Supplementary Information

Supplementary Table

Table S1 Measurement of Copper (Cu) and Zinc (Zn) content of WT and mutant *Ic*SOD represented as the quotient of moles of metal ions to protein (monomer) concentration.

Proteins	ICP-MS		Zincon		Bathocuproeine	
	Cu	Zn	Cu	Zn	Cu	Zn
Bovine SOD	0.98±0.02	0.99±0.05	0.89±0.04	-	0.92±0.03	-
WT	0.88±0.05	0.98±0.03	0.77±0.01	-	0.77±0.05	-
Mutant	0.89±0.04	0.98±0.02	0.74±0.03	-	0.88±0.04	-

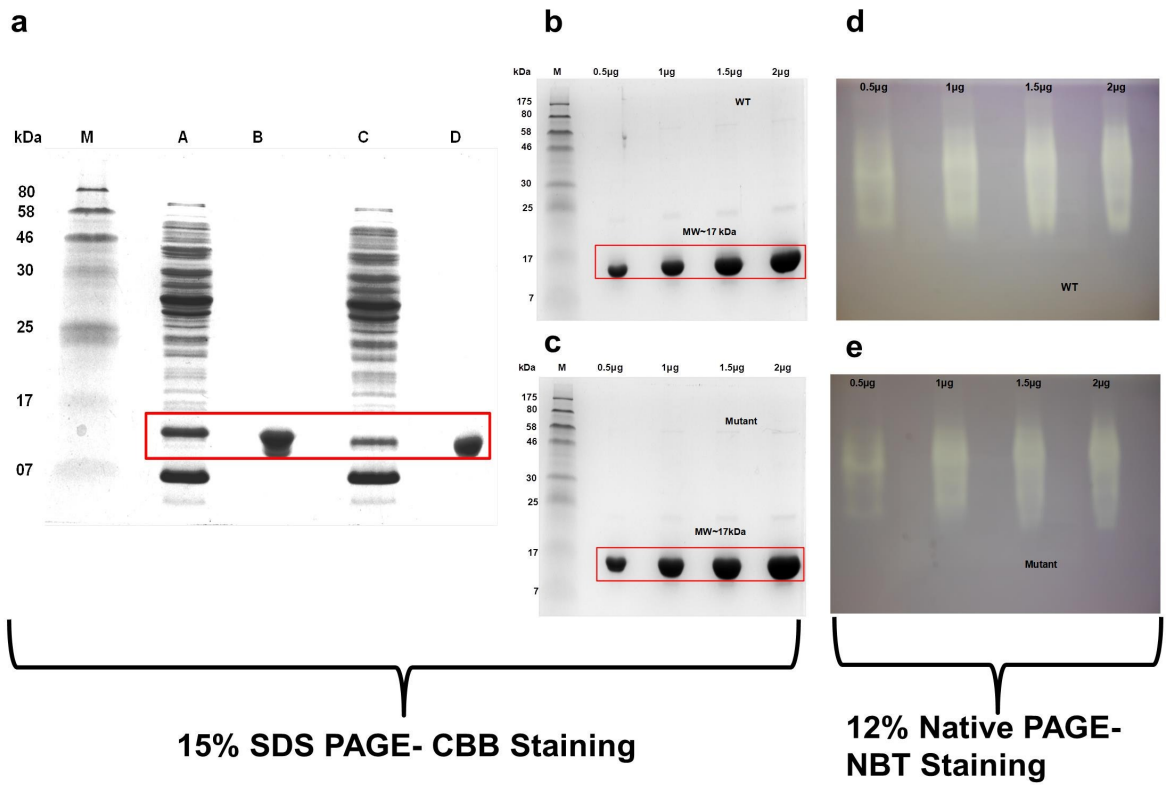
The metal content of the proteins was determined by ICP-MS at SGS India Pvt. Ltd., Gurgaon, India. The Cu content was also evaluated following Zincon and Bathocuproine as described earlier [1].

Table S2. The Common salt bridge interactions with their respective percentage occupancy throughout the simulation time between mutant and wild type protein.

