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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

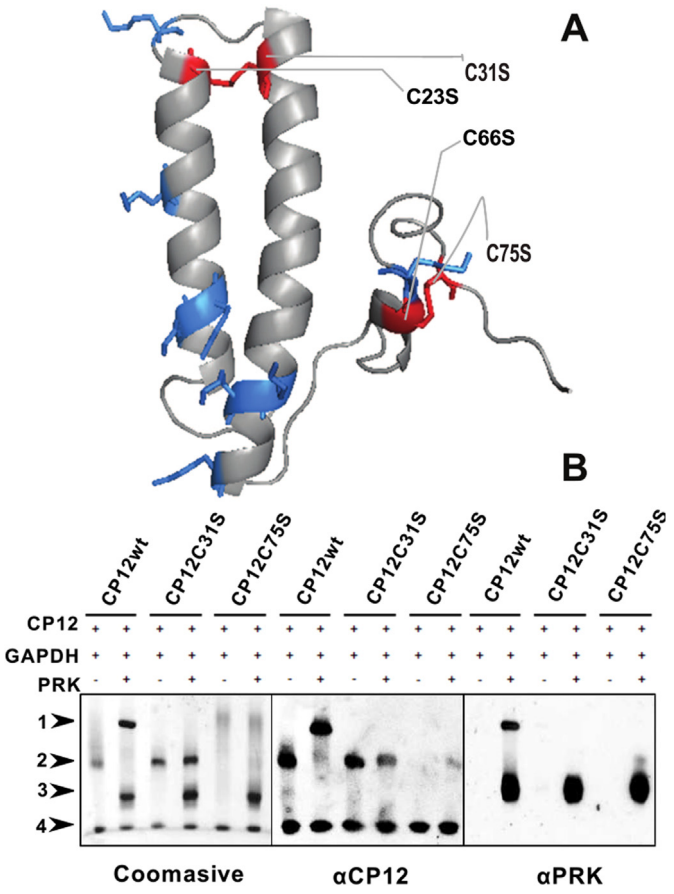

Fig. 1. Structure and interaction of CP12 with GAPDH and PRK. (A) 3D structural model of *Chlamydomonas reinhardtii* CP12. The highlighted lysine (blue) and cysteine residues (red) were used for donor and acceptor labeling in corresponding CP12 mutants respectively. (B) In vitro reconstitution of the GAPDH/CP12/PRK complex for the wild type and mutant CP12s. GAPDH (0.09 nmol) and wild type or mutant (C31S or C75S) CP12s (0.18 nmol) were incubated 12 h at 4 °C in 30 mM Tris, 4 mM EDTA, 0.1 mM NAD and 5 mM cysteine, pH 7.9 in the presence or the absence of PRK (0.09 nmol). The samples were separated on 4–15 % native PAGE (PhastGel, GE Healthcare) and proteins were subsequently revealed with Coomasive staining (left panel) or immunoblots using antibodies raised against CP12 or PRK (middle and right panels respectively). From top to bottom, the arrows indicate: (1) the GAPDH/CP12/PRK complex, (2) the GAPDH/CP12 complex, (3) free GAPDH and PRK and (4) free CP12. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

mutants, and retrieved low FRET efficiencies at high GuHCl concentration (5 M), confirming the flexibility and complete disordered state of CP12 mutants (data not shown).

In CP12C31S, PRK does not show any effect on the FRET histogram peak at $E < 0.1$, confirming that the complex CP12-PRK is not formed due to the lack of the N-terminal disulfide bridge on the CP12C31S mutant (Fig. 2B). However, in the presence of GAPDH, CP12C31S mutant shows a peak at intermediate $E \sim 0.26$, resulting from the formation of a somewhat fuzzy folded structure of CP12 in complex with GAPDH (Fig. 2C). Remarkably, in the presence of both GAPDH and PRK proteins CP12C31S shows the peak at $E \sim 0.33$, resulting in the formation of a more folded structure of CP12C31S protein (Fig. 2D). In the case of CP12C31S, and upon treatment with DTT that disrupts the unique bridge C66–C75 at the C terminus of CP12, even in the presence of GAPDH, a peak at $E < 0.1$ was observed, confirming that the complex was not formed between reduced CP12C31S and GAPDH (Fig. 2E).

