.** Circular dichroism was measured on a Jasco J-715 circular dichroism spectrometer (Japan). First, 0.14 mg/ml of SOD(Na2) and 0.4 mg/ml of other proteins including SOD(Na2), SOD(Na2), SOD(Tris), and SOD in 20 mM potassium phosphate buffer (pH 8.0) was placed in a 1 mm quartz cell. All spectra were recorded at a scanning rate of 100 nm/min with 0.1 nm wavelength steps from 190 to 250 nm and three accumulations.

**Structural modelling.** The structure of SOD(2225), the functional domain of SOD(2225) was modelled via the SWISS-MODEL server (http://swissmodel.expasy.org/).
The thermostability maintenance of SODs. However, whether the repeat-containing domains were thus proposed to involve Geobacillus but not in SODs from any species, these repeat-containing domains were thus proposed to be involved in the thermostability maintenance of SODs. However, whether the repeat sequence forms a special secondary structure remains unknown. The phylogenetic trees of both the N- and C-terminal domains of Geobacillus SODs exhibited highly similar topological structures (Fig. 1a,b), indicating that the sod genes potentially gained their extra NTDs before the divergence at nodes N1 and N2 occurred.

**Results**

Bioinformatic analysis of SODNG2215. SODNG2215 possesses a unique repeat-containing N-terminal domain (NTD) that differs from its closely related homologues from Bacillus. The functional domains (SODA) of SODNG2215-like SODs from Geobacillus are conserved (preliminary sequence identity: 84–93%) and are also highly similar to their counterparts from Bacillus (preliminary sequence identity: 64–71%). Alignment of the SODNG2215-like SODs from 13 thermophilic Geobacillus strains showed that these SODs have an extra N-terminal segment despite their various lengths (Supplementary Fig. S1). A blast analysis revealed that these segments share significant amino acid similarity with each other but not with other sequences in GenBank. Further alignment of these N-terminal segments revealed that the eight SODs (subgroup 1) harbour two repeat sequences within their extra N-terminal segments, whereas the other four (subgroup 2) only have a single repeat sequence (Fig. 1b, c). Due to the presence of these extra segments in SODNG2215-like SODs from all thermophilic Geobacillus but not in SODs from any Bacillus species, these repeat-containing domains were thus proposed to be involved in the thermostability maintenance of SODs. However, whether the.

**Figure 1** | Phylogenetic analysis of C-terminal (functional) fragments (a) and N-terminal fragments (b) of SODs from *G. thermodenitrificans* NG80-2 (YP_001126309), *B. subtilis* BSn5 (ADV92836), and their closely related homologs. The accession number of each SOD in GenBank is labelled prior to the species name. The proteins used for the activity test in this study are highlighted in red. The sequence logos of the repeat sequences were constructed from alignments of SOD N-terminal fragments from the 13 Geobacillus strains (c). The letter size is proportional to the degree of amino acid conservation. The deletion (gap) is represented by the letter “B”.

The structure of a closely related iron SOD from the strain *M. thermastotrophicus* Delta H, which has a 52% sequence identity and 67% sequence similarity with SODANe8215, was used as a template. The stereochemical and overall quality of the final model was assessed using PROCHECK and ProSA. In the resulting model, 89% of the residues were in the most favoured regions, and no residues were located in disallowed regions. The PROCHECK overall g factor evaluating all torsion angles and bond lengths was 0.08, indicating a high-quality model. The ProSA Z score of the SODANe8215 model was calculated as −6.03. Z scores for experimentally determined X-ray structures of proteins with a similar size (around 207 amino acids) lie in the range of −1 to −10.8. The Z score of this model was thus within the range of scores typical for similarly sized native proteins.

**Gene manipulation, expression, and purification of SOD variants.** To explore the role of the SODNG2215 NTD, we constructed five recombinant clones (Fig. 2a) to comparatively study the thermostability, temperature-induced conformational changes, and stress resistance of the corresponding recombinant proteins. First, the full-length sodNG2215 and the NTD-deleted form (sodANe8215) were separately cloned to study the effects of the NTD in its natural host. To further investigate its effects on other Fe/Mn SODs, the NTD was fused to a Fe/Mn SOD from a mesophilic strain of *B. subtilis* BSn5 (SODBSn5), which shares 66% amino acid sequence identity with SODNG2215. The resulting construct (sod1) contained the DNA segments encoding the SODNG2215 NTD and the SODA domain of SODBSn5. The SODs from *Bacillus* are the closest phylogenetically to the SODs from Geobacillus (Fig. 1a) and typically exhibit poor heat-resistance49. The full-length sodBSn5 and sodABSn5 genes encoding the C-terminal SODA domain were also cloned as negative controls.