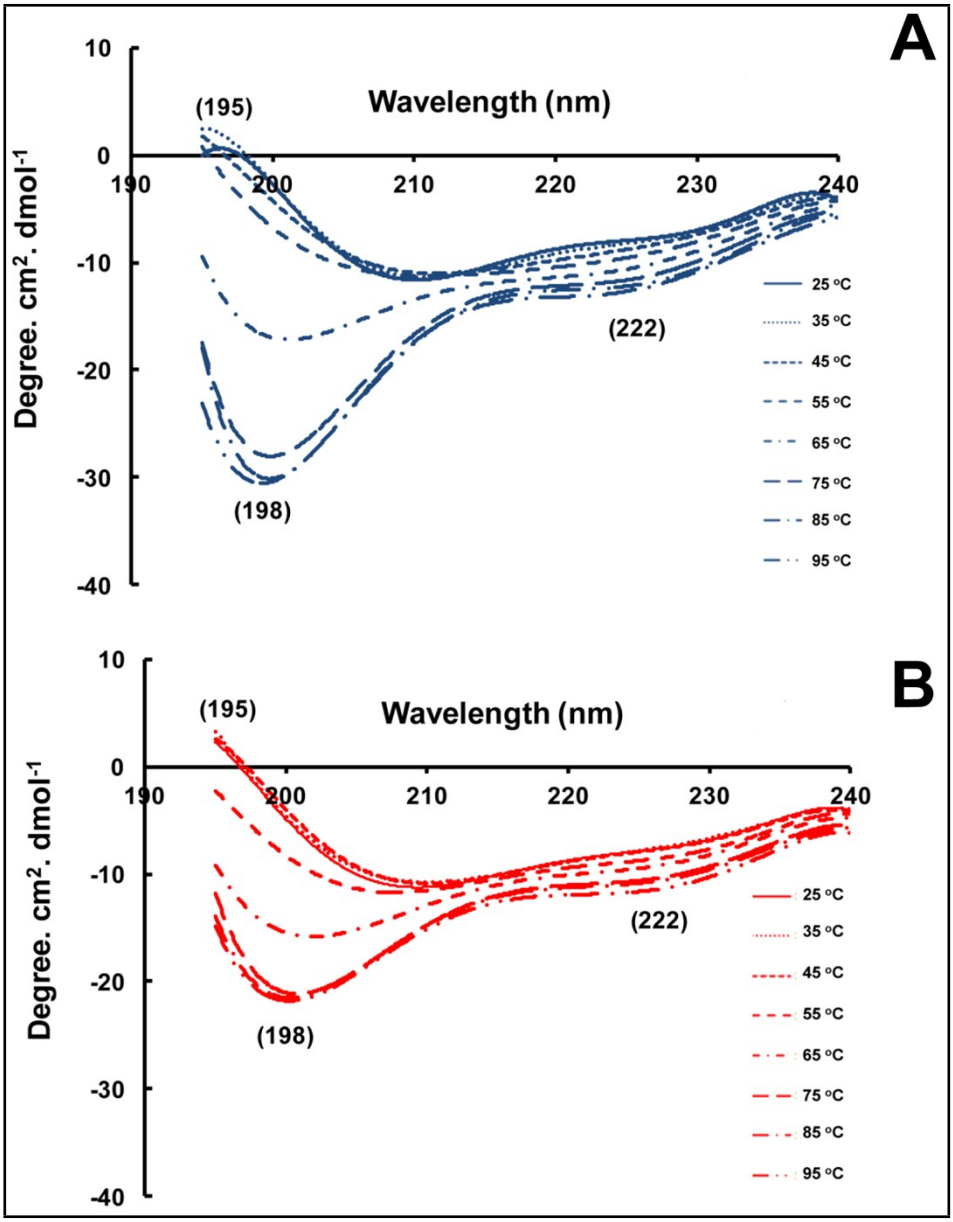
NAME	WILD	MUTANT
ASP100-ARG78	95.565	96.89
ASP252-ARG230	91.835	24.145

Table S3. Oligonucleotide primers used for PCR reactions. 'F' and 'R' stands for forward and reverse primers used in the specific experiments. The bold face lower case letters in the nucleotide sequence represent the specific mutational sites.

