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The hyperthermophilic nature of the metallo-oxidase from *Aquifex aeolicus*

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ABSTRACT

The stability of the *Aquifex aeolicus* multicopper oxidase (McoA) was studied by spectroscopy, calorimetry and chromatography to understand its thermophilic nature. The enzyme is hyperthermostable as deconvolution of the differential scanning calorimetry trace shows that thermal unfolding is characterized by temperature values at the mid-point of 105, 110 and 114 °C. Chemical denaturation revealed however a very low stability at room temperature (2.8 kcal/mol) because copper bleaching/depletion occur before the unfolding of the tertiary structure and McoA is highly prone to aggregate. Indeed, unfolding kinetics measured with the stopped-flow technique quantified the stabilizing effect of copper on McoA (1.5 kcal/mol) and revealed quite an uncommon observation further confirmed by light scattering and gel filtration chromatography: McoA aggregates in the presence of guanidinium hydrochloride, i.e., under unfolding conditions. The aggregation process results from the accumulation of a quasi-native state of McoA that binds to ANS and is the main determinant of the stability curve of McoA. Kinetic partitioning between aggregation and unfolding leads to a very low heat capacity change and determines a flat dependence of stability on temperature.

1. Introduction

The multi-copper oxidases (MCOs) constitute a family of enzymes whose principal members are ceruloplasmin (Fe(II) oxygen oxidoreductase, EC 1.16.3.1), ascorbate oxidase (L-ascorbate oxygen oxidoreductase, EC 1.10.3.3) and laccase (benzenediol oxygen oxidoreductase, EC 1.10.3.2) [1,2]. This family of enzymes is widely distributed throughout nature and members are encoded in the genomes of organisms in all three domains of life — *Bacteria*, *Archaea* and *Eukarya* [3]. MCOs that contain ~500 amino acid residues are composed of three Greek key β-barrel cupredoxin domains (domains 1, 2 and 3) that come together to form three spectroscopically distinct types of Cu sites, i.e. type 1 (T1), type 2 (T2), and type 3 (T3) [1,2]. The T1 Cu site is characterized by an intense Cys-S π to Cu^{2+} charge transfer absorption at about 600 nm responsible for the blue colour of these enzymes. The T2 Cu site is characterized by the lack of strong absorption features. The T3 Cu site is composed of two Cu atoms typically antiferromagnetically coupled, for example, through a hydroxide bridge. It is characterized by an intense transition at 330 nm originating from the bridging ligand that is apparent as a shoulder on the protein absorbance band at 280 nm. T1 mononuclear copper site is the primary acceptor site for electrons derived from the reducing substrate while the T2 and T3 sites form a trinuclear centre that is the site for O_2 reduction [1–3]. Multi-copper oxidases have broad substrate specificity and oxidise numerous aromatic phenols and amines. Only a few members present higher specificity to lower valent metal ions such as Mn^{2+}, Fe^{2+} or Cu^{1+}, being thus designated as metallo-oxidases [3]. The best studied metallo-oxidases are human ceruloplasmin, yeast Fet3p and bacterial CueO which are suggested to play an *in vivo* catalytic role in the maintenance of both copper and iron homeostasis in their respective organisms.

We had recently cloned, overproduced, purified and characterized a recombinant metallo oxidase (McoA) from *Aquifex aeolicus*, a hydrogen-oxidising, chemolitoautotrophic bacterium that grows between 58 and 95 °C and optimally at 89 °C, occupying the deepest branch of the bacterial phylogenetic tree [4,5]. We found that McoA is a copper-activated metallo-oxidase with spectroscopic properties typical of MCOs. However one particular aspect of McoA is the presence of a methionine rich region segment (residues 321–363) evidenced in the comparative model structure, reminiscent of those found in copper homeostasis proteins. Reaction kinetic analysis of the wild type enzyme and the deletion mutant (McoAΔP321-V363) without the methionine rich segment indicates that this region is near the T1 Cu catalytic centre and is most probable involved in the catalytic mechanism through copper binding [4].

The study of the hyperthermophilic nature of *A. aeolicus* McoA offers an opportunity to get insight into protein general mechanisms of thermostability and into stability mechanisms of this particular family of enzymes. Studies on the stability of MCOs have focused mainly on

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thermal stability measured by differential scanning calorimetry [6–10]. The study of unfolding pathways using chemical denaturants has been mostly performed for the small blue copper proteins which only have a T1 copper centre [11–15] besides a recent report with human MCO ceruloplasmin [16]. The stability of McoA was assessed in this work by using spectroscopic techniques, differential scanning calorimetry (DSC) and gel-filtration chromatography. Stopped-flow kinetics was performed to obtain additional information regarding the mechanism of unfolding and the role of copper in the stabilization of McoA. To our knowledge this is the first report on studies of MCOs based on unfolding kinetics. Their measurement quantified the increase in McoA stability due to copper binding and revealed the importance of aggregation processes on McoA stability. Among the different possible mechanisms responsible for its high thermostability McoA features an extremely flat dependence of stability on temperature.

2. Experimental procedures

2.1. Protein expression and purification

Purification of recombinant McoA from *A. aeolicus* was performed as described before [4]. Apo-McoA was obtained by incubating McoA in 20 mM Tris HCl buffer, pH 7.6 in the presence of EDTA (5 mM) for 1 h followed by dialysis. Protein copper content of McoA forms was determined using the trichloroacetic acid/bicinchoninic acid method of Brenner and Harris [17] and confirmed by atomic absorption spectroscopy (Instituto Superior Técnico, Universidade Técnica de Lisboa).

