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FRET analysis of CP12 structural interplay by GAPDH and PRK

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ABSTRACT

CP12 is an intrinsically disordered protein playing a key role in the regulation of the Benson–Calvin cycle. Due to the high intrinsic flexibility of CP12, it is essential to consider its structural modulation induced upon binding to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and phosphoribulokinase (PRK) enzymes. Here, we report for the first time detailed structural modulation about the wild-type CP12 and its site-specific N-terminal and C-terminal disulfide bridge mutants upon interaction with GAPDH and PRK by Förster resonance energy transfer (FRET). Our results indicate an increase in CP12 compactness when the complex is formed with GAPDH or PRK. In addition, the distributions in FRET histograms show the elasticity and conformational flexibility of CP12 in all supra molecular complexes. Contrarily to previous beliefs, our FRET results importantly reveal that both N-terminal and C-terminal site-specific CP12 mutants are able to form the monomeric (GAPDH-CP12-PRK) complex.

1. Introduction

Intrinsically disordered proteins (IDPs) are involved in a wide range of regulatory processes in the cell. Owing to their peculiar conformational flexibility, IDPs are highly represented in various proteomes [1] and display allostery to control the protein–protein interactions in crowded cellular environment [2]. IDPs show unique promiscuous binding behavior with multiple partners acting therefore as molecular interaction hubs [3]. CP12 is a prime example of intrinsically disordered protein molecular hub in photosynthetic organisms. It is a nuclear-encoded chloroplast protein with a molecular mass of 8.5-kDa. CP12 interacts with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and phosphoribulokinase (PRK), two enzymes of the Benson–Calvin cycle that finally forms a double unit of $(GAPDH–CP12–PRK)_2$ supramolecular complex well characterized in some photosynthetic organisms [4–9]. The CP12 protein has four conserved cysteine residues, which in their oxidized state form two disulfide bridges that play a crucial role in the assembly of the supra-molecular complex [10–12]. The N-terminal pair of cysteine residues of CP12 is involved in its interaction with PRK [13], while the C-terminal one is implicated in the interaction with GAPDH [14]. Using surface plasmon resonance technique and *in vitro* reconstitution assays, it was shown that CP12 primarily associates with tetrameric GAPDH, and further this GAPDH-CP12 complex, binds a dimer of PRK to form a sub molecular complex, that in turn dimerizes [15–18]. Several biochemical studies analyzed the interaction sites between the CP12-GAPDH and CP12-PRK complexes [19–24]. A recent study on CP12 by fluorescence correlation spectroscopy (FCS) quantified the hydrodynamic radii of both native and CP12 mutants and also of CP12 in interaction with GAPDH and PRK [25]. However FCS fails to quantify structural rearrangements of CP12 upon interaction with tetrameric GAPDH and dimeric PRK. Therefore, we engaged into Förster resonance energy transfer (FRET) experiments.

FRET is a powerful tool to understand the molecular function of CP12, that is crucial, due to CP12 high flexibility and lack of rigid structure, and to investigate CP12 structural modulation properties induced upon binding to other proteins. In this study, we used the wild-type CP12 (CP12wt) and its site-specific mutant proteins altered both at the N-terminus and at the C-terminus, which are

Abbreviations: IDP, intrinsically disordered protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PRK, phosphoribulokinase; GuHCl, guanidinium hydrochloride; E, energy transfer efficiency; FCS, fluorescence correlation spectroscopy; FRET, Förster resonance energy transfer.

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disturbing the interactions with PRK and GAPDH respectively. Fluorescence detection in combination with FRET is extremely well suited for studying the heterogeneous ensemble of CP12 structures upon interaction with unlabeled GAPDH and PRK.

2. Methods

2.1. Protein purification & labeling

Chlamydomonas reinhardtii CP12, GAPDH and PRK were obtained as described earlier [13]. In all experiments, His-tagged CP12wt and mutants proteins behave like the native protein, indicating that the His tag does not interfere in our experiments [13]. Lysine residues in the CP12wt and also in CP12 mutant proteins were labeled with the amine-reactive NHS-ester derivative of the Atto590 fluorophore. In both CP12 mutant proteins CP12C31S and CP12C75S were labeled with the thiol-reactive maleimide derivative of the fluorophore Atto647N by following the manufacturer protocol (Atto-Tec). Labeling followed by chromatography purification and Sample purity and specificity was performed as reported by Moparthi et al. [25].

2.2. In vitro reconstitution of the GAPDH–CP12–PRK complex

2.2.1. Western blot

Wild type and mutant CP12s (0.18 nmol) were incubated with GAPDH (0.09 nmol) in presence or the absence of PRK (0.09 nmol) 12 h at 4 °C in 30 mM Tris–HCl, 4 mM EDTA, 0.1 mM NAD, 5 mM cysteine, pH 7.9 in a final volume of 50 μl. The formation of the PRK-GAPDH-CP12 complex was visualized by native PAGE 4–15% gels (PhastGel, GE Healthcare, Little Chalfont, United Kingdom) and Western blots performed using anti-PRK and anti-CP12.