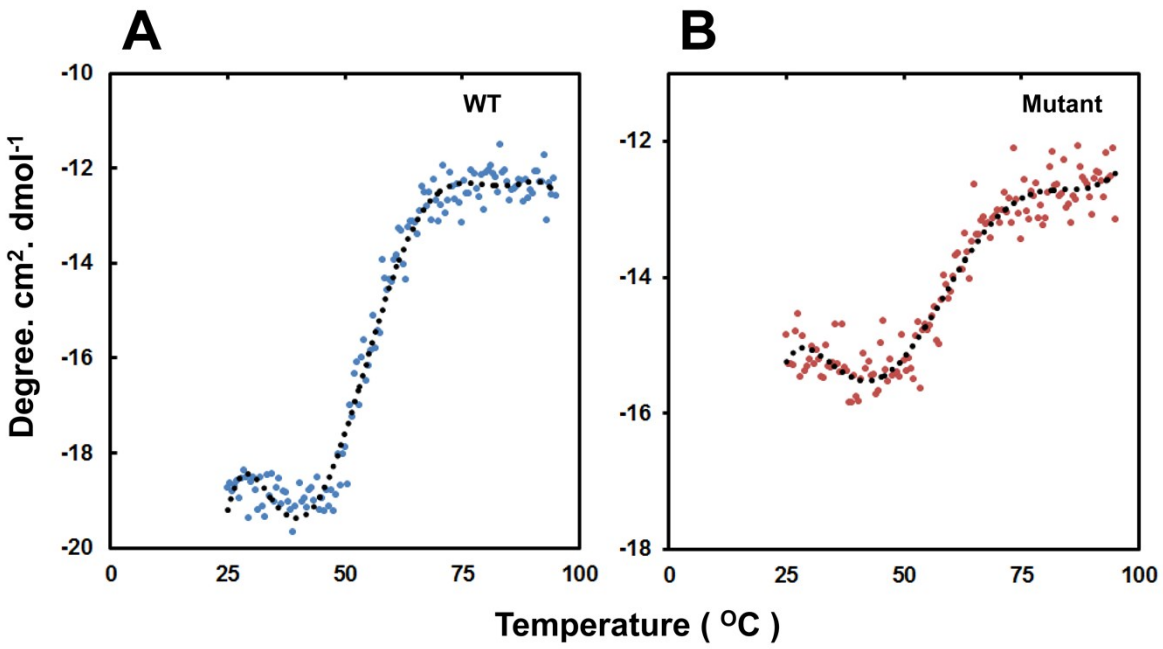
Name of the oligonucleotide	Sequence (5' to 3')	PCR Experiment
*Ic*SODF1	ATGGTGAAGGCTGTCGCAGTTC	Cloning of *Ic*SOD (WT) gene
*Ic*SODR1	TTAACCCTGCAGGCCAATGATAC	
S95 F	ATGGTACTGCT**tgt**TTCACCATCA	Mutagenesis
S95 R	TGATGGTGAA**aca**AGCAGTACCAT	
SP6	TATTTAGGTGACACTATAG	Sequencing
T7	TAATACGACTCACTATAGGG	
pQE F	ATATGGATCCCACGTGATATCCT	



Supplementary Figure S1: Overexpression and purification of *Ic*SODs. (a) SDS-PAGE analysis of **A**-induced WT soluble fraction, **B**-purified WT enzyme, **C**- induced soluble fraction of mutant *Ic*SOD, **D**-purified mutant enzyme. **M** denotes the marker lane. Enzyme preparation after Ni-NTA purification has been shown in **B** and **D** lane and boxed one show the purification of induced band. SDS-PAGE analysis of purified WT and mutant protein at different concentrations has been depicted in **b** and **c** respectively. **(d-e)** represents respectively the native page and in gel activity (NBT staining) of purified WT and mutant proteins.

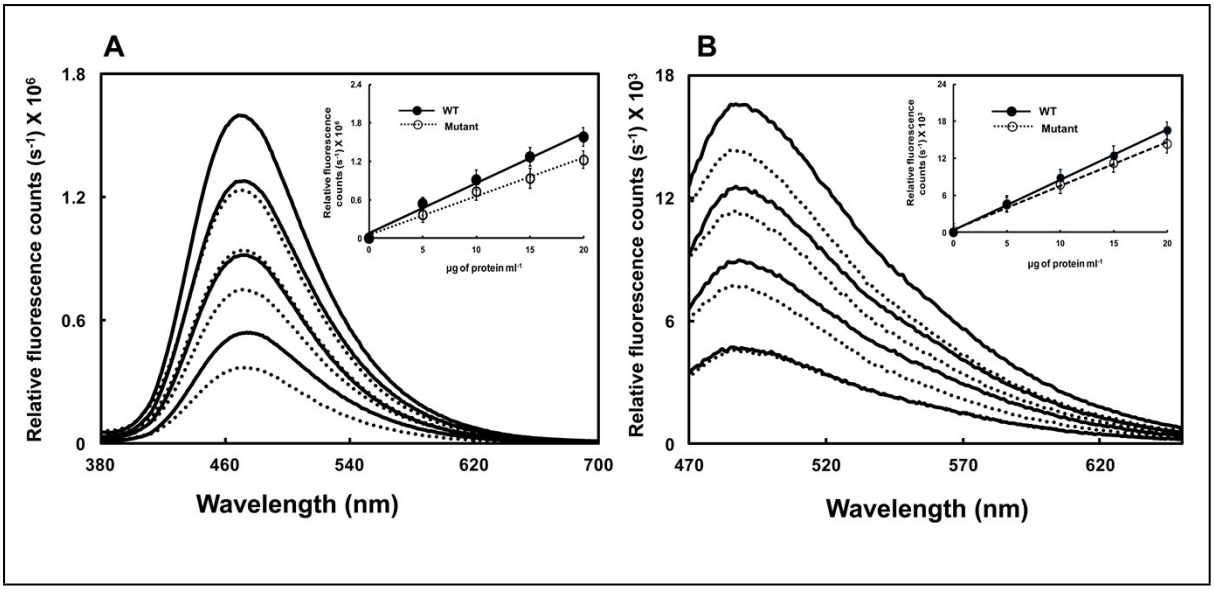


Supplementary Figure S2: CD spectral analysis at varied temperature of WT (A) and mutated *Ic*SOD (B). The presence of negative bands at 198 nm regions and at 224 nm, been shown in brackets. Transition to random coil can be marked as prominent negative band at 198 nm.

