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Using hydroxyl radical footprinting to explore the free energy landscape of protein folding

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ABSTRACT

Characterisation of the conformational states adopted during protein folding, including globally unfolded/disordered structures and partially folded intermediate species, is vital to gain fundamental insights into how a protein folds. In this work we employ fast photochemical oxidation of proteins (FPOP) to map the structural changes that occur in the folding of the four-helical bacterial immunity protein, Im7. Oxidative footprinting coupled with mass spectrometry (MS) is used to probe changes in the solvent accessibility of amino acid side-chains concurrent with the folding process, by quantifying the degree of oxidation experienced by the wild-type protein relative to a kinetically trapped, three-helical folding intermediate and an unfolded variant that lacks secondary structure. Analysis of the unfolded variant by FPOP–MS shows oxidative modifications consistent with the species adopting a solution conformation with a high degree of solvent accessibility. The folding intermediate, by contrast, experiences increased levels of oxidation relative to the wild-type, native protein only in regions destabilised by the amino acid substitutions introduced. The results demonstrate the utility of FPOP–MS to characterise protein variants in different conformational states and to provide insights into protein folding mechanisms that are complementary to measurements such as hydrogen/deuterium exchange labelling and Φ-value analysis.

1. Introduction

Mass spectrometry (MS)-based techniques are being employed increasingly to assist in the structural characterisation of biomolecules, such as proteins, protein assemblies and protein–ligand complexes [1,2]. Numerous methods have been developed for this purpose, including covalent labelling, hydrogen/deuterium exchange (HDX), and chemical crosslinking, which can be mapped at a residue-specific level using MS analysis [1–6].

Fast photochemical oxidation of proteins (FPOP) is an emerging MS-based technique with the potential to elucidate conformational changes in proteins resulting from folding/unfolding processes [7–15] and for probing changes in solvent accessibility that accompany ligand binding [16–18]. The FPOP approach requires the addition of small quantities of H_2O_2 (typically 0.02% (v/v)) to protein-containing solutions immediately prior to infusion through a capillary system and irradiation with a pulsed laser [19]. This procedure generates hydroxyl radicals that react with potentially any solvent accessible amino acid side-chain (although reaction rates for each amino acid differ significantly) [4], resulting typically in +16 Da mass additions, although additional modifications may also be observed [4,20]. Addition of quenching reagents such as glutamine or histidine reduces the hydroxyl radical lifetime to approximately 1 μs [17,12,21]. The labelling time after each laser pulse, therefore, is faster than most protein unfolding/refolding reactions, allowing each protein conformation to be labelled effectively instantaneously in an experiment. Hence, both spatial (amino acid labelling sites) and temporal information regarding protein folding can be obtained [7–15]. FPOP–MS serves as a complementary technique to the more established method of HDX-MS [22], but the covalent nature of the modifications in FPOP–MS permits more rigorous sample handling procedures downstream of the labelling process without loss of information.

Mutational analysis of protein folding and unfolding kinetics (Φ-value analysis) is one of the most powerful approaches used to dissect protein folding mechanisms [23]. The information from these analyses can then be used to introduce mutations that stabilise partially folded intermediates or more highly unfolded states of a protein at equilibrium, allowing their direct structural analysis. Here, we have chosen to study the four-helix, bacterial immunity protein, Im7 (Fig. 1) [24] and two mutant constructs. The first construct, L53A I54A Im7 (DM Im7), has two amino acid changes in helix III [25]. This species populates the on-pathway folding

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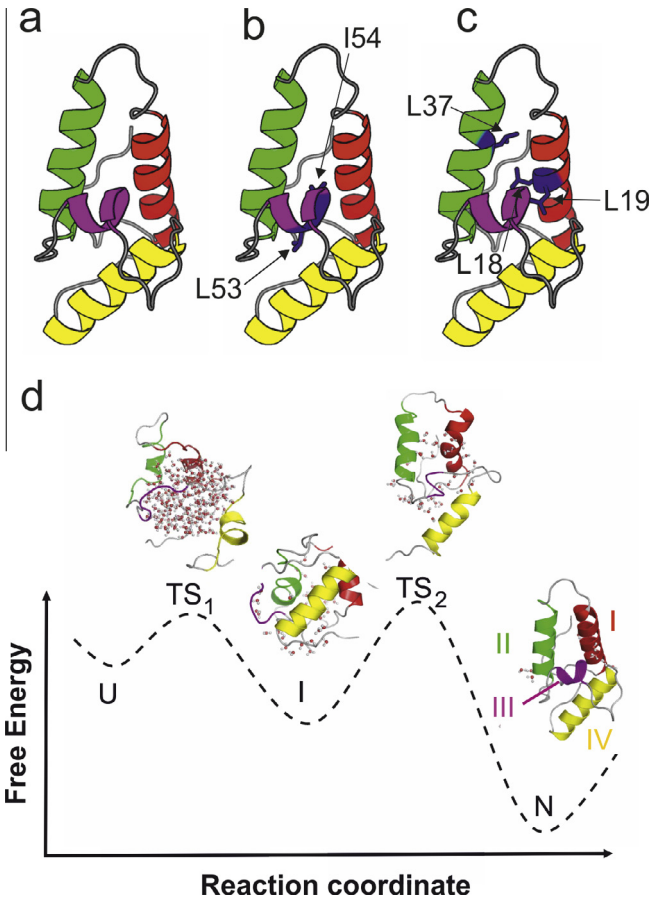

