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A knot in the protein structure: probing the near-infrared fluorescent protein iRFP designed from bacterial phytochrome

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Abstract

Substantial interest to a family of the bacterial phytochrome photoreceptors (BphPs) is caused by development of near-infrared fluorescent proteins and biosensors, molecularly engineered from BphPs. The near-infrared fluorescent proteins have allowed bioimaging of deep tissues and whole organs noninvasively in living animals. BphPs and derived near-infrared fluorescent proteins contain a structural element, called knot, in their polypeptide chains. Formation of knot structures in proteins was denied for a long time. Here, we studied denaturation and renaturation processes of the near-infrared fluorescent probe, iRFP engineered from RpBphP2, which utilizes a heme-derived tetrapyrrole compound biliverdin as a chromophore. iRFP contains a unique figure-of-eight knot. The denaturation and renaturation curves of the iRFP apoform coincided well, suggesting its efficient refolding. However, the iRFP holoform exhibited the irreversible unfolding and aggregation, associated with the bound chromophore. The knot structure in the apoform did not prevent its subsequent binding of biliverdin, resulting in the functional iRFP holoform. We suggest that the irreversibility of protein unfolding is caused by posttranslational protein modifications, such as chromophore binding, rather than the presence of the knot. These results are important for future design of BphP-based near-infrared probes and add important features to the fundamental problem of protein folding.

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AUTHOR CONTRIBUTION

K.K.T., V.V.V. and I.M.K. conceived the study. Olesya V.S., G.S.B., Olga V.S. and D.M.S. performed the experiments and analyzed the data. All authors took part in the results discussion and manuscript preparation. All authors read and approved the final manuscript.

Keywords

RpBphP2; knot; bacteriophytochrome; iRFP; IFP1.4; biliverdin

INTRODUCTION

Substantial interest in the study of a subfamily of phytochromes found in bacteria, called bacterial phytochromes (BphPs), is associated with recent development of near-infrared fluorescent probes for bioimaging of deep tissues and organs in whole living animals [1, 2]. These probes can be used for versatile basic biological applications and biomedical diagnostics. In general, phytochromes are the red/far-red photoreceptors that are found in plants [3], fungi [4–8] and several types of prokaryotes [9, 10]. Light absorption by chromophores of phytochromes induces photoreactions that are transmitted to an effector domain, resulting in activation of signaling. BphPs utilize a heme derived tetrapyrrole compound, called biliverdin IX α (BV), as a chromophore [11], and their output effector domains are usually consist of histidine kinase domains [12].

BphPs can exist in two stable states that absorb at 680–710 nm, which is known as the Pr-state or the red absorbing state, and at 740–760 nm, which is known as the Pfr-state or the far-red absorbing state. Most BphPs adopt the Pr-state as their ground state, whereas a few of them, namely the bathy BphPs, can be found in the Pfr-ground state [13]. The BphPs in the Pr- and Pfr-states differ by the conformation of BV: its D ring adopts either *cis*- or *trans*-configuration, respectively. The ground Pr-state (or Pfr-state) can be converted into the Pfr-state (or Pr-state) upon illumination with red (or far-red) light. This photo-induced state can be slowly reversed back by means of dark reversion or can be rapidly reversed upon illumination with far-red (red) light [11, 12].

BV binding occurs in the chromophore-binding domain (CBD) of the protein, which consists of the PAS and GAF domains in BphPs. The names of the PAS and GAF domains are acronyms of the protein names in which these domains were first identified: PER/ARNT/Sim in the case of the PAS domain and cGMPphosphodiesterase/Adenylcyclase/FhlA transcriptional activator in the case of the GAF domain. The PAS domain in BphPs contains at N-terminal patch a conserved cysteine residue that covalently attaches the BV, while the chromophore itself fits into the cleft in the GAF domain.