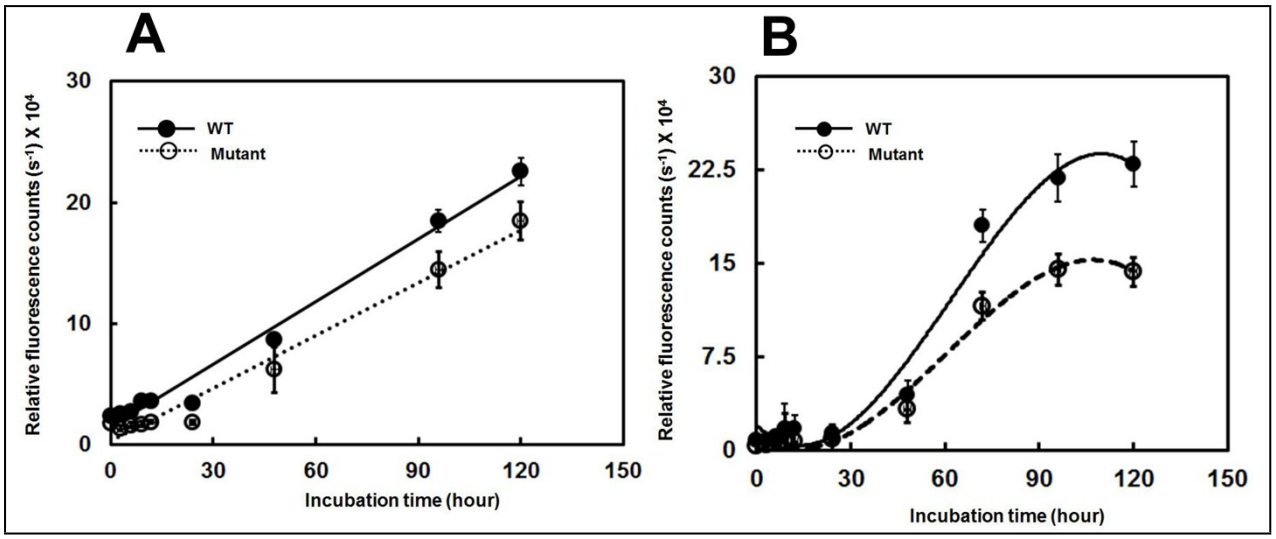


Supplementary Figure S3: Thermal denaturation of WT (A) and mutant (B) *Ic*SOD.