SDS-PAGE analysis showed that all five SOD proteins were expressed accordingly to their expected sizes (Fig. 2b).

**Removing the NTD does not alter the metal ion specificity of the host SOD.** To investigate the metal ion preference of SODNG2215 and its NTD-deleted form, SODANe8215, Fe2⁺- and Mn2⁺-reconstituted enzymes were prepared. As shown in Supplementary Table S2, an atomic absorption analysis demonstrated that each monomer of the
native SOD$_{NG2215}$ was associated with 0.55 ± 0.03 atoms of iron and 0.10 ± 0.01 atoms of manganese, whereas 0.73 ± 0.02 atoms of iron and 0.81 ± 0.03 atoms of manganese were associated with the Fe$^{2+}$- and Mn$^{2+}$-reconstituted SOD$_{NG2215}$ enzymes per subunit, respectively. The Fe$^{2+}$- and Mn$^{2+}$-reconstituted SOD$_{NG2215}$ exhibited specific activities of 769.1 ± 17.2 and 1158.4 ± 29.3 U/mg, respectively; the apoprotein was inactive. The Mn$^{2+}$-reconstituted SOD$_{NG2215}$ was approximately 40 to 50% more active than the native and Fe$^{2+}$-reconstituted SOD$_{NG2215}$, suggesting that SOD$_{NG2215}$ is a cambialistic SOD that can accommodate both Fe$^{2+}$ and Mn$^{2+}$ as cofactors with manganese preferred over iron in regard to enzymatic activity. The metal ion specificity study of SOD$_{NG2215}$ revealed similar characteristics only with less enzymatic activity than SOD$_{BSn5}$ (Supplementary Table S2). This result indicates that the NTD has no effect on the metal ion specificity of SOD.

Oligomerisation form and composition of SOD are not altered by the NTD. Analytical ultracentrifugation analysis (Fig. 3) indicates that either the full length SOD$_{NG2215}$ or the NTD-deleted form of SOD$_{NG2215}$ exist mainly in a tetrameric form. SOD$_{BSn5}$ and its counterparts, SOD$_{BSn5}$- and NTD-fused SOD$_{r}$, were found in dimeric and monomeric forms with no tetramer detected. The extremely increased peak area ratio of the tetramer compared with the monomer indicates most protein molecules of SOD$_{NG2215}$ and SOD$_{BSn5}$ tend to form multimers, whereas all three SOD$_{BSn5}$-derived proteins prefer to exist in monomeric forms. It thus seems that the functional SODA domain of the SOD variants determines the formation of oligomerisation, whereas the NTD has no effect on the oligomerisation form or the monomer-multimer ratio.

NTD contributes to host thermophilicity with a slight alteration in pH profiles. The optimum active temperature (OAT) was determined by testing the SOD activity at temperatures ranging from 20 to 80°C (Fig. 4a). The OAT for SOD$_{NG2215}$ was 70°C, which is close to that of other thermophile-derived SODs (50–70°C)$^{37,40}$ but lower than that of the hyperthermophile SODs (87–95°C)$^{11,15}$. SOD$_{NG2215}$, SOD$_{BSn5}$, and SOD$_{BSn5}$ exhibited mesophilic properties with OATs of 30°C, 35°C and 30°C, respectively. Intriguingly, SOD, exhibited optimal activity at 55°C, and retained 46% of its maximum activity even at 80°C. Clearly, the NTD-fused proteins of SOD$_{NG2215}$ and SOD$	ext{r}$ are considerably more thermophilic than their counterparts without the NTD (SOD$_{BSn5}$ and SOD$_{BSn5}$r).