The N-terminal region of 35 residues upstream of the PAS domain and the loop contributed by the GAF domain (residues 225–257) create a knot structure that bridges the PAS and GAF domains. Initially, this knot was misclassified as a trefoil knot, in which the polypeptide chain has three crossings [14, 15]. The high resolution structure of the CBD from the *D. radiodurans* bacterial phytochrome DrBphP showed that this knot was a figure-of-eight knot with four crossings of the polypeptide chain [16]. The function of the knot in BphPs remains unclear. However, it has been shown that the knot does not donate to CBD of DrBphP unusually high resistance to mechanical denaturation [17].

A number of possible functions have been attributed to the knot in BphPs including stabilizing the contacts between the PAS and GAF domains, reducing the undesirable energy

losses upon chromophore isomerization during photoconversion, associated with flexibility of the chromophore-binding site, and ensuring the correct positioning of the Cys residue for conjugation to the chromophore by restricting mobility of the N-terminal patch [14]. The other proposed function of knots is to prevent the degradation of the proteins in the cell [15, 18, 19].

Recently, several BphPs have been developed into the near-infrared fluorescent proteins, [20–22], which are able to bind BV present at low concentrations in mammalian tissues and, thus, to acquire near-infrared fluorescence without supplementing with a large excess of exogenous BV. The first protein in this series, termed iRFP (recently renamed into iRFP713), was engineered from the CBD of the *R. palustris* bacterial phytochrome RpBphP2 [20]. iRFP differs from the CBD of RpBphP2 by 13 amino acid substitutions (Figure 1 and “Material and methods”). However, similarly to parental RpBphP2 it has the figure-of-eight knot structure. The palette of the near-infrared fluorescent proteins have been expanded with four spectrally distinct probes developed from the CBDs of the *R. palustris* bacterial phytochromes RpBphP2 and RpBphP6 [22]. The most blue- and red-shifted of them, iRFP670 and iRFP720, have been successfully applied to visualize two different tumor in the live mouse using photoacoustic tomography [23]. It was also shown that a bimolecular near-infrared fluorescence complementation probe, named iSplit [24], which was designed from iRFP by splitting the PAS and GAF domains, has the decreased intracellular stability with respect to its ancestor. iSplit is formed by the complementation of separate PAS and GAF domains when they are brought into close proximity due to the interaction between their fusion partners. As the PAS and GAF domains are synthesized and likely prefold separately, the assembled from them iSplit can devoid the knot.

For a long time, it was believed that the presence of knots in the protein structure was impossible [25]. Our current knowledge of protein folding is summarized in the energy landscape model [26, 27]. It is thought that during evolution the protein sequences with cooperative and fast folding, that is the proteins whose folding are described by the funnel-like minimally frustrated energy landscape, are mainly selected [28]. In turn, during knot formation, the protein should overcome a high entropic barrier of threading one terminal through a loop in the polypeptide chain, and this process significantly slows down protein folding. Therefore, it is interesting that proteins with the knot topology have been preserved during evolution.

The mention of a knot formation in a polypeptide chain was first made almost 40 years ago [29]. Nevertheless, this hypothesis was considered improbable for a long time. Moreover, protein structures with knotted topology were discarded from the prediction of protein structure on the basis of the crystallographic data and simulations of protein folding [30, 31]. Currently, the existence of proteins with different types of knots has been proven [32–35]. The first discovered knot structure was the trefoil like knot of carbonic anhydrase [36]. Because the deletion of two or three amino acids at the C-terminus of carbonic anhydrase results in the disappearance of the knot, this protein is often not considered as a knotted protein. A deep trefoil knot was also found in RNA-methyltransferases [37, 38], in Rds3p from *S. cerevisiae*, which contains a Zn-finger structure [39], and in the nucleic acid binding protein in the ribbon-helix-helix (RHH) superfamily of DNA-binding proteins [40, 41]. To

date, the most complex knots have been detected in ubiquitin carboxyl-terminal hydrolases, which contain knots with five crossings of the polypeptide chain [15, 42], and in the haloaciddehalogenase DehI from *P. putida*, which contains a knot with six crossings of the polypeptide chain [40, 43].

