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Calorimetric evidence for different structural roles of Glu132 and Glu133 residues in human superoxide dismutase

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Abstract

DSC measurements have been performed on human superoxide dismutase (HSOD) and on its mutants on the Glu132 and Glu133 residues which have been alternatively modified to a Gln residue. In both cases, the substitution has dramatic effects on the thermal denaturation of HSOD as evidenced by the calorimetric experiments. In particular, replacement of the Glu residue in position 132 seems to have a greater effect on the stabilization of the protein, highlighting the key role played by this position. All the experimental data are qualitatively explained in terms of interactions between the groups which form the active site channel.

Keywords: Site-directed mutagenesis; Structural stability; Superoxide dismutase; Thermal denaturation

1. Introduction

Superoxide dismutase (SOD) is a dimeric enzyme of MW 32000, which contains a copper and a zinc ion in each identical subunit [1]. The physiological role of SOD, i.e. the ultimate reason for the intense research on this enzyme, is the dismutation of superoxide radicals which are produced during the oxygen metabolic cycle and which are extremely reactive towards cells [2].

Copper, which is essential for the catalysis, is at the bottom of a wide channel bearing numerous charged groups. Some of the residues present in the active site channel have been shown, by studies on mutants obtained through site-directed mutagenesis, to play

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an important role in the structural and catalytic properties of the enzyme [3–9]. Among the residues that form the active site channel, i.e. Arg143, Thr58, Lys136, Glu132 and Glu133, the last two groups, which bear negative charges, have been thought to have an important function in the correct orientation of the substrate at the entrance of the channel [10, 11].

SOD and some of its mutant forms have been the object of a large number of theoretical [12–17] and experimental papers [18–20], which all agree in highlighting the fundamental role played by electrostatic interactions in the enzymatic behavior. In contrast, no experimental data concerning the thermal behavior of SODs mutated at the above-mentioned strategical positions are available in the literature.

However, it is well known that differential scanning calorimetry (DSC) of mutated proteins provides a very powerful tool in understanding the ways in which the stability of a particular protein is altered by changes in the primary structure resulting from mutations [21, 22]. These studies are usually limited to overall conformation energies. In fact, a recent paper [23] illustrates very clearly the formidable difficulties involved in reaching a detailed understanding of the energetic results of a single amino acid replacement in a protein. Nevertheless such data have proven to be useful in assessing the effects resulting from amino acid replacement and in attempting to correlate these effects with observed changes in structure, stability and activity, as well as supplying the numbers with which the results of theoretical calculations can be compared.

In this work we have carried out DSC measurements on human SODs in which the negative Glu residue in position 132 and, alternatively, 133 is replaced through site-directed mutagenesis by Gln, a residue which is neutral and with approximately the same bulkiness as Glu.

As we will show, the apparent excess heat capacity curves of the mutated form of human superoxide dismutase (HSOD), compared to those of the wild-type SOD, show dramatic changes in the thermal stability of the enzyme. This confirms, on the one hand, the difficulties arising when one attempts to correlate primary structure to the overall stability of the enzyme, and on the other, the importance of the position of charged groups in stabilizing the enzyme structure.

2. Materials and methods

2.1. Chemicals

Wild-type superoxide dismutase from human erythrocytes (HSOD-WT) was purchased from Sigma Chemical Co. (St. Louis, USA) and used without further purification. The purity of this enzyme was approximately 98%. The two mutant forms of HSOD in which the Glu132 and, alternatively, the Glu133 residues are substituted by Gln, were prepared and expressed in *Escherichia coli* cells in the laboratory of Prof. I. Bertini using a previously-reported procedure [24]. The mutated protein was purified by ion exchange chromatography on DEAE Sepharose CL-6B (Pharmacia). Further details can be found in Refs. [3–7]. Protein concentration was determined from the dry weight of the pure protein powder for all the protein solutions. Potassium phosphate

and sodium chloride (analytical grade) were purchased from Fluka Chemie AG (Buchs, Switzerland).

2.2. Differential scanning calorimetry (DSC)

DSC scans were carried out with a Setaram (Lyon, France) micro-differential scanning calorimeter (microDSC) with stainless steel 1 ml sample vessels, interfaced with a Bull 200 Micral computer. The sampling rate was 1 point per second in all measuring ranges. All the protein samples (2.6 mg ml^{-1}) were dissolved in 50 mM phosphate buffer at pH 7.03. The ionic strength was adjusted to 0.1 M by NaCl. The same solution without protein was used in the reference vessel. Both the sample and reference were scanned at a heating rate of 0.5°C min^{-1}, from 30 to 100°C with a precision of ±0.08°C.

In order to obtain the apparent molar heat capacity (C_p) curves, buffer–buffer baselines were obtained at the same scanning rate and then subtracted from sample curves. The excess apparent molar heat capacity (C_{pexc}) curves were routinely obtained as previously reported [25]. In the experimental conditions reported, the average level of noise was about ±0.4 μW and the reproducibility at refilling was about 0.1 mJ K^{-1} ml^{-1}.

The experimental uncertainties, in enthalpy and temperature, calculated by repeating each experiment at least three times, are reported in Table 1.

Calibration in energy was obtained by giving a definite power supply, electrically generated by an EJ2 Setaram Joule calibrator, within the sample vessel.

