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Relevance of metal ions for lipase stability: Structural rearrangements induced in the *Burkholderia glumae* lipase by calcium depletion

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ABSTRACT

We have studied the accessibility of the structural calcium ion in the *Burkholderia glumae* lipase and the consequences of its removal on the protein conformation by different biophysical techniques (circular dichroism, fluorimetry, and mass spectrometry) and by molecular-dynamics simulations. We show that, in the native protein, calcium is not accessible unless specific flexible loops are displaced, for example, by a temperature increase. Such movements concern the whole calcium-binding pocket and particularly the environment of the coordinating aspartate residue 241. As a consequence of metal depletion the protein unfolds irreversibly and undergoes aggregation. The removal of the metal ion causes major structural transitions and leads to an increase in β-structure, in particular in protein regions that are largely unstructured in the native protein and encompass the calcium coordination residues.

1. Introduction

Binding of metal ions is exploited by proteins to perform a variety of different functions i.e. enzyme catalysis, cellular signalling, control of redox processes and structural stabilization. As a matter of fact, about 40% of known proteins are reported to contain metal cofactors (Dudev and Lim, 2008). Among these, calcium is very common thanks to its abundance inside the cell and to the flexibility of its coordination chemistry. In higher organisms, calcium binding mediates key signal transduction pathways, where it modulates conformation, activity and interactions of numerous proteins (Capozzi et al., 2006; Swulius and Waxham, 2008; Williams, 2006; Gerke et al., 2005). A more general function, conserved throughout evolution and shared with other metal ions, is the stabilization of protein regions and domains, *via* the restriction of local flexibility and susceptibility to unfolding (Gregory et al., 1993). This structural role is broadly documented and is further substantiated by the observation that thermolabile proteins bind metals with lower affinity than their more stable homologues (Siddiqui and Cavicchioli, 2006).

The effects evoked in a polypeptide by the association of Ca^{2+} can be complex, even in simple globular proteins, where minor structural rearrangements may be induced and may influence both the stability and the activity in an interplay where the two events can hardly be dissected. Bacterial lipases provide a good case study for deepening such issues, as several of them bind Ca^{2+} ions at different locations within the enzyme structure (Kim et al., 1996; Schrag et al., 1996; Simons et al., 1999; Nardini et al., 2000; Tyndall et al., 2002; Angkawidjaja et al., 2007). Lipases are carboxylesterases active on the ester bonds of long-chain acylglycerides. Although very diverse in amino acid sequence, they all share a catalytic mechanism based on a Ser-His-Glu/Asp active site and an overall structural organization which conforms to the α–β hydrolase fold (Schrag and Cygler, 1997). A peculiar feature of most lipases is the presence of an α-helical flexible "lid" element that covers the active site and participates in substrate recognition. The solved 3D structures of lipases from *Pseudomonas* and *Burkholderia* sp., which belong to the closely related families I.1 and I.2 of bacterial lipases (reviewed in Arpigny and Jaeger, 1999), reveal one calcium atom bound relatively tightly to the protein, as shown by the low B factors of the side chains of coordination residues and of the metal ion (Kim et al., 1996; Nardini et al., 2000). Based on the evidence provided by crystallographic data, it was suggested that Ca^{2+} plays a role in the stabilization of the overall protein structure and that it contributes to the correct positioning of a protein loop carrying the catalytic histidine (Kim et al., 1996; Schrag et al., 1996; Nardini et al., 2000; Lang et al., 1996). Consistent with this view, it was shown that treatment with metal chelators destabilizes lipases

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towards chemical and heat denaturation (El Khattabi et al., 2003) and the thermosensitive *Pseudomonas fragi* lipase was predicted to bind calcium with lower affinity than its thermoresistant homologues (Alquati et al., 2002).

Despite the availability of structural information, the mechanistic details of calcium binding and dissociation in this group of lipases is still unclear, neither it has been determined under which conditions the metal ion is susceptible to be released from the protein structure, which would help interpreting the results of experiments on its role. This kind of studies requires knowledge of the species distribution as for bound calcium and folding state and the characterization of even minor and local conformational transitions in the enzyme upon Ca^{2+} binding and release. In this study, we apply a combination of biophysical techniques, to study the accessibility of Ca^{2+} in the *Burkholderia glumae* lipase (BGL) and the consequences of its removal upon EDTA treatment or acidification, and interpret the experimental results by molecular-dynamics simulations. We show that Ca^{2+} is not accessible for chelation unless some flexible loops are displaced, suggesting that a slight local denaturation (relaxation) is necessary for metal release. Furthermore, our results suggest that, upon metal chelation, the protein irreversibly unfolds through a highly co-operative transition and undergoes aggregation. Besides shedding new light on the contribution of Ca^{2+} to the conformational stability of BGL, this information may be of relevance also in view of molecular approaches towards the obtainment of robust lipases for use in biocatalysis by protein engineering.