With CP12C75S, the presence of PRK induces a significant shift of the FRET histogram peak to $E \sim 0.49$, indicating the formation of a more compact structure of CP12 in interaction with PRK (Fig. 2G). In contrast, GAPDH does not induce any structural modulation of CP12C75S, confirming the involvement of the C-terminal disulfide bridge in the complex formation (Fig. 2H). However, in the presence of both GAPDH and PRK, CP12C75S shows a narrower peak at $E \sim 0.41$ than in the presence of PRK alone, indicating a significant change in the energy transfer efficiency, clearly demonstrating that the presence of GAPDH does affect CP12C75S structure in the presence of PRK (Fig. 2I). Upon treatment with DTT, even in the presence of both PRK and GAPDH, CP12C75S shows low FRET efficiencies, confirming the lack of interactions between reduced CP12C75S and PRK (Fig. 2J).

3.1.2. Inter FRET approach

To probe the intermolecular interaction between CP12wt and PRK we labeled CP12wt with the Atto590 donor dye and PRK with Atto647N acceptor dye. Atto 590 and Atto 647N fluorophores were linked *via* lysine and cysteine residues in CP12wt and PRK respectively. Fig. 3 shows confocal FRET histogram of labeled CP12wt in the presence and absence of PRK. In the energy transfer efficiency (E) histograms, the peak at $E \sim 0$ shifted to $E \sim 0.28$, which confirms the average distance between donor and acceptor is > 85 Å in CP12-PRK complex (Fig. 3A). Further addition of unlabeled GAPDH to the CP12-PRK complex, shows the peak at $E \sim 0.23$, indicating a moderate change in the energy transfer efficiency when compared to presence of PRK alone (Fig. 3B). Upon treatment with DTT, even in the presence of PRK and GAPDH the FRET histogram has low efficiencies <20%, confirming that the complex was not formed between CP12wt and PRK (Fig. 3B).

4. Discussion

FRET studies can be used to assess the compactness of proteins at equilibrium and also to study the folding at the single molecule level [27–29]. IDPs show highly heterogeneous and dynamic ensembles of conformations due to their structural flexibility, and fold only on binding to their cellular targets, although some IDPs remain highly flexible even bound to their partner and form what is called "fuzzy complexes" [30]. CP12 is found in most photosynthetic organisms [11], and acts as a linker between PRK and GAPDH; it is of paramount importance in the cell, regulating both enzyme activities. It is a member of the IDP family [22], and deciphering its folding and binding to target proteins is expected to be essential for understanding its role in the regulation of CO_2 assimilation. Our study focuses for the first time on CP12 interactions with GAPDH and PRK during the formation of supra molecular or multi-enzyme complex by FRET. CP12 structure is still unknown, and a 3D model showed that oxidized CP12 is mainly composed of alpha helix, and coil segments while reduced CP12 is mainly disordered [26]. Since there is no structural information for the algal complex, our current study attempted to elucidate for the first time, CP12 structural transitions in *C. reinhardtii*, in terms of energy transfer efficiency to understand its mechanism of binding to structurally different key regulated enzymes, namely GAPDH and PRK.

For the first time our results show that CP12 mutants *in vitro* can form an intermediate "monomeric" unit (GAPDH–CP12–PRK) complex. Remarkably, the disruption of both the C-terminal and N-terminal disulfide bridges on CP12 does not prevent the formation of the monomeric unit (GAPDH–CP12–PRK). Our data does not show any sign of the formation of CP12C75S-GAPDH complex, suggesting that PRK acts as a strong modulator and strengthens the interaction of this mutant with GAPDH. Similarly, the absence of CP12C31S FRET modification in the presence of PRK and the clear