Fig. 1. (a) The native structure of Im7, which has a four-helix fold (PDB accession 1AYI [41]). The locations of the amino acid substitutions with Ala are shown for (b) DM Im7 and (c) TM Im7. (d) Schematic of the folding energy landscape of Im7 showing the species populated during folding (U, unfolded state; I, intermediate state; N, native state; TS_1 and TS_2, the first and second transition states). Structures generated from molecular dynamics simulations are shown for TS_1, I and TS_2 [27,38]. Water molecules are shown (red spheres) that are displaced from the core as the protein folds. The four helices present in native, wild-type (WT) Im7 are coloured (I) red, (II) green, (III) purple, and (IV) yellow.

intermediate at equilibrium, and has been shown previously to retain native-like secondary structure in helices I and IV, with partial formation of helix II and complete loss of helix III [25–27]. The second variant, L18A L19A L37A Im7 (TM Im7), was employed because these amino acid substitutions prevent Im7 folding and stabilise the unfolded state at equilibrium. This unfolded variant has been shown to be compact, retaining few fixed tertiary interactions and lacking persistent helical structure [28].

Here, we use MS-based techniques to interrogate the structures of Im7, DM Im7 and TM Im7. Initial studies utilising native electrospray ionisation-ion mobility spectrometry–mass spectrometry (ESI-IMS–MS) failed to distinguish between native Im7, the intermediate DM Im7 and unfolded TM Im7. We show that when the proteins were labelled using the FPOP technique, a correlation between the extent of structural destabilisation and the degree of covalent modification was observed. This demonstrates that FPOP can be implemented successfully to probe differences in the conformational properties and dynamics of proteins. Furthermore, localisation of oxidation sites shows that destabilisation of secondary structure in a particular region leads to an increase in the levels of oxidative labelling in the specific region of the sequence involved. This is consistent with FPOP permitting discrimination of the conformational dynamics of specific amino acid side-chains in regions of protein structure, producing data that are complementary to information about the main chain provided by HDX.

2. Materials and methods

2.1. Materials

Im7 constructs containing an N-terminal hexahistidine tag ($ME(His)_6$) were overexpressed in *Escherichia coli* and purified as described previously [24,29].

2.2. Circular dichroism

Far-UV circular dichroism (CD) spectra were recorded on a Chirascan CD spectrophotometer (Applied Photophysics, Leatherhead, Surrey, UK) using a 1 mm path length cuvette. Solutions contained protein at a concentration of 0.1 mg/mL in 10 mM sodium phosphate buffer, pH 7.0. Spectra shown are the average of three scans that were acquired over the range 190–260 nm with a bandwidth of 1 nm and a scan speed of 20 nm min^{-1}. The buffer contribution was subtracted from the spectrum of each sample.

2.3. Native electrospray-ion mobility spectrometry–mass spectrometry

Lyophilised proteins (5 μM) were dissolved in 50 mM ammonium acetate buffer, pH 6.9 and desalted using Micro BioSpin 6 (Bio-Rad, Hemel Hempstead, UK) columns. ESI-IMS–MS experiments were performed using a Synapt HDMS mass spectrometer (Waters Ltd., Wilmslow, Manchester, UK). Sample introduction was achieved by nano-ESI using in-house prepared gold-plated borosilicate capillaries. Typically, a capillary voltage of 1.4 kV was applied, the cone voltage was set to 40 V, and a backing pressure of 4.5 mBar was used. IMS separation was achieved using a wave height of 5 V, and a wave velocity of 250 ms^{-1}. Drift times were calibrated using experimentally determined CCSs of native proteins and applying a procedure described in detail elsewhere [30–33]. CCSs were calculated from coordinates deposited in the Protein Data Bank using a scaled projection approximation (PSA) [34]. Aqueous CsI was used for *m/z* calibration. Data were processed using MassLynx v4.1 and Driftscope v2.5 software (Waters Ltd., Wilmslow, Manchester, UK).

2.4. Fast photochemical oxidation

Samples contained protein (10 μM) in 10 mM sodium phosphate buffer, pH 7.0 and were supplemented with 20 mM L-glutamine. 0.02% v/v H_2O_2 (final concentration) was added immediately prior to laser irradiation from a 30% v/v stock solution. The sample was infused through a fused silica capillary (inner diameter 100 μm), which had a window etched with a butane torch, at a flow rate of 20 μL/min. Hydroxyl radicals were generated by exposing the sample (through the etched window) to irradiation from a Compex 50 Pro KrF excimer laser operating at 248 nm (Coherent Inc., Ely, UK) with frequency of 15 Hz and a laser beam width of <3 mm at the point of irradiation. These solution, flow and laser pulse conditions ensure each bolus of protein-containing solution is exposed only once to laser irradiation and that conformational averaging during labelling does not occur, as the labelling reaction is on a faster time scale than any unfolding event which may occur, due to the presence of the quenching reagent L-glutamine [12,19,21]. The capillary outflow (100 μL) was collected in a 1.5 mL tube that contained 20 μL of a 100 mM L-methionine/1 μM catalase solution in 10 mM sodium phosphate buffer, pH 7.0 to degrade residual H_2O_2 and quench any hydroxyl