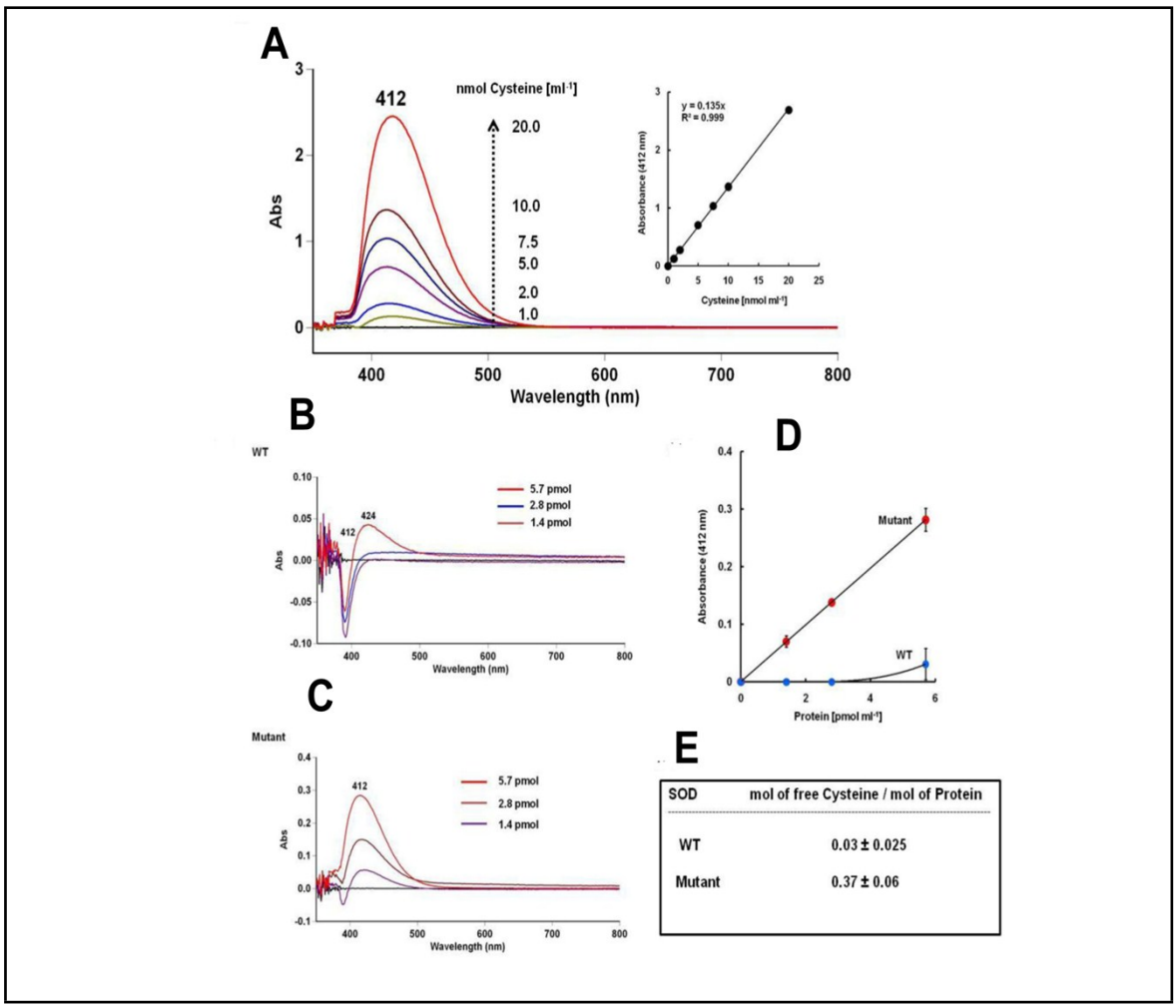
The melting profile was measured at 209 nm in the temperature range of 25^0C to 95^0C. The time constant for rise of temperature was maintained at 0.5^0C min^{-1}.



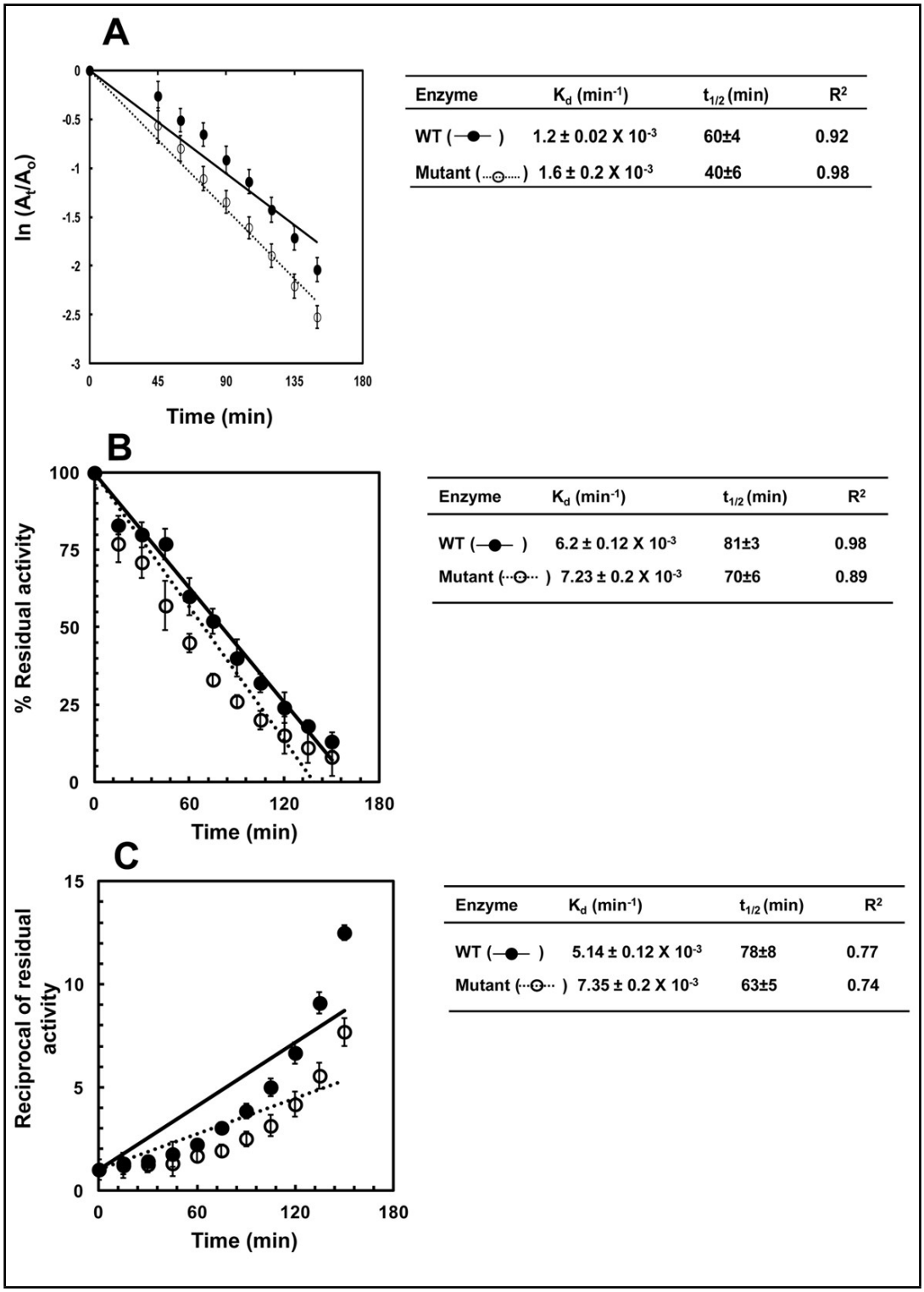
Supplementary Figure S4: Measurement of relative surface hydrophobicity by ANS (A) and aggregation by ThT fluorescence (B). In both A and B inset represents the relative fluorescence counts at different protein concentrations.