Moreover, a high conservation of knot structure among the members of the protein family was shown [44]. This result implies that knots can donate some functional advantages to protein despite the difficulty to fold the protein with such topologically complex element. Theoretical studies of the folding of knotted proteins allowed shielding some light on the knotting mechanism [45]. These investigations argue for the knotting to be a rate-limiting step during protein folding and suggest the mechanism of knot formation via twisted loop and slipknot intermediates [46–48]. The formation of the most complex knot in DehI has been proposed to involve the flipping step [40].

Here, we studied the guanidine hydrochloride (GdnHCl)-induced denaturation and renaturation processes of iRFP [20] in its holoform (in the complex with the BV chromophore) and in its apoform (the chromophore free) states. The effects that the BV chromophore and the knot in the iRFP structure have on protein folding are discussed.

RESULTS

Spectral features of iRFP in the holoform

iRFP in a holoform in buffer solution had an absorption band with a maximum at 280 nm, which can be attributed to aromatic residues, and two bands with maxima at 390 and 690 nm caused by chromophore absorption (Figure 2A). The absorption band of BV in the holoprotein with a maximum at 690 nm was approximately two times more intense than that with a maximum at 390 nm. The far-red absorption band also had a shoulder at 640 nm. The absorption spectrum of free BV in buffer solution is also given in Figure 2A. This spectrum contained two absorption bands that peaked at 376 and 680 nm, with the absorption peak at 376 nm being the most pronounced in this case. The iRFP holoprotein acquired its characteristic near-infrared fluorescence with a maximum at 715 nm under excitation with light at a wavelength of 690 nm. The excitation spectrum of iRFP in holoform recorded at the maximum of chromophore emission included two bands at 390 and 280 nm, in addition to the band at 690 nm; all of the excitation bands matched with the absorption ones (Figure 3).

The iRFP holoprotein has a pronounced CD spectrum with a two-peaked negative band in the far-UV region, which is a typical of proteins with mixed α/β secondary structures (Figure 2B). The near-UV CD spectrum of iRFP in holoform was characterized by a marked negative band (Figure 2E). The visible CD spectrum of iRFP had a clearly distinguishable negative band at 690 nm, with a shoulder at 640 nm, and a positive band at 390 nm, which is typical of bacterial phytochrome in the Pr-state (Figure 2F).

The tryptophan fluorescence spectrum of iRFP in holoform was blue-shifted, with a maximum at 330 nm (Figure 2C and Table 1). The holoprotein was characterized by a high value of fluorescence anisotropy ($r=0.15$; $\lambda_{\text{ex}}=297$ nm, $\lambda_{\text{em}}=365$ nm) and a short lifetime of

tryptophan fluorescence (1.2 ns) (Table. 1). The fluorescence of the tryptophan residues of the iRFP holoprotein in its native state was quenched. We analyzed the microenvironment of the tryptophan residues of iRFP using X-ray data of the CBD of bacterial phytochrome RpBphP2 (4E04.ent file) [49], keeping in mind the substitutions that were made to develop iRFP. iRFP contains three tryptophan residues: Trp108, Trp280, and Trp310. All of these residues should impact the total fluorescence of the protein, as possible fluorescence quenchers are not present in their microenvironment. The density of the microenvironment of the tryptophan residues is quite high; there are as many as 72, 75 and 78 atoms in a 7 Å radius from the indole ring of Trp108, Trp280, and Trp310, respectively. In comparison, the microenvironment of the single tryptophan residue Trp48 of azurin, which has a unique blue fluorescence spectrum with a maximum at 308 nm, is composed of only 69 atoms [50]. The high value of fluorescence anisotropy is also indicative of a rigid microenvironment of the tryptophan residues of iRFP. The residue Trp108 is submerged in the inner part of the PAS domain and is evidently inaccessible to the solvent. Residues Trp280 and Trp310 are located closer to the periphery of the GAF domain and are, therefore, partially exposed to the solvent. Both of these residues have five molecules of bound water in their microenvironment. Residue Trp310 belongs to the α -helix of the GAF domain, which constitutes the dimeric interface of iRFP. As a result, the microenvironment of Trp310 from one monomer of the iRFP dimer includes Trp310 of the other monomer.