Although the relative activities were used for the comparison of the thermophilicities of the variant SODs, the real activities of these recombinants are quite different. The initial enzymatic activities of SOD$_{NG2215}$, SOD$_{BSn5}$, SOD$_{BSn5}$r, SOD$_{BSn5}$ and SOD$_{BSn5}$r, investigated at 20°C are 800.9 ± 18.3, 837.1 ± 19.2, 682.3 ± 14.6, 598.8 ± 16.2 and 678.3 ± 15.1 U/mg, respectively, whereas the maximum at their individual OATs rise to 1132.2 ± 20.6 (70°C), 915.9 ± 18.9 (30°C), 933.0 ± 19.1 (35°C), 752.5 ± 17.5 (30°C), and 1055.1 ± 20.3 (55°C) U/mg, respectively.

The pH profiles of the SOD activity are presented in Figure 4b. SOD$_{NG2215}$ and SOD$_{BSn5}$ exhibited maximum activities at pH 4 and 5–6, respectively, indicating that they are both acidophilic. Outside their optimum pH ranges, the activities of both enzymes quickly decreased. To our knowledge, SOD$_{BSn5}$ is the most acidophilic, thermophilic, cambialistic SOD that has been reported to date. In contrast, SOD$_{BSn5}$- and the NTD fused SOD$_{r}$ were all neutrophilic with an optimum pH of around 8. Therefore, the NTD showed negligible effects on the pH preference of SOD activities.

NTD plays an important role in maintaining the thermostability of SODs. An optimum active temperature assay demonstrated that the presence of the NTD alters the thermostability of SODs. Therefore, we further examined the role of the NTD in SOD thermostability.

As shown in Figure 5, all enzymes were very stable at a low temperature (20°C) and almost fully active after 60 min. However, the enzymes exhibited variable stability when the temperature was greater than 40°C. SOD$_{BSn5}$, SOD$_{BSn5}$r and SOD$_{BSn5}$r, which are mesophilic, also demonstrated poor thermostability when the temperature was greater than 40°C. All three enzymes lost approximately 90% of their activity after 60 min above 60°C and were almost inactive after 10 min at 80°C.

In contrast, the thermophilic SOD$_{NG2215}$ exhibited excellent thermostability over a range of temperatures below 70°C and still remained 70% active after 60 min at 70°C. Even at 80°C, SOD$_{NG2215}$ remained 52% active after 10 min and almost maintained at this level until 60 min. SOD$_{r}$, the NTD fused SOD$_{BSn5}$r, also exhibited good thermostability (retained 80% and 61% active at 50°C and 60°C, respectively, after 60 min), indicating that this enzyme acted more like a moderate thermophilic Mn-SOD from B. licheniformis$^{20}$.
Meanwhile, the half-lives and D-values deduced from thermo-
stability assays clearly showed that the NTD fused SODs were much
more stable than their counterparts without the NTD especially at
high temperatures (Supplementary Table S3).

CD spectroscopy analysis reveals the enhanced conformational
stability of NTD-fused SODs. Far-UV CD spectra of five SODs
exhibited a positive maximum at 192 nm and double-lobed
negative peaks at 208 nm and 222 nm, which are characteristic of
the high α-helix content of the protein 41,42 (Fig. 6). However,
temperature-dependent CD measurements clearly indicated that
the conformational changes of SOD_{NG2215} and SOD, are quite
small compared with the other three counterparts, which is
consistent with the results from the temperature-dependent
activity assays. The CD signal intensity almost did not decrease
further when the temperature rose from 70 to 90 °C. Additionally,
strikingly reduced ellipticity signals at 192 nm and 208 nm for
SOD_{NG2215}, SOD_{BSn5}, and SOD_{BSn5} resulted in a sharp
isodichroic point at 202 nm, indicating a two-state helix-coil
transition 43. In contrast, the isodichroic points for SOD_{NG2215}
and SOD, are not obvious. When the Spectra was measured at 5 °C
intervals (Supplementary Fig. S4), an apparent isodichroic point
was observed at 201 nm for both SOD_{NG2215} and SOD,. In
addition, the magnitudes at 222 and 208 nm decreased very
slightly when the temperature crossed from 70 °C to 85 °C.

