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Native mass spectrometry and ion mobility characterize the orange carotenoid protein functional domains

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ABSTRACT

Orange Carotenoid Protein (OCP) plays a unique role in protecting many cyanobacteria from light-induced damage. The active form of OCP is directly involved in energy dissipation by binding to the phycobilisome (PBS), the major light-harvesting complex in cyanobacteria. There are two structural modules in OCP, an N-terminal domain (NTD), and a C-terminal domain (CTD), which play different functional roles during the OCP–PBS quenching cycle. Because of the quasi-stable nature of active OCP, structural analysis of active OCP has been lacking compared to its inactive form. In this report, partial proteolysis was used to generate two structural domains, NTD and CTD, from active OCP. We used multiple native mass spectrometry (MS) based approaches to interrogate the structural features of the NTD and the CTD. Collisional activation and ion mobility analysis indicated that the NTD releases its bound carotenoid without forming any intermediates and the CTD is resistant to unfolding upon collisional energy ramping. The unfolding intermediates observed in inactive intact OCP suggest that it is the N-terminal extension and the NTD–CTD loop that lead to the observed unfolding intermediates. These combined approaches extend the knowledge of OCP photo-activation and structural features of OCP functional domains. Combining native MS, ion mobility, and collisional activation promises to be a sensitive new approach for studies of photosynthetic protein-pigment complexes.

1. Introduction

Cyanobacterial photosynthesis contributes dramatically to the global carbon and nitrogen cycle [1–3]. In cyanobacteria, solar energy is mostly captured by the phycobilisome (PBS), a light-harvesting antenna complex that is anchored to the stromal side of the thylakoid membrane. The energy is then transferred to membrane-embedded reaction centers Photosystems I and II (PSI and PSII) where photochemical reactions take place [4–7]. Regulation of energy transfer between the antenna and reaction centers is extremely important for energy allocation to the two photosystems and cellular adaptation as well as to changing light conditions in the environment. Under strong light conditions, many cyanobacteria exhibit a self-protection mechanism called non-photochemical quenching (NPQ), a process in which extra energy collected by the PBS is dissipated as heat [8,9]. The orange carotenoid protein (OCP) acts as a sensor and practitioner in the NPQ regulatory process. OCP is in its inactive orange form under low-light or dark conditions. Under strong-light conditions, however, inactive OCP can be activated to its active red form and consequently is recruited to bind to the PBS. The carotenoid molecule intercepts energy from the PBS and prevents over-energization of photosystems, especially PSII where toxic singlet oxygen species are inevitably produced by PSII photochemistry [10].

OCP photo-activation has been intensely studied [11–16]. Although high-resolution structural models for inactive intact OCP and truncated active OCP N-terminal domain (NTD) expressed in *E. coli* have been reported [17,18], detailed information about the photo-induced conformational changes and the carotenoid-protein interactions are still limited for the intact active OCP. One challenge is the quasi-stable feature of active OCP that tends to relax to its inactive form, making currently available analytical characterization extremely difficult. It was observed, however, that the NTD alone could bind the carotenoid in its red form and is conformationally stable and functionally effective in PBS energy quenching [19].

Partial digestion or limited proteolysis experiments have been used in many structural biology studies [20,21]. In a typical limited proteolysis experiment, proteins are digested by proteases under native conditions. The enzyme cleavage sites exposed on the protein surface or in flexible regions are available for enzymatic cleavage, while those sites buried inside the interior of a protein are not accessible for enzyme attack. The partial digestion results in large protein fragments that represent intact domains or stable structural modules of a protein. When partial digestion analysis is combined with mass spectrometry (MS), a

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rapid and sensitive tool, a wealth of structural information can be obtained [22–25].

Other sensitive MS-based approaches in protein characterization have already been employed in studies of OCP photo-activation [17, 26–28]. For example, we and others have analyzed the global conformational changes of OCP upon photo-activation by using MS-based protein footprinting [26,28]. Native MS is a relatively new approach to characterize protein structure under conditions in which the native protein conformation is maintained in the gas phase for MS analysis [29–34].

Many mass spectrometers are equipped with ion mobility (IM) analysis [32,35], which may have applications in systems such as OCP. In brief, the protein–ion travel time through the drift tube under an applied electric field against the carrier buffer gas is a function of the protein ion's mass-to-charge ratio, as well as its size and shape (conformation). Based on the traveling time (drift time, DT) recorded in IM analysis, the collisional cross-section (CCS), which is a measure of ion size and conformation, can be determined [36,37]. The combination of native MS with IM has become a fast-growing research field in the last decade to determine protein conformational information [38].

Here, we report an extension of our previous native MS studies on intact OCP [27]. We analyzed OCP functional domains (NTD and CTD) from partial digestion of active OCP by using IM and collisional unfolding [39] under native MS conditions. Two OCP functional domains were compared with inactive intact OCP. Information on the conformation and carotenoid interaction of each functional module were elucidated.

2. Material and methods

2.1. Chemical and reagents

HPLC-grade water and ammonium acetate were purchased from Sigma–Aldrich (Sigma–Aldrich, St. Louis, MO). LC-MS grade water with 0.1% formic acid, acetonitrile with 0.1% formic acid, and formic acid were purchased from Fisher Scientifics (Pittsburgh, PA).

