SHORT COMMUNICATION

Effect of Lys→Arg mutation on the thermal stability of Cu,Zn superoxide dismutase: influence on the monomer–dimer equilibrium

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The thermal stability of two single (K3R, K67R) and one double (K3R-K67R) mutants of Xenopus laevis B Cu,Zn superoxide dismutase has been studied to test Lys→Arg substitution as an ‘electrostatically conservative’ strategy to increase protein stability. The K3R mutant displays an increased thermostability with respect to the wild-type enzyme, whilst a decreased stability was observed in the case of the K67R and K3R-K67R mutants. Concentration dependence of the apparent inactivation constant (k_{app}) of the latter mutants, as compared to that of the wild type enzyme and K3R mutant, indicates that their higher sensitivity to heat inactivation is due to a perturbation of the dimer association. These results are confirmed also by fluorescence anisotropy measurements of the internal probe Tyr149. The possible role of Arg67 in perturbing the dimer dissociation equilibrium toward the monomeric form is discussed.

Keywords: fluorescence anisotropy/heat stability/long-range interactions/molecular modelling/monomer–dimer equilibrium

Introduction

Increasing the thermostability of proteins has long been of interest to biochemists. An approach to the understanding of the chemical basis for the thermal stability of biological macromolecules has been the comparative analysis of homologous proteins from mesophilic and thermophilic organisms (Argos et al., 1979). One of the conclusions that has been derived from this kind of analysis is that the ratio of Arg: (Lys + Arg) in proteins is related to the optimum growth temperature of the organisms from which the proteins are isolated (Merker et al., 1981). Experimental evidence for the stabilizing effects of the Lys→Arg substitution has been recently reported for three structurally unrelated proteins, namely xylose isomerase, glyceraldehyde-3-phosphate dehydrogenase and human Cu,Zn superoxide dismutase (Mrabet et al., 1992).

In this work, we have investigated the stability of two single and one double Lys→Arg mutants of Xenopus laevis B Cu,Zn superoxide dismutase B (XSODB) at positions 3 and 67 (K3R, K67R and K3R-K67R) by irreversible thermal denaturation and fluorescence anisotropy. XSODB, a highly stable dimeric enzyme (Bannister et al., 1987), which contains a copper atom and a zinc atom in the active site (Tainer et al., 1982), has been cloned and expressed in our laboratory (Battistoni et al., 1992) and its structure has been solved at 1.5 Å resolution (Djinovic-Carugo et al., 1996). The results indicate that K3R XSODB is more stable than the wild type enzyme and K3R-K67R to thermal denaturation, while the opposite effect is observed in the case of K67R. In K67R and K3R-K67R, the mutation surprisingly affects the dimer dissociation equilibrium, even if Lys67 is not directly involved in the interaction between monomers. This finding should caution towards Lys→Arg substitution as an indiscriminate strategy to increase protein stability and points out the role of long-range interactions between residues in determining the properties of protein molecules.

Materials and methods

Choice of the mutants

Lysines 3 and 67 have been chosen for replacement by arginine from the nine lysine residues present in the XSODB monomer, when analysing the recently solved 1.5 Å resolution structure of the enzyme (Djinovic-Carugo et al., 1996). It must be noted that Lys3 or XSODB is not homologous to the Lys9 residue mutated into arginine in a similar study carried out on the human enzyme (Mrabet et al., 1992). Inspection of the XSODB structure indicated that mutation of lysines 3 and 67 to arginine could potentially lead to the formation of new charge–charge interactions between the inserted guanidinium group and neighbouring polar and charged atoms. Lys3 lays near the dimer interface and is involved in a salt bridge with the C-terminal Pro150. The Glu side chain, whose carboxyl group is ~6 Å away from the Lys3 Ne, is located on the opposite side; mutation of Lys3 to Arg, therefore, is expected to give rise to a stronger interaction with Glu21. Lys67 is located in the second part of loop 6,5 (the zinc-binding loop); it is highly solvent accessible and does not strongly interact with either the nearby chain carboxyl groups of Glu65 and Asn66 or with the carboxyl groups of Glu65 and Asp78 (both ~6 Å distant from the Ne of Lys67).