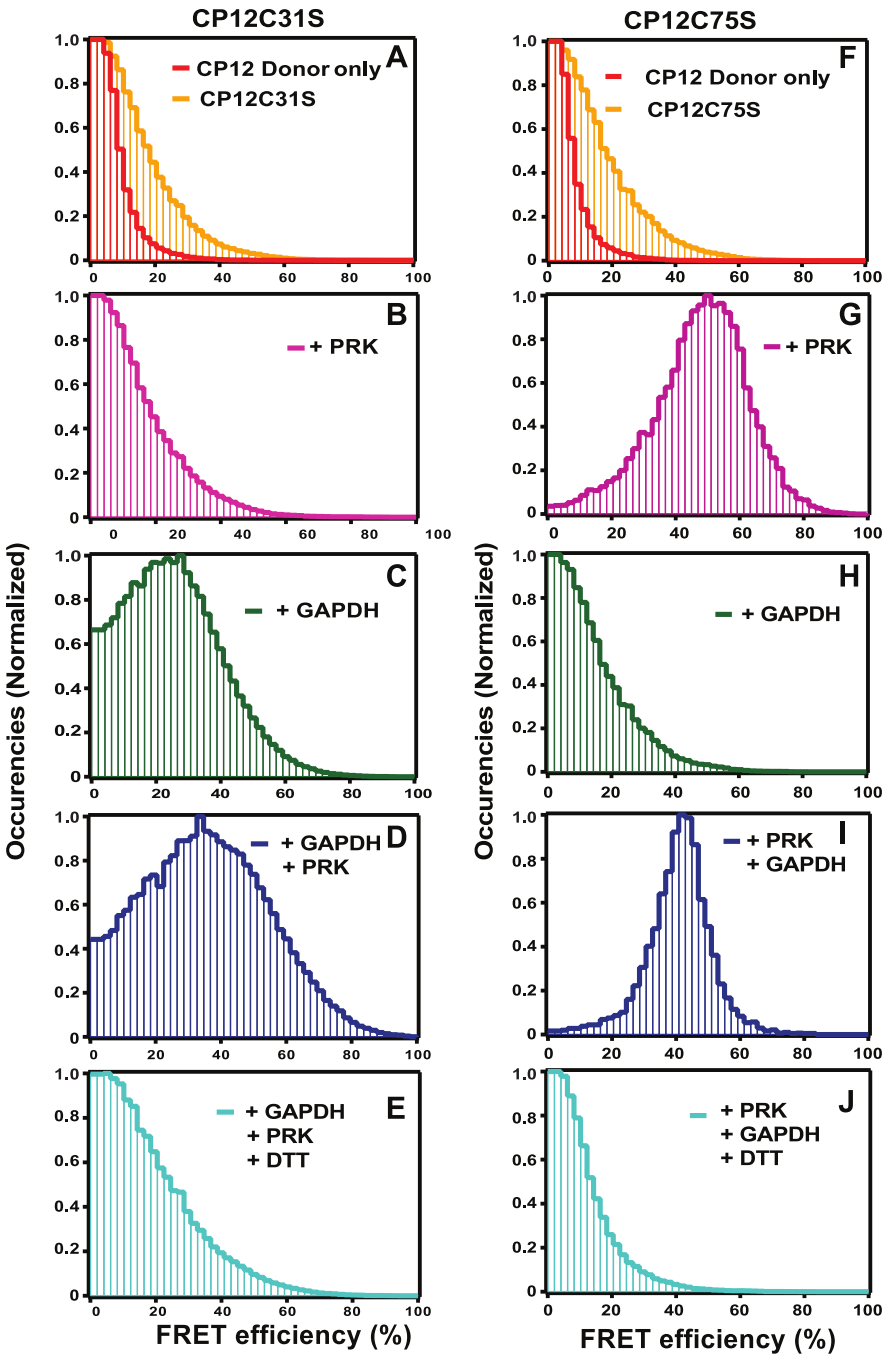

Fig. 2. FRET efficiency histograms of dual-labeled CP12C31S and CP12C75S. All experiments were carried out at 21 °C using reconstitution buffer at pH 8.0 in the presence or absence of unlabeled 50 nM GAPDH tetramer and/or, 50 nM PRK dimer. The higher value in FRET histogram indicates the degree of compactness of the protein. 1 mM DTT was used as a reducing agent, and corresponding samples were incubated for 2 h at room temperature.