The NTD enhances the host proteins’ abilities to resist inhibitors,
detergents, and denaturants. The effects of various inhibitors,
detergents, and denaturants on SOD activity were examined using
EDTA, β-ME, SDS, urea, and guanidine hydrochloride (Fig. 7 and
Supplementary Table S4). SODs fused with the NTD are
considerably more resistant to these stresses than their
counterparts lacking the NTD. Of these stresses, the NTD confers
an extraordinary ability to resist denaturants. When tested with urea
or guanidine hydrochloride at a final concentration of 2.5 M, 72–
93% of the relative activities of SOD_{NG2215} and SOD, were
maintained. In contrast, only 43–64% of their activities remained
in their counterparts without the NTD. Additionally, SOD_{NG2215}
and SOD, maintained greater than 45% of their relative activities
in SDS at a concentration of 1%, whereas the other SODs were
completely repressed.

Structural modelling and analysis reveal a conserved backbone
structure shared by SOD_{NG2215} and its homologs. The structures
of several SODs, including those from M. thermautotrophicus (1MA1,
tetrameric), A. pernix (3AK3, tetrameric), P. aerophilum (3EVK,
tetrameric), S. acidocaldarius (1B06, tetrameric), and S. solfataricus
(1WB8, dimeric), which fall under the same branch as SOD_{NG2215},
have been reported.

The structure of SOD_{NG2215}, the functional domain of
SOD_{NG2215}, modelled based on the structure of a closely related iron
SOD from M. thermautotrophicus Delta H, which has 52% sequence
identity and 67% sequence similarity with SOD_{NG2215}. The model
of SOD_{NG2215} demonstrated good superimposition with 1MA1,
3AK1, 3EVK, 1B06, and 1WB8 with rmsd values of 0.061, 0.513,
0.501, 0.697, and 0.763, respectively. In addition to the similar back-
bone structure, the metal binding residues (Fig. 8) are also conserved
among the thermophilic Fe-SODs.
Discussion

Three types of SODs (Fe/Mn-SOD, Mn-SOD and Cu/Zn-SOD) are found in thermophilic Geobacillus, of which Mn-SOD was identified as being considerably thermostable. In this study, we investigated the thermostability of SODNG2215, a cambialistic SOD from the facultative anaerobic Geobacillus thermodenitrificans NG80-2, which can function with both Fe$^{2+}$ and Mn$^{2+}$ as cofactors. Cambialistic SODs can be divided into two groups. One group exhibits approximately equal activity in the Mn$^{2+}$ and Fe$^{2+}$ forms, whereas the other group prefers manganese over iron in regard to enzymatic activity. The former group is often found from anaerobes and the latter is from aerobes or facultative anaerobes. Clearly, SODNG2215 belongs to the latter group.

Geobacillus is a phenotypically and phylogenetically coherent genus of thermophilic bacilli and was recently separated from the genus Bacillus. Sequence analysis has revealed that the SODNG2215-like proteins from Geobacillus all contain unique NTDs that are not present in other mesophilic Bacillus species. The NTDs are thought to be involved in maintaining the thermostability of these SODs. Of considerable interest is a 40-amino-acid sequence that is repeated either once or twice within the NTDs and is exclusive to SODNG2215 homologs from Geobacillus. The phylogenetic analysis of currently known thermostable Fe-, Mn- or Fe/Mn- SODs from various microorganisms showed that these SODs were roughly split into three major branches except for a Mn-SOD from Thermostosynechococcus elongates and a Fe-SOD from Thermosyntheticus sibiricus, with that the phylogeny and metal specificity of these thermophilic SODs are not congruent.

The Fe/Mn-SOD (SODNG2215) and Mn-SOD (GTNG_2400) from G. thermodenitrificans NG80-2 are located in two distinct subgroups. Given their differing protein sizes and ion requirements, it appears that these two SODs may have divergently evolved. SODNG2215 and one of its closely related homologs from Geobacillus sp. EPT3, which was previously identified as a Mn-SOD, cluster into a same branch with five other SODs derived from different species. However, SODNG2215 and Mn-SOD from EPT3 may adopt completely different thermophilic adaptation strategies compared to five other SODs because the NTD deleted SODNG2215 is more like a mesophilic enzyme. In this study, we provided strong evidence that the NTD containing two repeat sequences contributed to SOD thermostability. Further work to determine the crystal structure and characterise the NTDs containing only a single repeat sequence will help to elucidate the specific role that these sequences play in thermostability.