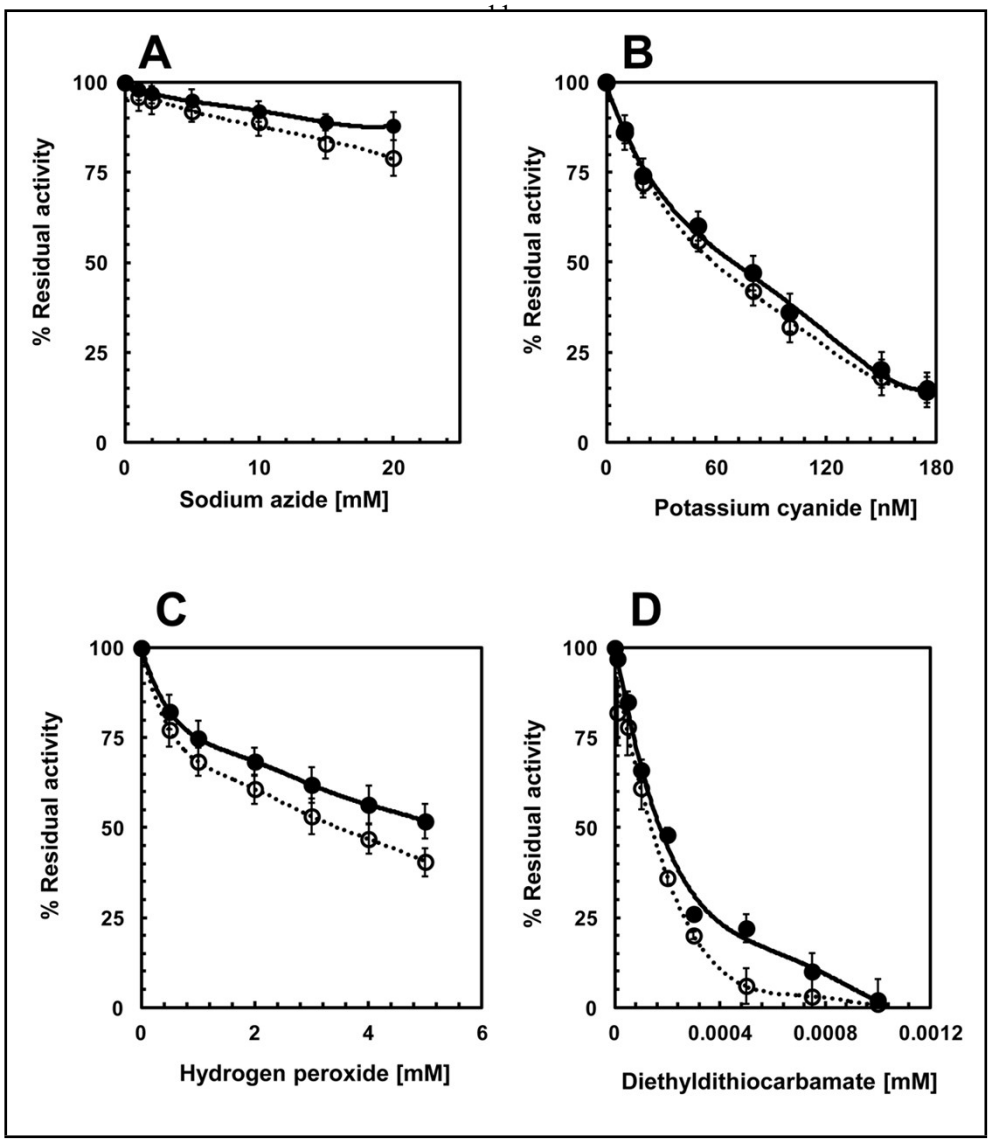
Supplementary Figure S5: Measurement of surface hydrophobicity by ANS (A) and aggregation pattern by ThT (B) fluorescence as function of incubation time. Both the samples were aged at 4°C for same time period (48 hour) and filtered using 0.2 micron filter. 10µg of protein ml^{-1} was used with 20 µM ANS and 10 µM ThT in PBS for ANS and ThT fluorescence. All the samples were incubated at 37°C to study the time kinetics.