2.2.2. FRET

All *in vitro* reconstitution sub-complexes of CP12wt and also CP12 mutants (C31S or C75S)-GAPDH or -PRK, or the ternary complex GAPDH-CP12 (wt or C31S or C75S)-PRK were performed as previously described [25] at 21 °C with 0.1% Tween-20 (Sigma). In the case of intra FRET experiments, 10 nM of labeled CP12C31S or CP12C75S was mixed with 5X molar excess unlabeled tetrameric GAPDH and unlabeled dimeric PRK separately or in combination in reconstitution buffer for 12 h. In the case of inter FRET experiments, 10 nM of labeled CP12wt was mixed with 50 nM of labeled PRK dimer and 50 nM of unlabeled GAPDH tetramer. In corresponding samples, 1 mM DTT was used as a reducing agent and samples were incubated for 2 h.

2.3. Experimental setup

FRET detection was performed on a confocal inverted microscope with a Zeiss C-Apochromat 63x 1.2NA water-immersion objective, and an iChrome-TVIS laser (Toptica GmbH) operating at 550 nm and delivering 3 ps pulses at 40 MHz repetition rate used as an excitation source. Filtering the laser excitation was achieved by a set of two bandpass filters (Chroma ET525/70M and Semrock FF01-550/88). Dichroic mirrors (Chroma ZT594RDC and ZT633RDC) separate the donor and acceptor fluorescence light. The detection was performed by two avalanche photodiodes (Micro Photon Devices MPD-5CTC with 50 μm active surface) with 620 ± 20 nm (Chroma ET605/70M and ET632/60M) and 670 ± 20 nm (Semrock FF01-676/37) fluorescence bandpass filters for the donor and acceptor channels respectively. The photodiode signal was recorded by a fast time-correlated single photon counting module (Hydraharp400, Picoquant GmbH) in time-tagged time-resolved (TTTR) mode. The excitation power at the diffraction limited spot was set to 40 μW for all experiments.

2.4. FRET analysis based on acceptor fluorescence bursts

All fluorescence bursts above the background noise were recorded separately by the acceptor channel and donor channel. Conceptually, the apparent FRET efficiency of each burst was calculated according to the ratio of acceptor counts n_A over all detection events $n_A + n_D$, which is the sum of donor and acceptor counts. We also took into consideration the differences in the quantum yields of fluorophores (φ_A and φ_D), fluorescence detection efficiencies (η_A and η_D), direct excitation of the acceptor by the laser light (n_A^{de}), donor emission crosstalk into the acceptor channel (α). Using the commercial software Symphotime 64 (Picoquant GmbH) and by considering all these parameters the FRET efficiency is computed according to the formula below:

$$E = \frac{n_A - \alpha n_D - n_A^{de}}{n_A - \alpha n_D - n_A^{de} + \gamma n_D} \quad (1)$$

where $\gamma = \eta_A\varphi_A/\eta_D\,\varphi_D$ accounts for the differences in quantum yields (φ_A and φ_D) and fluorescence detection efficiencies (η_A and η_D) between the acceptor and donor.

We estimate $\gamma = 0.9$, and $\alpha = 0.28$ for the current setup.

3. Results

In vivo, the inherent flexibility and structural adaptability of CP12 makes it an adapter between the tetrameric GAPDH and the dimeric PRK in Calvin cycle. A 3D model of CP12 based on bioinformatics approach shows the four cysteine residues and all lysine positions (Fig. 1A) [26]. *In vitro* reconstitution assays showed that CP12 mutants were unable to form the dimeric unit $(GAPDH–CP12–PRK)_2$ complex (Fig. 1B). We also observed that the addition of 1 mM DTT in the reconstitution buffer affected the formation of the ternary complex (data not shown).

3.1. Binding-induced modulation of CP12 analyzed by FRET

To probe the intramolecular distance distributions of CP12, Atto 590 and Atto 647N as donor and acceptor fluorophores were linked to lysine and cysteine residues respectively, for that reason, oxidized wild -type CP12 that does not possess any free sulfhydryl group was not used. The Förster distance between the selected FRET pair is 74 Å. CP12 FRET was calculated to obtain information on the structural modulation of CP12 protein by GAPDH alone, PRK alone and in concert. The FRET histograms from all these conditions are compiled in Figs. 2 and 3. A change in compactness corresponds to variation in the FRET efficiency *i.e.,* an increase of the FRET efficiency indicates a compaction of the protein while a decrease indicates a swelling/expansion of the protein.

3.1.1. Intra FRET approach

Fig. 2(A–E) shows confocal single-molecule FRET histograms of labeled CP12C31S in the presence and absence of GAPDH, of PRK or with both enzymes. In all cases, only one peak was observed in the energy transfer efficiency (E) histograms (Fig. 2). The FRET histogram obtained with CP12 molecules labeled only with the donor dye is used as a reference. All FRET histograms with the acceptor are significantly different from the isolated donor reference. The FRET efficiency for both CP12 mutants is below 40%, confirming that both the donor and acceptor dyes are on distances higher than 80 Å (Fig. 2A and F). We also measured the GuHCl effect on both CP12