2.2. UV–visible spectra

UV–visible spectra were acquired using a Nicolet Evolution 300 spectrophotometer from Thermo Industries.

2.3. Enzyme assays

The enzymatic activities were routinely assessed at 40 °C using syringaldazine (SGZ) as substrate. The assay mixtures contained 0.1 mM SGZ, 20 mM Tris–HCl buffer pH 7.6 and the reactions were followed at 530 nm (ε_{530}=65,000 M^{-1} cm^{-1}). The protein concentration was measured by using the absorption band at 280 nm (ε_{280}=75,875 $M^{-1}cm^{-1}$).

2.4. Equilibrium unfolding studies

Steady-state fluorescence was measured in a Cary Eclipse spectrofluorimeter using 2 μM of McoA and 296 nm as excitation wavelength. Increased guanidinium hydrochloride (GdnHCl) concentrations were used to induce proteins unfolding at pH 7.6 (20 mM Tris–HCl buffer) and pH 3 (50 mM glycine buffer). To monitor unfolding of McoA, a combination of fluorescence intensity and emission maximum was used as described by Durão et al. [7]. Unfolding of McoA was difficult to quantify through total emission (by integrating the fluorescence emission at different wavelengths) or single wavelength fluorescence emission as the difference between the emission from folded and unfolded states is not very significant. The combination of fluorescence intensity and emission maximum decreases data scatter and gives very similar stability parameter values compared to the ones obtained from total fluorescence emission.

The thermodynamic stability of McoA monitored by fluorescence was analyzed according to a two-state process using the following equations:

$$y = y_F f_F + y_U f_U \quad (1)$$

$$K_{(U-F)} = f_U / f_F \quad (2)$$

$$\Delta G_{(U-F)} = -RT \ln K_{(U-F)} \quad (3)$$

$$\Delta G_{(U-F)} = \Delta G_{(U-F)}{}^{water} - m_{(U-F)}[GdnHCl] \quad (4)$$

$$[\mathrm{GdnHCl}]_{50\%} = \Delta G_{(U-F)}{}^{water} / m_{(U-F)} \quad (5)$$

where F and U are folded and unfolded states of McoA, respectively, y is the fluorescence signal, f is the fraction of McoA molecules with a given conformation, K is the equilibrium constant, ΔG is the standard free energy, $m_{(U-F)}$ is the linear dependence of ΔG on GdnHCl concentration and $[\mathrm{GdnHCl}]_{50\%}$ is the GdnHCl concentration for ΔG=0. y_F and y_U were calculated directly from the pre- and post-transition regions according to a linear dependence. Copper bleaching/depletion and loss of activity induced by GdnHCl were performed at 40 °C as described by Durão et al. [7] and quantified by using Eqs. (1–5) but assuming an equilibrium that describes copper bleaching/depletion from the folded state. Copper bleaching resulting from reduction of the T1 Cu site cannot be distinguished from copper depletion and were used indistinctly.

2.5. Quenching of fluorescence by acrylamide

Quenching of tryptophyl fluorescence was carried out by titrating a McoA solution with a stock solution of acrylamide at room temperature in 20 mM Tris–HCl, pH 7.6 with 200 mM NaCl. Excitation was at 296 nm and total fluorescence emission (F) was integrated from 305 to 420 nm. The total fluorescence was then corrected to account for acrylamide absorbance as described elsewhere [18]. Data was fitted to the Stern–Volmer equation:

$$F_0/F = 1 + K_{SV}[Q] \quad (6)$$

where F_0 and F are the total fluorescence emission in the absence and presence of quencher, respectively, K_{SV} is the Stern–Volmer quenching constant and [Q] is the acrylamide concentration.

2.6. Thermal denaturation

DSC was carried out in a VP-DSC from MicroCal at a scan rate of 60 °C/h. The experimental calorimetric trace was obtained with 0.3 mg/mL of protein at pH 3 (50 mM glycine buffer) and a baseline obtained with buffer alone was subtracted from the experimental trace. The resulting McoA DSC trace was analyzed with the DSC software built within Origin spreadsheet to obtain the transition excess heat capacity function (a cubic polynomial function was used to fit the shift in baseline associated to unfolding). The excess heat capacity can only be accurately fitted using a non two-state model with three transitions (equation in the data analysis software).

2.7. Gel filtration chromatography

Gel filtration chromatography was carried out in a Superose 12 HR10/30 column (GE Healthcare) using 20 mM Tris–HCl, pH 7.6, 200 mM NaCl as eluent. For runs in the presence of GdnHCl the column was previously equilibrated with the same buffer containing the desired concentration of GdnHCl. Samples of McoA at 0.5 mg/mL concentration were incubated in 0–4 M GdnHCl for 1 h at room temperature before injection. Ribonuclease (13.7 kDa), chymotrypsinogen A (25 kDa), ovalbumin (43 kDa), albumin (67 kDa), aldolase (158 kDa) and catalase (232 kDa) were used as standards.

2.8. Stopped-flow kinetics

Kinetic experiments were carried out on an Applied Photophysics Pi-Star 180 instrument with fluorescence intensity detection. The