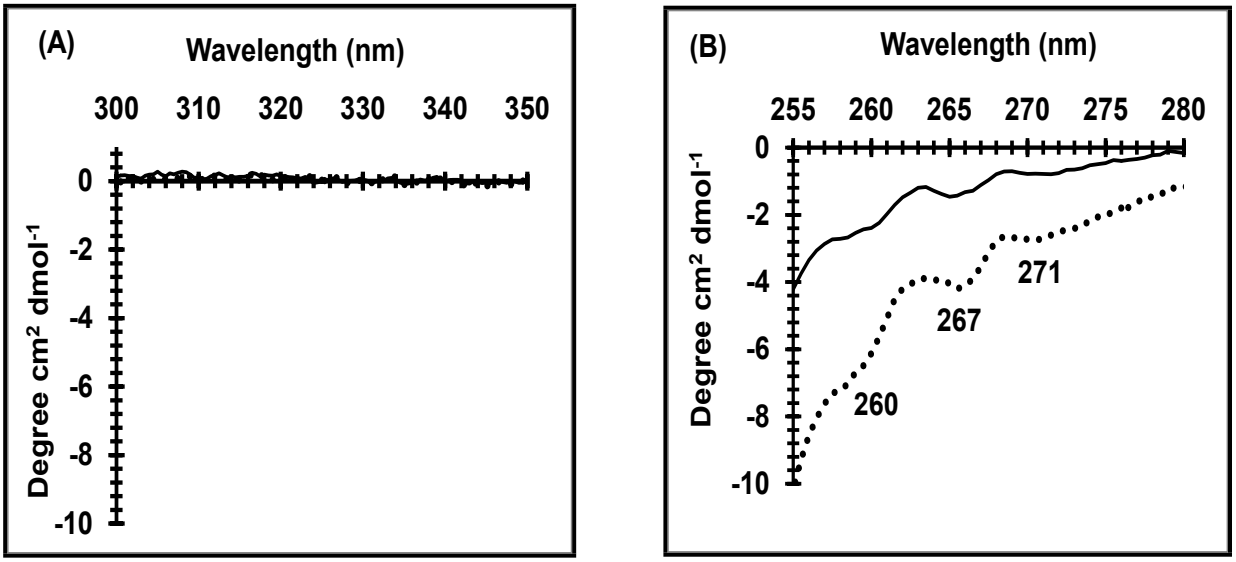
Supplementary Figure S6: Estimation of free thiol (cysteine) in *Ic*SOD proteins. (A) Analysis of cysteine content at varied cysteine concentration as measured at 412 nm. Inset depicts the standard graph made using defined concentrations of cysteine. **(B)** Cysteine content of WT **(C)** Cysteine content of mutant protein. **(D)** Comparative analysis of cysteine content of both i.e. WT and mutant. **(E)** Mole of free cysteine content/mol of protein.



Supplementary Figure S7: Thermal inactivation kinetics of *Ic*SODs. The data were fit as first **(A)**, zero **(B)**, and second order reactions **(C)**, wherein the first order reaction gave the most linear relationship and as reflected by R^2 value shown in the inset.



Supplementary Figure S8: Concentration dependent effect of various inhibitors on WT and mutated *Ic*SOD superoxide dismutase activity. Inhibitors used were (**A)** Sodium azide (**B)** Potassium cyanide (**C**) Hydrogen peroxide **(D)** Diethyldithiocarbamate. Closed circle (●) with solid line represents the WT while open circle (○) with dotted line represents mutant form of *Ic*SOD. The residual activity was calculated taking 1 unit of SOD as 100%.



Supplementary Figure S9: Near UV-CD spectral analysis of WT and mutated *Ic*SOD. Room temperature (25^{o}C) near UV-CD spectral analysis of WT (dotted line) and mutated *Ic*SOD (solid line). The presence of negative bands at 260 and 267 denotes for phenyl alanine and for tyrosine at 271 nm (B). As expected no signature of tryptophan (generally at 280 and 293 nm) is discernible since the protein is devoid of this amino acid. The data indicate that as compared to wild the CD intensity is reduced for the mutant dimer (A).