All of the tryptophan residues have several polar groups in their microenvironment. The microenvironment of Trp108 contains the polar groups of Arg110 and bound water. The polar groups of residues Arg173, Arg275 and Glu316 are found in the microenvironment of Trp280, and the polar groups of Gln144 and Gln311 are located in the vicinity of Trp310. Usually, blue-shifted spectra are typical of tryptophan residues that are in microenvironments formed mainly by hydrophobic residues, as is the case of the single tryptophan residue Trp48 of azurin [50]. The same fluorescence properties can be observed for tryptophan residues with microenvironments that are predominantly composed of polar residues if the microenvironment is rather rigid [51]. The analysis of the location and characteristics of the tryptophan microenvironments in iRFP showed that its blue-shifted tryptophan fluorescence was caused by the rigidity of the microenvironment, even though these microenvironments contained polar groups.

The unfolding–refolding of iRFP in the holoform

Quasi-equilibrium curves of the different characteristics of iRFP in holoform, including the intensity of tryptophan fluorescence at the emission wavelength of 320 nm, parameter *A* and fluorescence anisotropy, chromophore fluorescence intensity at the emission wavelength of 715 nm and the ellipticity at 222 nm, are shown in Figure 4 as a function of the final GdnHCl concentration. Quasi-equilibrium curves of the GdnHCl-induced unfolding and refolding of the holoprotein were recorded after the protein was equilibrated in the presence of a desired denaturant concentration for 24 hours. The prolonged equilibration time did not result in a noticeable change in the detected characteristics. As the drastic change of the red absorption band of iRFP in holoform was clearly observed in the entire range of GdnHCl concentrations (Figure 5A and Figure 4B, insert), we corrected the chromophore fluorescence to the change in the total optical density of the solution at the excitation

wavelength according to the procedure previously developed in our laboratory [52–54]. It was shown that the denaturation of iRFP in holoform was irreversible. Indeed, all of the recorded characteristics of the holoprotein did not recover to the level of the native protein, even under strong refolding conditions where the final concentration of GdnHCl was less than 0.4 M (Figures 4 and 5). The accumulation of partially folded protein in 2 M GdnHCl was observed during the refolding of the iRFP holoprotein. This state was characterized by a less pronounced secondary structure compared to the native state (Figure 4E). The chromophore fluorescence was still quenched in this state, indicating that the protein core was not organized around BV (Figure 4B). Conversely, the structure around the tryptophan residues was already formed (Figure 4A, C and D). Still, the characteristics of tryptophan fluorescence in the partially folded state differed from those of native iRFP. As stated previously, iRFP contains three tryptophan residues; Trp108 is located in the PAS domain, and Trp280 and Trp310 are located in the GAF domain. In its partially folded state, the chromophore must be incorporated into the cleft of the GAF domain has not yet been packed into the protein globule; therefore, it is tempting to speculate that in the partially folded state the PAS domain has collapsed, while the GAF domain remains unstructured. This assumption requires experimental confirmation.

Decreasing the GdnHCl concentration below 1.5 M led to the aggregation of molecules in the partially folded state, as indicated by a significant increase in light scattering (Figure 4F). It was previously revealed that, under certain conditions, GdnHCl can induce the aggregation of proteins in a partially folded state [55]. The decrease in the CD in the far-UV region at GdnHCl concentrations less than 1.5 M (Figure 4E) was most likely due to protein aggregation, as light scattering can distort the CD spectra of macromolecules [56]. Therefore, we wanted to know what element of protein assembly, the chromophore or the knot, interfered with protein folding. To answer this, we expressed and purified the iRFP apoprotein, which is devoid of the chromophore.