FRET shift for the GAPDH-CP12C31S-PRK indicates that GAPDH acts as a strong controller that strengthens CP12C31S and PRK interaction. One can conclude that CP12 is able to interact more or less strongly with PRK alone and GAPDH alone but much more strongly with GAPDH and PRK during the formation of the monomeric unit. A similar result had been observed in the cyanobacterium *Synechococcus sp*. PCC7289, its CP12 not displaying the N-terminal disulfide bridge but still interacting with PRK [31]. These complexes however are unable to dimerize as does the wild type, suggesting the disulfide bridges must play an essential role in the formation of the complex dimeric unit, probably through redox properties.

For the first time, our results show that the broad distribution in the FRET histograms suggests that CP12 exhibits multiple conformations in the presence of both GAPDH and PRK separately and in concert. In Fig. 2, both CP12 mutants CP12C31S and CP12C75S show significant differential binding induced structural modulation with GAPDH or PRK respectively. Given the importance of CP12 mutant conformational modulation, it is surprising that the PRK-CP12 complex is more compact than the GAPDH-CP12 complex. However while crystal structures have been solved of the higher plant *Arabidopsis thaliana* and the cyanobacterium *Synechococcus elongatus*, in both organisms a fuzzy complex was found for the GAPDH-

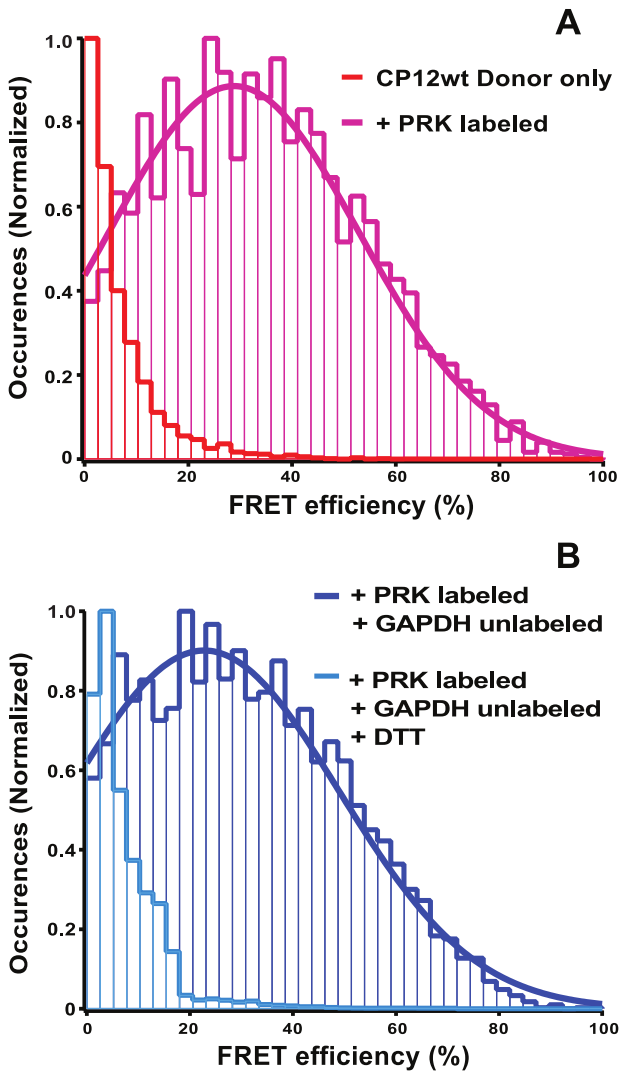

Fig. 3. Inter molecular FRET between CP12wt and PRK. FRET histograms of Atto 590 labeled CP12wt in the presence or absence of Atto647N labeled PRK alone (A), and in concert together with unlabeled with GAPDH (B). Gaussian distributions are used to fit the data (solid lines). Corresponding samples were incubated in 1 mM DTT for reducing the di-sulfide bridges to disrupt the formation of GAPDH/CP12/PRK complex.

