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Temperature and pressure dependence of azurin stability as monitored by tryptophan fluorescence and phosphorescence. The case of F29A mutant

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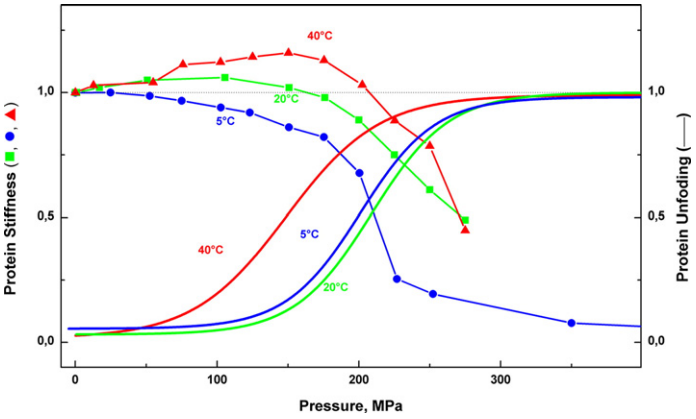
HIGHLIGHTS

- Thermodynamic characterization of F29A azurin unfolding induced by pressure.
- The engineered cavity appears filled, at least partially, with water molecules.
- Pressure decreases F29A flexibility at high temperature.
- Protein hydration dominates on compaction at lower temperatures.
- The position of the engineered cavity is not a key factor for protein stability.

GRAPHICAL ABSTRACT



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ABSTRACT

The effects of a single-point, F29A, cavity-forming mutation on the unfolding thermodynamic parameters of azurin from *Pseudomonas aeruginosa* and on the internal dynamics of the protein fold under pressure were probed by the fluorescence and phosphorescence emission of Trp48, deeply buried in the compact hydrophobic core of the macromolecule.

Pressure-induced unfolding, monitored by the shift in the fluorescence spectrum, led to a volume change of 70–90 ml mol^{-1}. The difference in the unfolding volume between F29A and wild type azurin was smaller than the volume of the space theoretically created in the mutant, indicating that the cavity is, at least partially, filled with water molecules. The complex temperature dependence of the unfolding volume, for temperatures up to 20 °C, suggests the formation of an expanded form of the protein and highlights how the packing efficiency of azurin appears to contribute to the magnitude of internal void volume at any given temperature. Changes in flexibility of the protein matrix around the chromophore were monitored by the intrinsic phosphorescence lifetime. At 40 °C the application of pressure in the predenaturation range initially decreases the internal flexibility of azurin, the trend eventually reverting on approaching unfolding. The main difference between wild type and the cavity mutant is the inversion point which happens at 300 MPa for wild type and at 150 MPa for F29A. This suggests that, for the cavity mutant, pressure-induced internal hydration is more dominant than any compaction of the globular fold at relatively low pressures.

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1. Introduction

Understanding the mechanism underlying pressure-induced protein unfolding should provide more information regarding protein folding, stability and dynamics. It is commonly accepted that water and protein cavities play a fundamental role in this process, however the exact details of pressure unfolding are still under debate. It has been proposed that the mechanism underlying pressure-induced unfolding is the hydration of the protein core [1]. On the other hand, the data obtained by Rouget et al. [2] indicate that exposure of the surface area and the change in hydration are not the main factors responsible for pressure effects on proteins. More recently, Roche et al. [3] presented data suggesting that pressure unfolds proteins primarily because of cavities in the native structure. The authors suggest that the volumetric properties and the responses of proteins to pressure perturbation are determined largely by internal solvent excluded volumes, their anisotropic distribution and their tendency to expand with temperature. The protein interiors are in fact tightly packed, however the packing is not uniform [4]. Packing defects exist ubiquitously in proteins in the form of interior cavities of very different sizes, sometimes as large as 200 $Å^3$, as found by X-ray crystallography [5–8] and by theoretical methods [9–11]. Extensive site-directed mutagenesis experiments have identified that the internal cavities can affect both the biological function [12,13] and structural stability [14–17] of proteins. To determine whether cavities are empty or filled with water is important in clarifying their role in protein denaturation. The extent to which naturally occurring or engineered cavities, sufficiently large to accommodate one or more water molecules, are empty or hydrated depends on several factors (size, hydrophobicity, etc.) and is currently the subject of active debate [18–23]. In addition little is known about how the capacity of the proteins to expand, depending on the strength of the interactions, contributes to the magnitude of the internal void volume at any given temperature. It has also been hypothesized that the contribution of a cavity to the volume difference between the unfolded and folded states depends on the cavity's position within the protein structure, i.e. cavities closer to the surface are likely to contribute less to volume change [24].

In this study we applied high pressure to a cavity-mutant protein, F29A azurin mutant, to examine how a relatively large cavity, created just outside the protein core, could affect both the thermodynamic parameters of pressure unfolding and the pressure modulation of protein dynamics.