Molecular modelling of mutant proteins

Three-dimensional structures of the mutant enzymes have been computer modelled using the program Sybyl by Tripos Associates. In all the mutants no steric clashes have been observed between the inserted side chain and the surrounding protein environment. The model structures, including the crystallographic water shell, have been energy minimized using the program MOIL (Elber et al., 1995). After minimization of the K3R mutant, the side chain of Glu21 bends and moves in both subunits at only 3 Å distance from the Arg3 Ne, making the interaction between the two side chains stronger. In the case of the K67R mutant, the Arg67 side chain is in an extended and solvent-accessible conformation and no relevant change in the protein region surrounding this residue is observed upon minimization.

Site-directed mutagenesis and protein purification

The nucleotide sequences containing the two single and the double mutations were subcloned in the expression plasmid pKK233–2. Wild type and mutant enzymes were produced in
Enzyme concentration (5 uM) all the samples show very similar enzyme concentration (Figure 2), indicates that at a high A plot of the RfcjppS of the four samples as a function of the from altered kinetic barriers or from thermodynamic effects. The inactivation stability between the wild type and mutant enzymes results however it is not possible to determine whether the difference degree of stability: K3R > K3R-K67R = wt > K67R.

(\(k^{*}\), order rate constant) allows us to derive the following reaction scheme for the inactivation process:

\[
D \leftrightarrow 2M \rightarrow 2U
\]

where D is the dimer, M the monomer and U the unfolded protein.

Solving the first equilibrium one can obtain

\[
K_d = [M]^2[D]
\]

from which

\[
[M] = (K_d[D])^{1/2}
\]

The unfolding rate is defined as

\[
dU/dt = k_u[M]
\]

and, hence,

\[
dU/dt = k_u(K_d[D])^{1/2}
\]

Since the inactivation rate, defined as \(-dA/dt = k_{app} A\), is proportional to the unfolding rate of the enzyme, at a given dimer concentration, \(k_{app}\) is proportional to \(k_u(K_d)^{1/2}\).

Fluorescence spectroscopy
Fluorescence anisotropy experiments have been performed using an ISS PCl photon-counting spectrofluorometer (ISS Inc., Champaign, IL). Data were recorded at 25°C, with a bandpass of 4 nm in excitation and no monochromator in emission. Scattered light was eliminated with a WG 305 cut-off filter and by blank subtraction. To minimize the overlapping of Raman scattering from water with the emission spectrum of tyrosine, the excitation wavelength was set to 270 nm. The protein concentration was determined by absorption at 258 nm (\(\varepsilon_{285} = 10.3 \text{ mM}^{-1} \text{cm}^{-1}\); Rotilio et al., 1972).

Results and discussion
The effect on protein stability of Lys→Arg mutations was studied by irreversible thermal denaturation experiments (Matthews et al., 1987; Mrabet et al., 1992). The relative activity at 70°C of the wild type and mutant XSODBs is reported in Figure 1 as a function of the incubation time. For all the samples the relative activity decreases during the treatment following first-order kinetics. Evaluation of the apparent first-order rate constant (\(k_{app}\)) allows us to derive the following degree of stability: K3R > K3R-K67R = wt > K67R. However it is not possible to determine whether the difference in stability between the wild type and mutant enzymes results from altered kinetic barriers or from thermodynamic effects. A plot of the \(k_{app}\)s of the four samples as a function of the enzyme concentration (Figure 2), indicates that at a high enzyme concentration (5 μM) all the samples show very similar apparent first-order kinetics. At a lower enzyme concentration ([XSODB] < 1 μM), the \(k_{app}\) of the K67R mutant rapidly increases; such an increase is lower for the double mutant, but still higher than that of the wild type. The \(k_{app}\) of the K3R mutant is the lowest one. The concentration dependence of the thermal inactivation of XSODB indicates, in agreement with recent unfolding studies (Mei et al., 1992), that denaturation of the protein occurs in at least two steps, the first of which involves the dimer to monomer equilibrium:

\[
D \leftrightarrow 2M \rightarrow 2U
\]

According to the scheme described above, the apparent first-order rate constant, \(k_{app}\), is proportional to \((K_d^{1/2}k_u)\) (see Materials and methods). Different values of \(k_{app}\) for the mutants, with respect to the wild type, may be due to differences in \(K_d\) in \(k_u\) or in both.

Figure 2 shows that the K67R and double mutants have higher \(K_d\) and/or \(k_u\) values (higher \(k_{app}\) values) with respect to the wild type; the opposite effect is observed in the K3R mutant (lower \(k_{app}\) values).