The unfolding–refolding of iRFP in the apoform

First, we studied the properties of iRFP in apoform (Figure 2). The absorption spectrum of the apoprotein exhibited a dominant absorption band with a maximum at 280 nm, which can be attributed to aromatic residues, and two minor bands with maxima at 390 and 690 nm caused by the absorption of bound BV (Figure 2A). This was evidence of slight contamination of the apoprotein solutions by the holoprotein. The tryptophan fluorescence spectra of the apoprotein and holoprotein overlap perfectly (Figure 2C). The other characteristics of tryptophan fluorescence (anisotropy and parameter *A*) were similar for the two forms of the protein (Table 1). The tryptophan fluorescence of iRFP in holoform was strongly quenched (Figure 2D). Furthermore, the lifetime of the tryptophan residues in the excited state of the apoprotein was three times higher compared to that of the holoprotein (3.7 and 1.2 ns, respectively). We suppose that the dissipation of tryptophan fluorescence in the holoprotein occurred through an effective non-radiative energy transfer to the chromophore of iRFP. iRFP in the apo- and holoforms have nearly identical secondary structures, as confirmed by the coincidence of their CD spectra in the far-UV region. As expected, the CD of the iRFP apoprotein in the visible region was not pronounced because this CD was determined by the absorption of the chromophore. Moreover, the CD of the

apoprotein in the near-UV region was not expressed. Apparently, the pronounced CD spectrum of the holoprotein in the near-UV region arose from the chromophore, which has minor absorption bands in the UV region in addition to the dominant absorption bands with maxima in the visible region (390 and 690 nm). Thus, the spectral features of iRFP in apoform argue that it retains the secondary and tertiary structures inherent to the native protein. Next, we tested the ability of iRFP in apoform to bind BV (Figure 6). These experiments were conducted using equilibrium microdialysis analysis, which utilizes a device divided into two microdialysis cells that are connected by a membrane permeable for substances of low-molecular-weight. In our experiment, one cell of the microdialysis device was filled with the apoprotein solution with a concentration of 8 μ M. A molar excess of BV (in the range of 25 to 70 μ M) was added into the second cell of the microdialysis device. The concentration of free BV was allowed to reach equilibrium in both cells. The binding of BV to iRFP included the covalent attachment of the dye to the protein, meaning that the chromophore irreversibly interacts with iRFP, and this process is non-equilibrium. Consequently, the BV binding constant to iRFP cannot be calculated. Still, this approach allowed us to establish a portion of apoprotein molecules that were bound to the chromophore. BV was incorporated into iRFP at a molar ratio of 1:1, and full saturation of the apoprotein with BV was reached. This is probably indicative of an absence of misfolded protein molecules. BV binding to apo-iRFP also resulted in a characteristic change of the visible absorption of the complex and in the acquisition of near infrared fluorescence, as well as pronounced CD in the visible region (Figure 6). These data show that the apoprotein was able to properly bind biliverdin, and this indicates that the conformation and microenvironment of BV in the apo-iRFP/BV complex is likely similar to the native iRFP.