Azurin from *Pseudomonas aeruginosa* was chosen as a model system due to the wealth of structural (crystallographic, spectroscopic, and theoretical), thermodynamic, and kinetic data available on both native and mutated forms. Theoretically, the introduction of Ala in place of a bulky Phe creates a cavity of about 100 $Å^3$ per molecule. The mutation makes the protein less stable to guanidinium hydrochloride denaturation [25]. The pressure unfolding equilibrium was monitored by an accompanying large change in the fluorescence spectrum of Trp. On the other hand, the influence of pressure on the dynamics of the protein core was probed by the phosphorescence lifetime of Trp48 [26]. The phosphorescence emission of Trp48 in copper-free azurin is strong and long-lived even in buffer at ambient temperature [27]. This emission has been proved to be remarkably sensitive to the flexibility of the structure surrounding the chromophore induced by a wide range of experimental conditions such as metal binding [27], freezing [28], dehydration [29], high pressure [30], sugar addition [31], and pH [32].

Our results in terms of both stability and flexibility responses to pressure obtained with the single-point mutant, F29A, were similar to those reported for other cavity mutants of azurin where a space is inserted in the inner core of the protein. These findings demonstrate that water molecules do fill the nonpolar cavity of azurin, and suggest that this hydrated cavity leads to the further internal hydration of the macromolecule, thus acting as a nucleation site for unfolding.

2. Materials and methods

All chemicals were of the highest purity grade available from commercial sources and were usually used without further purification. Water, doubly distilled over quartz, was purified by Milli-Q Plus system (Millipore, Bedford, MA). All glassware used for sample preparation was conditioned in advance by standing for 24 h in 10% HCl suprapur (Merck, Darmstadt, Germany). Tris(hydroxymethyl)-aminomethane (Tris) and ethylendiamino-tetracetic acid (EDTA) were from Merck (Darmstadt, Germany). QuickChange kit (Stratagene, LaJolla, CA) was used to construct the F29A mutant of azurin from *P. aeruginosa* [25]. Details about site-directed mutagenesis and protein expression have been previously described [33,34]. Isolation and purification of mutant protein were performed following the procedure described for the native enzyme [35]. Apo-protein was obtained from holo F29A azurin by removing Cu^{++} by potassium cyanide [35].

Before spectroscopic measurements, F29A azurin was extensively dialyzed in 50 mM Tris/1 mM EDTA, pH 7.5. In all experiments, protein concentration was typically 5–10 μM.

2.1. Fluorescence and phosphorescence measurements

Spectroscopic measurements under pressure were carried out by placing the sample cuvette in a pressure cell (SITEC, Zurich, Switzerland) provided with sapphire windows and employing water as pressurizing fluid. Details of the sample cuvette and procedure to avoid leakages between sample and pressurizing fluid during pressure cycles have been reported before [26]. Fluorescence spectra and phosphorescence decays of F29A azurin were monitored at equilibrium as function of pressure, from 0.1 to 650 MPa, at different temperature ranging from −13 to 50 °C. Particular care was taken to assure temperature equilibration of the sample after each pressure variation, which required at least 5 min. The reversibility of the pressure-induced changes in the emission was checked at the end of each pressure cycle.

Measurements were conducted on a homemade apparatus equipped with pulsed excitation (λ_{ex} = 289 nm, pulse duration of 5 ns, pulse frequency up to 10 Hz, and energy per pulse varying from 0.5 to 1 mJ) provided by a frequency-doubled Nd/Yag-pumped dye laser (Quanta Systems, Milan, Italy) [25]. Fluorescence emission collected at 90° from the excitation was monitored by a back-illuminated 1340 × 400 pixels CCD camera (Princeton Instruments Spec-10:400B (XTE), Roper Scientific, Trenton, NJ) cooled to −60 °C. Phosphorescence decays were monitored by collecting the emission at 90° from excitation through a filter combination with a transmission window of 405–445 nm (WG405, Lot-Oriel, Milan Italy; plus interference filter DT-Blau, Balzer, Milan, Italy). The photocurrent amplified by a current-to-voltage converter (SR570, Stanford Research Systems, Stanford, CA) was digitized by a 16 bit speed (1.25 MHz) multi-function data acquisition board (NI 6250 PCI, National Instrument Italy, Milan, Italy) supported by LabVIEW software capable of averaging multiple sweeps. Prompt fluorescence from the same pulse was collected through a 310–375 band pass filter combination (WG305 nm plus Schott UG11) and detected by an ultraviolet-enhanced photodiode (OSD100-7, Centronics, Newbury Park, CA). The prompt fluorescence intensity was used to account for possible variations in the laser output among measurements as well as to obtain fluorescence normalized phosphorescence intensities. All phosphorescence decays were analyzed by a nonlinear least-squares fitting algorithm (DAS6, Fluorescence decay analysis software, Horiba Jobin Yvon, Milan, Italy). For phosphorescence measurements, it is paramount to rid the solution of all O_2 traces. Deoxygenation of protein samples was obtained by adding to the samples, in nitrogen atmosphere, sodium dithionite to get a final concentration of 100 μM [36].

Each spectral and lifetime determination was repeated at least three times.