To obtain further information concerning the monomer–dimer equilibrium of the wild type and mutant XSODBs, fluorescence anisotropy of the different samples, as a function

![Figure 1](image1.png)

Fig. 1. Plot of the thermal inactivation of wild type and mutant XSODBs. The lines represent the best fit of the experimental data. The inactivation experiments were carried out at 70°C, with 1 μM enzyme in 10 mM phosphate buffer, pH 7.4. Aliquots of the samples were withdrawn at intervals and assayed for residual superoxide dismutase activity at 30°C using the pyrogallol method (Marklund and Marklund, 1974).

![Figure 2](image2.png)

Fig. 2. Plot of the inactivation constant (\(k_{app}\)) versus the XSODB concentration for the wild type, the two single mutants (K3R and K67R) and the double mutant (K3R-K67R). The values of \(k_{app}\) were obtained by best fitting of the logarithm of the residual activity as a function of incubation time: \(k_{app} = -d\log A/dt\).
of protein concentration, has been measured by following the fluorescence emission of the only tyrosine residue present in the XSODB monomer (Tyr149). A lower fluorescence anisotropy is expected for the monomeric with respect to the dimeric form of the protein, assuming a constant fluorescence intensity lifetime (Lakowicz, 1983). At 5 μM protein concentration, the fluorescence anisotropy of the four samples is similar (Figure 3) and only slightly higher for the K67R and K3R-K67R proteins, likely due to perturbation of the tyrosine environment. As the protein concentration decreases, the fluorescence anisotropy is constant for the wild type and K3R mutant, whilst it rapidly decreases for the K67R and double mutants. In all four samples the fluorescence intensity lifetime remains constant at any concentration, as indicated by the relative fluorescence quantum yields (data not shown). These results indicate that, in the concentration range investigated, the wild type and K3R mutant are always in the dimeric form whilst the K67R and double mutants undergo monomerization under the micromolar concentration range, clearly indicating that the mutation of Lys67 into Arg favours the formation of the monomeric form of the enzyme. Nevertheless, despite the different monomer–dimer equilibrium displayed by the wild type with respect to the K67R mutant, their activity is identical (data not shown), suggesting that the monomeric species is fully active. A comparable Kd constant may be inferred for the K67R and K3R-K67R mutants, because of the identity of their fluorescence anisotropy values (Figure 3), implying that the differences observed in k_{app} between the two mutants are due to a lower value of k_d for the K3R-K67R mutant. Analogous considerations apply to the wild type and the K3R mutant which have similar K_d values (Figure 3) but different k_{app} values (Figure 2), again because of a lower k_d for the mutant.

In XSODB, the Lys3→Arg substitution does not perturb the monomer–dimer association equilibrium but does decrease the unfolding rate of the protein, probably because of the formation of a stronger electrostatic interaction between Arg3 and Glu21, as observed after minimization of the K3R mutant (see Materials and methods). On the other hand, the Lys67→Arg mutation increases the dimer dissociation constant, K_d, explaining the higher k_{app} values displayed by K67R with respect to the wild type. No conclusion can be inferred about the effect of mutation on k_d. Data obtained on the double mutant confirm the effect of the Lys67 mutation, since the K3R-K67R mutant displays the same K_d as K67R. Moreover, the k_{app} for the double mutant is lower than that of K67R, indicating a lower k_d for the double mutant and confirming the stabilizing effect of the Lys3→Arg mutation.

The increase in K_d in the K67R and K3R-K67R mutants is surprising in that Lys67 is located far away from the monomer–monomer interface; however, this residue is located in the second part of loop 6,5 (the zinc-binding domain) stabilizing the loop conformation via a double hydrogen bond between its main chain polar groups and the side chain of Asn63. Mutation of Lys67 is likely to introduce a local perturbation of this hydrogen bonding network making the zinc-binding sub loop less ordered. This perturbation can affect the dimer association modifying the two closest intersubunit contacts, due to the hydrogen bonds between the invariant residues Gly49 and Gly112 of one monomer and the main chain of Tyr149 of the other monomer. An X-ray study is in progress in order to understand the fine structural details of K67R mutation.

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References

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Fig. 3. Steady-state fluorescence anisotropy of Tyr149 in the wild type (full circles), K3R (open circles), K67R (open squares) and K3R-K67R (open diamonds) XSODBs as a function of protein concentration.