Different structural probes (tryptophan fluorescence and the ellipticity at 222 nm) were used to examine the GdnHCl-induced unfolding and refolding of iRFP in its apoform (Figure 7). The comparison of the denaturation curves of tryptophan fluorescence intensity, parameter *A* and ellipticity at 222 nm recorded during the unfolding of iRFP in the apo- and holoforms is shown in Figure 7. Experimental data imply that, despite the same secondary and tertiary structure of the apo- and holoproteins, the chromophore significantly stabilizes iRFP and makes the denaturation transition more cooperative. It is important to note that we normalized the fluorescence intensity at the emission wavelengths of 320 nm and 365 nm for both proteins according to the fluorescence of its unfolded state. We supposed that, in the unfolded protein, there were no conditions for the energy transfer from the tryptophan residues to the chromophore, and the tryptophan fluorescence of the apo- and holoproteins in the unfolded state could be considered equal. The normalization procedure described above allowed us to compare the denaturation curves of tryptophan fluorescence intensity obtained during the unfolding of iRFP in the apo- and holoforms (Figure 8A, B). It was obvious that the intrinsic fluorescence of the holoprotein was quenched in its native state, and an increase in fluorescence occurred at high denaturant concentrations after disruption of the protein structure.

DISCUSSION

In the past few decades, our understanding of protein folding has accumulated from studies that mainly address the folding of small globular proteins. Proteins that possess complex

structural elements can enrich our understanding of protein folding. Thus, the study of knotted proteins is important to understand protein folding in general. Most theoretical and experimental studies of protein folding with knotted topology have been performed on YibK and YbeA from a class of RNA methyltransferases that contain a deep trefoil knot [57–60]. These studies have shown that the *in vitro* unfolding of both YibK and YbeA induced by chemical denaturants is reversible [58, 59], while refolding these proteins is a complex process that involves the accumulation of intermediate states [57]. These studies have also shown that the newly translated polypeptide chain spontaneously folds in a cell-free translation system and that its correct folding does not require the assistance of molecular chaperones [61].

We studied the GdnHCl-induced denaturation and renaturation processes of the near-infrared fluorescent protein iRFP, which was developed from the truncated bacterial phytochrome RpBphP2 (Figure 1). iRFP retains the PAS and GAF domains that interact in the figure-of-eight knot. It was shown that the figure-of-eight is a feature of PAS-containing phytochromes. The knot is tied by the N-terminal region upstream of the PAS domain and the loop from the GAF domain [62, 63]. The knot region contains a highly conserved among PAS-containing phytochromes residues, e.g. Ile35 and Gln36 [64]. The residue of Gln36 is involved in hydrogen bond that is supposed to strengthen the knot. The knot function is not well established. It has been assumed that the knot of bacterial phytochromes can minimize the energy losses upon chromophore isomerization during photoconversion, impact to productive conformational changes in photosensory core module (it is formed by PAS, GAF and PHY domains) during chromophore isomerization, and/or facilitating the efficient binding of the chromophore [14, 17].

It was revealed that iRFP denaturation was irreversible, and protein renaturation was complicated by the aggregation of partially folded protein molecules. At the same time, the denaturation of iRFP in apoform (BV free) was fully reversible, meaning that the knot does not prevent efficient protein folding. According to these data, we concluded that the irreversibility of iRFP denaturation and protein aggregation is associated with the bound chromophore instead of the knot structure.

We next tested an iRFP mutant bearing a substitution of Cys15 to Ser. This protein is not able to make covalent bond with the chromophore but able to bind it into GAF cleft. The similar experiments as above on unfolding–refolding for this mutant showed the reversibility of its denaturation in the presence of BV. The integrity of protein structure was tested by near infrared fluorescence of the chromophore and parameter *A* (Figure 9). We observed the recovery of these characteristics at decreasing denaturant concentrations. It is interesting to note that chromophore fluorescence of the mutant iRFP decreases significantly in the presence of small GdnHCl concentration (Figure 9B) where the protein structure is preserved (Figure 9A). This indicated that the addition of small amount of denaturant induces the dissociation of non-covalently bound BV from the protein. Moreover, the stability of mutant iRFP is comparable of those for iRFP in apoform (Figure 8D and 9A). Contrary, covalently bound chromophore was shown to increase the protein stability and make the denaturation transition more cooperative (Figure 8D). Thus, in the absence of

covalently bound BV the protein refolds into native-like structure which is ready to bind the chromophore.