3. Results and discussion

In Fig. 1, we report the apparent molar excess heat capacity (C_{pexc}) profiles relative to the thermal denaturation of wild-type human superoxide dismutase (HSOD-WT), and the Glu132→Gln and Glu133→Gln mutant proteins (Glu132→Gln is HSOD mutated at position 132, and Glu133→Gln is HSOD mutated at position 133).

The corresponding excess molar calorimetric denaturation enthalpy (ΔH_{exc}) values, calculated by integration of the peaks, and the temperature corresponding to the maximum of the C_{pexc} profiles (T_m) are reported in Table 1.

Our calorimetric data demonstrate two important features: (i) The replacement of a Glu residue by a Gln, either in position 132, or in position 133, involves noticeable effects on the thermal stability of each species in respect to HSOD-WT; (ii) The thermal stability of the enzyme depends on the location of the mutation.

In particular, this mutation increases in any case the thermal stability of the enzyme: for the Glu133→Gln mutant, T_m is 9°C higher than HSOD-WT, and for the Glu132→Gln form, it is 12°C higher than HSOD-WT.

Moreover, after mutation, either in position 132 or 133, the cooperativity of the transition increases, as can be noted from the sharpness of the corresponding calorimetric peaks. These results underline the fact that in a complex and cooperative system, an effect placed in a single residue can involve dramatic changes in the whole

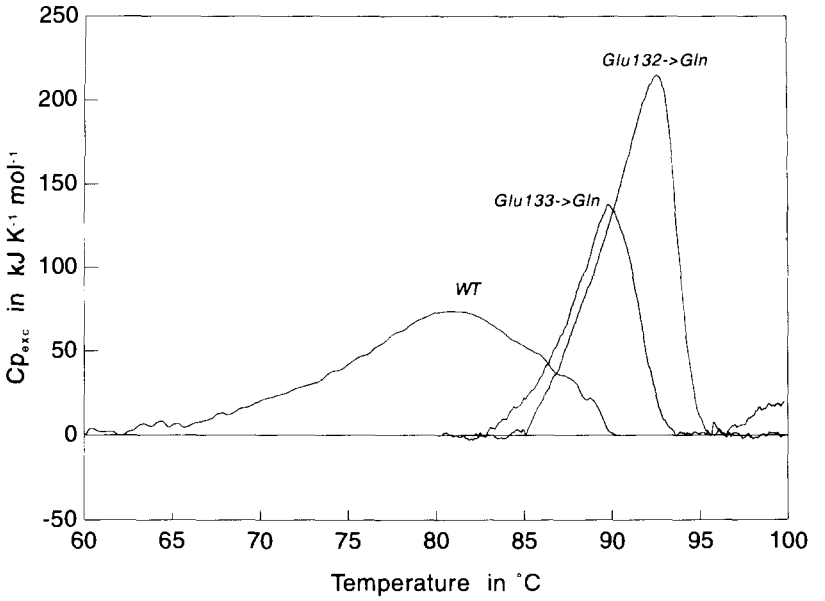

Fig. 1. Excess apparent molar heat capacity (C_{pexc}) of HSOD-WT and its mutants Glu132→Gln and Glu133→Gln.

Table 1
Excess calorimetric enthalpies (ΔH_{exc}) and maximum heat capacity temperatures (T_m) for the thermal denaturation of HSOD-WT and the mutant forms Glu132→Gln and Glu133→Gln

	ΔH_{exc}/kJ mol^{-1}	T_m/°C
HSOD-WT	1007 ± 56	80.7 ± 0.1
Glu132Gln	1136 ± 61	92.5 ± 0.1
Glu133Gln	535 ± 28	89.6 ± 0.1

structure. This is much more evident if we compare the calorimetric curves of the two mutants Glu132→Gln and Glu133→Gln.

The calorimetric excess enthalpies differ noticeably depending on the position of the substitution. In particular, looking at Table 1, we note a small increase in ΔH_{exc} when the substitution is placed in position 132, and a dramatic decrease when the substitution is placed in position 133.

A detailed understanding of the molecular origin of these macroscopic effects is beyond the aims of this short communication; nevertheless, on the basis of a recent study [12] and our data, we can affirm that the elimination of the carboxylate group of the Glu can involve, depending on position, important changes in the interactions with the other residues that form the active site channel.

Molecular dynamics (MD) studies carried out on SOD-WT and some of its mutants [12] have shown that all the amino acid residues in the range 119–143 show great

mobility. Among these, Glu132, Glu133, Lys136 and Arg143, all charged residues present in the active site channel, undergo large movements, allowing them to interact each other, with the ions, or with the solvent. This high flexibility of the active site channel, combined with the local electrostatic fields, is thought to be determinant for a correct orientation and access of the substrate to the catalytically active zone. In particular, the residues Glu132 and Glu133 have an important role in determining the shape and the flexibility of the channel. In fact it has been shown that Lys136 interacts with the negative carboxylate group of Glu132. Moreover, the carboxylate group of Glu133 is H-bonded to the OH moiety of Thr137. All these interactions are believed to create a network of H-bonds and electrostatic interactions which are essential not only for the activity of the enzyme, but also in determining the structure, and, as evidenced in the present communication, the stability of the native enzyme.

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