Figure 6 | Temperature-dependent CD spectra of SODNG2215 (a), SODBSn5 (b), SODBSn (c), SODBSn (d), and SOD (e).

Figure 7 | Effects of inhibitors, detergents, and denaturants on SOD activity. The enzyme was incubated with each inhibitor, detergent, or denaturant at various final concentrations in 50 mM sodium phosphate buffer (pH 7.8) at 25 °C for 30 min. Residual activities were measured by the standard assay as described in the Methods section. The reaction mixture without inhibitor, detergent, or denaturant was used as a control and defined as 100%. The values are the mean ± standard deviation from three separate replicates. No enzymatic activities of SODBSn5 and SODBSn were detected with 1% SDS.
The finding that the optimum active temperature of the NTD-deleted SOD\textsubscript{NG2215} decreased dramatically from 70 to 30 °C suggests that the NTD plays an essential role in maintaining the high thermostolerance of SOD\textsubscript{NG2215}. In contrast, when the NTD was fused with SOD\textsubscript{Bsn5}, a mesophilic SOD, the resulting enzyme (SOD\textsubscript{NG2215}Bsn5) exhibited remarkably enhanced thermostability. The detailed assessment of thermostability also demonstrated that the thermostability of SOD\textsubscript{NG2215} is significantly enhanced compared with SOD\textsubscript{Bsn5} and SOD\textsubscript{NG2215}. All these findings indicate that the NTD of SOD\textsubscript{NG2215} is capable of enhancing the thermostability as well as the thermostability of other mesophilic SOD homologs. Futhermore, the comparison of five SOD proteins’ Teq values calculated according to the Equilibrium Model (Supplementary Fig. S5) indicated that the NTD also increased the estimated optimum working temperature range\textsuperscript{[64]}\textsuperscript{[67]}. It is worth mentioning that although SOD\textsubscript{Bsn5} has a small N-terminal peptide containing 78 residues that is absent in SOD\textsubscript{NG2215}Bsn5, it only slightly affects the thermostability and enzymatic activity.

Some previous studies on cambialistic SODs found that they were more thermostable when reconstituted with manganese rather than iron\textsuperscript{[48]}. The thermostability of Mn\textsuperscript{2+}- or Fe\textsuperscript{2+}-reconstituted SOD\textsubscript{NG2215} and SOD\textsubscript{NG2215}Bsn5 were also investigated in the present work (Supplementary Fig. S3). The trends chart of the thermostability of both enzymes at 70 °C revealed that the Mn\textsuperscript{2+}-reconstituted proteins are considerably more stable than the Fe\textsuperscript{2+}-reconstituted SODs and as stable as the native SODs. Importantly, when the NTD is deleted, the Mn\textsuperscript{2+}-reconstituted SOD\textsubscript{NG2215} still exhibited poor stability at high temperatures, and its thermostability was even worse than the Fe\textsuperscript{2+}-reconstituted SOD\textsubscript{NG2215}, which contains the NTD. Thus, the effect of the NTD is much more important than the ion constitution in regards to the thermostability of SOD\textsubscript{NG2215}.