2.2. OCP protein purification and partial digestion

The growth and the OCP isolation were described elsewhere [10,27]. The OCP partial digestion was carried out as previously described [19].

2.3. LC-MS analysis

The partially digested OCP sample was analyzed by a Waters Synapt G2 mass spectrometer coupled with Waters nanoAquity UPLC system (Waters Corporation, Milford, MA). The sample was loaded into a custom-packed capillary column (Polymer Resin Phase, 100 Å, 5 μm, 5 cm × 100 μm, Sepax Technologies, Newark, DE), and the proteins were trapped and desalted by 85% solvent A (water with 0.1% formic acid) for 15 min prior to the elution. The sample was eluted by increasing solvent B (acetonitrile with 0.1% formic acid) from 15 to 85% over 25 min at a flow rate of 0.7 uL/min. The eluted sample was directly introduced into the mass spectrometer through a nano-electrospray source. The mass spectrometer was operated under the resolution mode ('V' optics for TOF analyzer, TOF resolution is 20,000 FWHM at m/z 956) with mass range from 50 to 1995 *m/z*. The spray voltage as 2.8 kV; cone voltage was 30 V and extraction voltage as 4 V. The trap region collisional energies was 6 V. The data were manually analyzed by using MassLynx (Waters Corporation, Milford, MA) and Massign software to assign the molecular weight [40].

2.4. Native MS and ion mobility analysis

The partially digested OCP sample was washed by 200 mM ammonium acetate solution (pH 6.8) in a 3 kDa molecular weight cut off filter (Vivaspin, Sartorius AG, Goettingen, Germany). The original buffer and salts were removed during 10–15 cycles of washing. The OCP sample was introduced into the mass spectrometer source by using off-line nano-electrospray tips. The native MS and ion mobility experiments were conducted in the Waters Synapt G2 mass spectrometer. The mass spectrometer was operated in the "sensitive mode" ('V' optics for TOF analyzer, TOF resolution is 10,000 FWHM) with a mass range from 100 to 10,000 *m/z*. The backing pressure (pressure read out from the first pirani gauge at the source region) was adjusted to 5–6 mBar for transferring intact protein ions. The collision voltage for transfer region was 20 V. The collision voltage for trap region was adjusted from 5 to 200 V to unfold the protein ions [41,42]. For ion-mobility experiments, the gas flow rate was 35 mL/min, the IMS wave height was 20 V, and the IMS wave velocity was 500 m/s. The data were output from MassLynx and plotted by Origin (Origin Lab Corporation, Northampton, MA). The intact OCP sample was also analyzed by native MS and ion-mobility experiments. The ion-mobility experiment was calibrated to give cross-sections with standard proteins based on published protocols [36,43]. The drift time information from the native MS ion-mobility experiment was converted into collisional cross-section [36,43].

3. Results and discussion

3.1. Partial digestion and analysis of OCP

When we performed SDS-PAGE analysis of limited digestion OCP, we found two major polypeptide fragments with apparent molecular weights (MW) of 14 and 17 kDa, respectively (Fig. 1), consistent with a previous report [19]. We then analyzed the partially digested OCP samples by regular LC-MS and found large peptides covering the NTD and CTD. Because regular LC-MS is run under denaturing conditions, the non-covalent interactions between the protein and carotenoid were lost, as no peptide-carotenoid complex could be detected in the LC-MS experiment. The OCP sequence was analyzed for in-silico trypsin digestion. We assigned peaks from the LC-MS experiment to corresponding digestion products based on the MW. There are two major groups of digestion products observed in LC-MS experiments (peaks were assigned to N-terminal (at retention time 18 min) and C-terminal digestion product/fragments (at retention time 17.5 min) separately (Fig. 2)). We also observed that each group includes a series of peptide fragments. For example, in the first group of peaks (retention time), the major species has molecular weight 13,974 Da and was assigned to the C-terminal fragment (from amino acid residue 186–310; theoretical MW of 13,974.03 Da). The second group of peaks contains multiple, co-eluting species in the MW range from 16.1 to 17.3 kDa. They were assigned to N-terminal fragments. The major peak of 17,222 Da is the N-terminal fragment from amino acid residue 10-170, the theoretical MW is 17,222.8 Da. Our data showed the detailed, heterogeneous partial digestion peptide patterns that otherwise cannot be detected by traditional SDS-PAGE.

We conducted partial digestion under light irradiation (active form of OCP or "red OCP"). The most exposed cleavage sites are between residues 161–196, which includes the loop region between the NTD and the CTD of OCP. The LC-MS experiment identified two major fragments

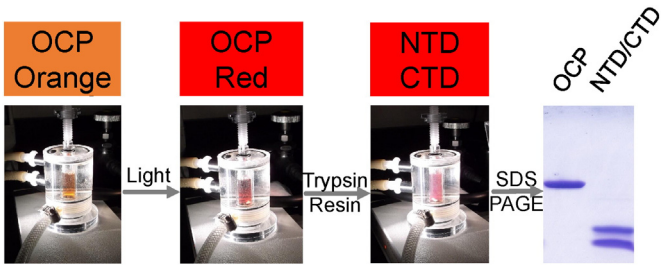

Fig. 1. Partial proteolysis of photoactivated red OCP under low temperature (4 °C) by trypsin.