Supplementary methods:

Overexpression and purification of WT and variant (S95C) recombinant *Ic*SOD protein

E. coli M15 cells harboring the pQE30- *Ic*SOD WT and the variant plasmid were grown in 2xYT medium (tryptone 16g, yeast extract 10g and NaCl 5g L^{-1}, pH 7.0), supplemented with 100 µg ml^{-1} ampicillin and 25 µg ml^{-1} kanamycin on an orbital shaker (240 rpm) at 37 ^{o}C. When the cell density of the culture reached a value of 0.6 absorbance at 600 nm, isopropyl β-D-Thiogalactopyranoside (IPTG) was added to a final concentration of 1mM and the culture was allowed to grow for another 5-h. Presence of the recombinant protein in the soluble fraction was checked following the protocol as described earlier[1] (Supplementary Figure S1). Supplementation of $CuSo_4$ and $ZnSo_4$ in the growth media during induction period didn't cause increase in activity or protein yield of the *Ic*SOD proteins. To purify the proteins in native confirmation, IPTG induced culture was harvested by centrifugation (8000 x g for 20 min), and the protein was affinity purified using Ni-NTA Fast Start columns with slight modification from manufacturer's instructions. Briefly, the pellet was re-suspended in lysis buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 1mg ml^{-1} lysozyme, 25 unit ml^{-1} of benzonase nuclease and protease inhibitor cocktail specific for histidine tagged proteins. The suspension was sonicated at ice temperature to lyse the cells and the clear lysate obtained after 14,000 x g centrifugation (30 min at 4^{0}C) was loaded onto Ni-NTA column, and washed with washing buffer containing 20 mM imidazole. The enzyme was finally eluted with solution containing 250 mM imidazole. The enzyme sample was de-salted and exchanged with low concentration phosphate buffer (5 mM K-PO_4, pH-7.2) using Zeba Desalt Spin Columns, Dialysed further for 48 hour and concentrated using Amicon Centriprep YM-10 centrifugal filter devices (Millipore).

UV-Visible and CD measurements

Room temperature (25 ^{0}C) UV-Vis absorbance characteristics of the purified protein (1mg ml^{-1}in 5 mM K-Po_4 buffer, pH 7.2) was recorded from 800 nm to 200 nm with a Varian Cary 300 Bio double - beam spectrophotometer.

The CD spectra were recorded on a Jasco J-715c spectropolarimeter with a peltier type cell holder. For wavelength scan measurements were recorded with 2.5 µM (far UV) and 41 µM (near UV) of protein samples in a 1-mm path length quartz cuvette with a scan rate of 50 nm min^{-1}. The response time and the bandwidth were maintained at 4s and of 2 nm respectively. Buffer spectra were also acquired under identical conditions and subtracted from the protein spectra before analysis. The thermal unfolding of the protein (from 25^{o}C to 95^{o}C) was

monitored using Jasco PTC-348WI spectropolarimeter at 209 nm. Protein concentration was 10 μM, time constant was 4 s, and the scan rate was 0.5 C/min. T_m was calculated by fitting the thermal unfolding data as described earlier[2,3].

Differential scanning calorimetry **(**DSC) experiments were performed using micro-calorimeter. Protein samples were dissolved at 1mg ml^{-1} concentration, dialyzed against 5 mM potassium phosphate (pH 7.2), and de-aerated under mild vacuum for 30 min before loading in the sample cell. The reference cell was filled with de-aerated dialysis buffer. A scan rate of 60 ^0C h^{-1} was used in all the experiments. At each run the value of buffer as base line was subtracted from the sample. The reversibility of thermal transitions was checked by a second heating cycle of the same sample immediately after cooling the sample. Data analysis was performed with the software package (Origin) after subtracting a progress line. Analyses of the DSC scans were carried out assuming the calorimetric transition to be single two-state transition with subunit dissociation.

All spectral analysis was made from the average of 3 to 4 individual scanning with 3 different protein preparations.

Effects of temperature and pH on SOD activity

Effects of elevated temperatures on the enzyme activity were examined by incubating 50 units of each protein at different temperatures (25 ^oC - 75 ^oC) in 5 mM K-PO_4 buffer (pH-7.2). 5 units equivalent aliquots were withdrawn after 1hour, kept immediately on ice for 10 minutes, centrifuged and the residual enzymatic activity was assayed taking 1 unit of enzyme. Similarly, The effect of pH was examined by incubating 50 units of the enzyme in 50 mM buffers of different pH value(pH 4.0-5.0-6.0 citric acid-citrate; pH 6.0-7.0-8.0 K-PO_4; pH 9.0-10.0 glycine-NaOH) for 30 min and assayed using 1 unit of enzyme[1].

References:

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3 C. N. Pace, B. A. Shirley and J. A. Thomson, *Oxford IRL Press Limited*, 1989, 311-330.