Enzymes isolated from thermophilic (50–80 °C) or hyperthermophilic (>80 °C) microorganisms are generally more thermostable and more resistant to enzyme inhibitors, protein detergents, pH, and other denaturing agents compared with SODs from mesophilic (25–50 °C) or psychrophilic (<25 °C) microorganisms\textsuperscript{[48]}.\textsuperscript{[50]}. These enzymes therefore have the potential to be widely used in industrial capacities. Factors contributing towards thermostability of proteins are numerous and complex, such as certain types of amino acids\textsuperscript{[51]}, residue contributions to VdW interaction energies\textsuperscript{[52]}, increase in hydrophobicity\textsuperscript{[53]}\textsuperscript{[54]}, differences in compactness\textsuperscript{[55]} and packing density\textsuperscript{[56]}, significant changes in hydrogen bonding networks\textsuperscript{[57]}, enhancement of secondary structure propensity\textsuperscript{[58]}, extensive ion pair interactions\textsuperscript{[59]}, differences in the number of amino acids comprising the loops\textsuperscript{[60]}, and even a single amino acid mutation far from the active site\textsuperscript{[61]}\textsuperscript{[62]}\textsuperscript{[63]}\textsuperscript{[64]}. Therefore, it is extremely difficult to bioengineer a specific enzyme with improved thermostability by using a “universal” method. The discovery that fusion with the NTD from SOD\textsubscript{NG2215} can significantly improve the thermostability of host SODs provides an easy and feasible method to generate heat-resistant SOD proteins. A similar example has been reported that the N-terminal domain of a multi-functional cellulase EGXA increased the enzyme’s thermostability (only tested on 45 °C) to a slight extent, while the mechanism might be different since the N-terminal domain was identified as a cellulose-binding domain and only selectively enhanced enzymatic activity and thermostability towards some of substrates\textsuperscript{[35]}. In addition to thermostability enhancement, the NTD also significantly improved the stress resistance of the host SOD proteins. For all of the tested stresses of inhibitors, detergents, and denaturants in this study, SOD\textsubscript{NG2215} and the NTD-fused SOD, both demonstrated enhanced resistance compared with their counterparts without the NTD (Fig. 7 and Supplementary Table S4).

All known thermophilic Fe-SODs except the SOD\textsubscript{NG2215}Bsn5-like proteins from Geobacillus are similar in protein size (20 kD), backbone structure, and metal binding residues (Fig. 8 and Supplementary Fig. S2). Despite such similar features, their thermal stability is remarkably different. It was believed that α-helical content, β-helix length, and even the salt bridges formed by the charged residues, such as Arg and Glu, are important factors for Fe-SOD thermostability. In contrast, the β-strand is negatively correlated via a principal component analysis of five thermophilic SODs and six mesophilic counterparts\textsuperscript{[25]}. Superimposing this model with SOD structures (1MA1, 3AK1, 3EVK, 1B06, and 1W8B) revealed that these tertiary structures are well conserved, especially the regions around the ion binding site. Evidence from metal reconstitution analysis and density gradient centrifugation indicated that the NTD of SOD\textsubscript{NG2215} did not affect the metal ion specificity and polymerisation of the protein. Additionally, both temperature-dependent CD analysis and temperature-dependent activity assays demonstrated that this domain greatly contributes to the thermo-tolerance of the host protein. We thus assumed that the NTD might provide an outer envelope that covers the temperature-sensitive hydrophobic residues or cavities on the surface of the active SOD “core” and improves the formation of hydrogen bonds or polar interactions between the monomers without affecting the interactions in the inner SOD “core”, which contributes the metal binding site and is important for tetramer formation. Additionally, artificially fusing the NTD to the mesophilic SOD\textsubscript{Bsn5}, a close homolog of SOD\textsubscript{NG2215}Bsn5, led to a moderately thermophilic enzyme (OAT changed from 30 to 55 °C). In contrast, SOD\textsubscript{NG2215} can tolerate higher temperatures, indicating that the NTD has evolved to be a well-matched heat-resistant partner to SOD\textsubscript{NG2215} over a long period.

In conclusion, to our knowledge, various factors determine protein thermostability. Some proteins even evolved more than one strategy to maintain their thermal tolerance. Herein, a new mechanism of protein thermostability defined by a unique peptide was discovered. The ability of the SOD\textsubscript{NG2215} NTD to improve the heat and stress resistance of host proteins could be highly useful in generating thermostable SODs for industrial applications. Work to determine the crystal structure of these enzymes is underway and could help to clearly reveal the mechanism of the improved thermostability conferred by the NTD to SODs.

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**Figure 8** | The model of SOD\textsubscript{NG2215} generated based on an iron SOD from M. thermautotrophicus Delta H (a). Superposition of the SOD\textsubscript{NG2215} model with 1MA1, 3AK1, 3EVK, 1B06 and 1W8B (b) as well as structures (1ISA and 1MSD) of SODs from E. coli and humans (c).
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**Author contributions**

W.W. and T.M. contributed equally to this work. W.W. and J.C. designed all the research and analyzed the data. W.W., T.M. and J.C. performed the experiments and wrote the manuscript. B.Z., N.Y., M.L., L.C., G.L. and Z.M. carried out some experiments and analyzed some data. All authors reviewed and approved the manuscript.

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