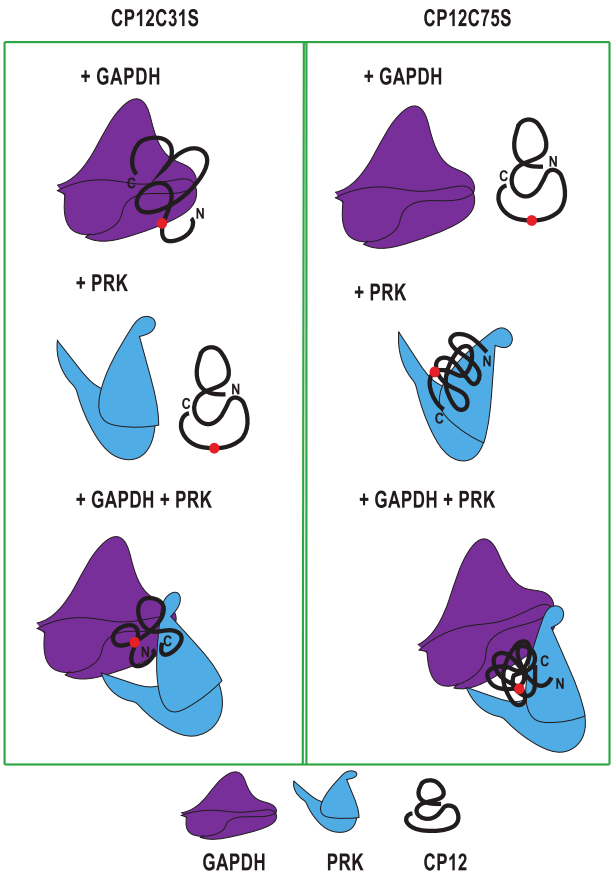

Fig. 4. Schematic representation of the CP12 structural interplay by GAPDH and PRK. Both CP12 mutants (CP12C31S and CP12C75S) show an ensemble of distorted structure with some residual structure. N and C mark the location of the protein termini. The GAPDH alone imprint effect on CP12C31S induces a structural rearrangement toward a fuzzy state. The PRK interaction on CP12C75S triggers a conformational change toward a more compact state. The GAPDH alone, and PRK alone does not form complex with CP12C75S and CP12C31S respectively. Both GAPDH and PRK together induce various conformational changes on both CP12 mutants, resulting in the formation of the "monomeric" unit (GAPDH–CP12–PRK).

CP12 complex with the fifty first residues of CP12 not visible in the density map of the crystals [21,32,33].

The CP12 protein has a strong negative charge potential due to an enriched content in negatively charged residues [33]. Highly charged IDPs exhibit pronounced collapse due to electrostatic repulsions between residues during folding [34,35]. It was established that alterations in the electrostatic potential properties of charged residues leads the subsequent protein–protein interactions [36]. In the present study, CP12 low FRET efficiency can be at least partly mediated by electrostatic repulsions of the highly charged residues in the absence of GAPDH and PRK. The significant increase in the FRET histograms suggests that CP12 intra molecular repulsions were neutralized by GAPDH and PRK. These features suggest that the formation of GAPDH-CP12 or CP12-PRK or GAPDH-CP12-PRK complexes involves coupled binding and folding of CP12.

Fig. 4 summarizes the molecular dynamics of the GAPDH-CP12-PRK complex. We hypothesize that the partially folded CP12 within the GAPDH-CP12 or PRK-CP12 complex generates extensive negative charge potential on its surface, which may recruit and attract positively charged surfaces on PRK or GAPDH *via* electrostatic forces (Fig. 4). Thermodynamically, a population of molecules is distributed over all the energy states associated with the different conformations according to the Boltzmann law, very often linked to the number of residues of polypeptide chains [37]. In the case of CP12, the number of residues is small but linked to its IDP feature; CP12 molecules can be distributed to many energy levels. Here we show that PRK and GAPDH "rigidify" CP12, decreasing the number of populated energy levels and that PRK seems to be the major actor to dictate a conformation to CP12.

Conflict of interest

None.

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