2. Materials and methods

2.1. Enzyme preparation and assay

The enzyme used in this study was *B. glumae* lipase 437,707 from CalBioChem. BGL was purified at room temperature by gel-filtration chromatography on Sephacryl S-100 pre-packed columns (1.6×60 cm) from Amersham (Piscataway, NJ, USA). Twenty milligrams of commercial powder were dissolved in 5 ml of 100 mM Tris–HCl, pH 7.5, loaded on the column and eluted with the same buffer. Lipase activity was determined by measuring the increase in absorbance at 410 nm produced by the release of *p*-nitrophenol during the hydrolysis of 10 mM *p*-nitrophenyl laurate (dissolved in isopropanol) in 100 mM Tris–HCl, pH 7.5 and 0.005% Triton X-100 at room temperature. To start the reaction, 0.5–1 μM lipase was added to a volume of 1 ml of the assay solution. Hydrolysis was followed for 3 min. Measurements were performed in triplicate. One unit was defined as the amount of enzyme which releases 1 μmol of *p*-nitrophenol/min under the test conditions.

2.2. Fluorimetry

Fluorimetric data were recorded with a Luminescence Spectrometer 45 (Perkin-Elmer, Wellesley, USA) equipped with a mercury lamp. 600 nM samples was measured in 100 mM Tris–HCl buffer, pH 7.5, after 1 h incubation with or without 10 mM EDTA at the indicated temperature. The excitation wavelength was set to 280 nm and an emission scan was performed at 100 nm/min from 260 to 400 nm. The "aggregation index" was determined as R_{ag} = I280/I340 according to Nominé et al. (2001).

2.3. Circular dichroism (CD)

Far-UV CD spectra were recorded on a J-815 instrument from Jasco (Easton, MD), equipped with a Peltier unit for temperature control, in a 1-mm quartz cuvette. BGL was dissolved in 10 mM ammonium acetate pH 7.5 at a final concentration of 10 μM with or without 10 mM EDTA. Spectra were recorded between 190 and 260 nm. Spectra deconvolution was performed by the program CDSSTR, ContinII, SELCON3 using a reference set of 43 folded and 5 unfolded soluble proteins (Sreerama and Woody, 2000).

2.4. ESI-MS

BGL samples were analyzed by electrospray-ionization mass spectrometry (ESI-MS) at a final protein concentration of 5 μM in 10 mM ammonium acetate, pH 7.5 with or without 1 mM EDTA. The pH of the solutions was adjusted by ammonium hydroxide or formic acid before protein addition. Mass spectra were obtained with a Qstar Elite time-of-flight spectrometer from Applied Biosystems (Darmstadt, Germany) equipped with a nano-spray ion source. Nano-ESI Au/Cd coated borosilicate capillaries (inner diameter of the emitter tip ~ 1 μm) were purchased from Proxeon (Odense, Denmark). The instrument setting was: spray tip potential 1400 V, declustering potential 80 V, interface temperature 50 °C, curtain-gas flow rate 1.13 L/min. Samples were sprayed at room temperature. Reported spectra are averaged over a 90-s acquisition with an accumulation time of 1 s per spectrum. Each measurement was repeated at least three times and representative results for each condition are shown. Data were analyzed with the software Analyst QS 2.0 (Applied Biosystems).

2.5. Molecular dynamics (MD) simulations

MD simulations were performed using the 3.3 version of the GROMACS software (www.gromacs.org), implemented on a parallel architecture, using the GROMOS96 force field. The X-ray structure of native BGL (PDB entries 1CVL (Lang et al., 1996) was used as starting point for MD simulations. Initial structure of the apo-form was obtained according to the following procedure: the Ca^{2+} ion was removed from the PDB file and the resulting apo-lipase structure was optimized by molecular mechanics (1000 steepest descent cycles followed by 10,000 conjugate gradient steps). This structure was then submitted for MD calculation.

Holo- and apo-structures, including crystallographic water molecules, were soaked in a dodechadral box of SPC (Simple Point Charge water model) water molecules (Berendsen et al., 1981) and simulations were carried out using periodic boundary conditions. All the protein atoms were at a distance equal or greater than 0.5 nm from the box edges. The ionization state of residues was set to be consistent with neutral pH and the tautomeric form of histidine residues was derived using GROMACS tools and confirmed by visual inspection. Further details on MD setup and solvent equilibration, thermalization and pressurization steps are reported elsewhere (Papaleo et al., 2008). Productive MD simulations were performed in the isothermal–isobaric ensemble at 30, 50, 60, 70 and 80 °C, using an external bath with a coupling constant of 0.1 ps. Pressure was kept constant (1 bar) by modifying the box dimensions and the time-constant for pressure coupling was set to 1 ps (Berendsen et al., 1984). The LINCS (Hess et al., 1997) algorithm was used to constrain hydrogen-heavy atom bonds, allowing the use of a 2 fs time-step. Electrostatic interactions were calculated using the Particle-mesh Ewald (PME) summation scheme (Darden et al., 1993). Van der Waals and Coulomb interactions were truncated at 0.8 nm. The non-bonded pair list was updated every 10 steps and conformations were stored every 2 ps.

It is well known that multiple trajectories help to identify recurring features and to avoid artefacts arising from the simulation procedure (Caves et al., 1998; Papaleo et al., 2008). To improve the conformational sampling, two 12 ns simulations were carried out at each temperature for both the apo- and holo-BGL, initializing the MD runs with different initial atomic velocities. In the following, MD trajectories collected for the same system at the same