We can propose that covalently bound to iRFP BV makes some non-native interactions with partially folded intermediate which accumulation we have observed during refolding of iRFP in holoform and thus interferes with correct protein folding. The other possibility of the irreversibility of iRFP unfolding is that the covalently bound BV inhibits the knot formation during refolding where N-terminal region bearing BV have to thread through a loop in the polypeptide chain.

Recent studies argue for the existence of knotted regions in the chemically denatured proteins [65, 66]. Mallam and colleagues developed a method to trap a knot in the unfolded state of protein [65]. They constructed mutant variants of YibK and YbeA by inserting Cys residues at both the C- and N-termini; these constructs were circularized in their native state through disulfide bond formation. These oxidized YibK and YbeA variants were unable to untie in their unfolded states. Because the oxidized YibK and YbeA variants refolded with rate constants comparable to those of their reduced non-circularized counterparts, it was concluded that wild-type YibK and YbeA could exist in a knotted conformation in their urea denatured states [65]. Single-molecule Forster resonance energy transfer experiments of doubly labeled TrmD tRNA methyltransferase from *E. coli* revealed that this protein also preserves the knot when denatured by GdnHCl [66]. The knotted conformation of the chemically denatured proteins is thought to be responsible for their successful refolding *in vitro*. Experimental data obtained for YibK and YbeA in the cell-free translation system, which is believed to mimic *in vivo* translation in the best way, demonstrated that knotting is an initial step after polypeptide chain synthesis, and this process is greatly accelerated by the presence of chaperones [61]. The knotted intermediate state is thought to be identical to the chemically denatured knotted structures observed for YibK, YbeA and TrmD. Further folding of the knotted intermediate is chaperone-independent [61].

Notably, the denaturation curves recorded during the unfolding and refolding of iRFP in apoform completely coincided. It is assumed that the functional regions and complex structural elements of the natural proteins can complicate the energy landscape by creating deep traps and can lead to hysteresis of the unfolding and refolding curves of the protein [67]. A well-known example of a protein with hysteretic behavior in the unfolding and refolding pathways is green fluorescent protein [68–70]. In the case of green fluorescent protein, an apparent hysteresis was presumed to arise from the green chromophore that must be locked in the protein core in the correct active form; the process that requires the precise isomerization of the chromophore. A hysteretic behavior of the unfolding–refolding curves was recently observed for 1O6D RNA methyltransferase. This behavior was explained by the decoupling of the unfolding events from the untying of the knotted polypeptide chain [67]. Thus, a non-hysteretic behavior of the unfolding–refolding curves of iRFP in apoform can be indicative of the lack of deep traps in the folding pathway of this protein. We should note that the unfolding and refolding curves of YibK, YbeA and TrmD did not exhibit a hysteretic behavior either [57].

In conclusion, while the denaturation of the iRFP apoform was fully reversible, the denaturation of its holoform was irreversible. This suggests that the knot does not inhibit protein folding. Moreover, our data showed that the irreversibility of iRFP denaturation and protein aggregation was associated with the bound BV chromophore, not with the presence of the knot in the protein. These results and studies of other proteins containing knots suggest that knotting can potentially increase an efficiency of protein folding. Indeed, the knotting of the polypeptide chain will decrease the available conformations of the non-folded protein. If we consider an energy landscape for the knotted protein, its top will be much narrower compared to the unknotted protein. We can assume that the formation of the knot will limit the folding traces that the unfolded knotted chain can follow. Then, the formation of the knot will also increase the probability of correct folding into the native state and reduce the probability of forming misfolded or aggregated states. In this way, the knots can be considered intrinsic “chaperones” that assist the protein in proper folding [61].

MATERIALS AND METHODS

Protein expression and purification

E. coli LMG194 host cells bearing two plasmids, the kanamycin-resistant pBAD/His-B-iRFP encoding infrared fluorescent protein iRFP and the ampicillin-resistant pWA21h encoding hemeoxygenase for BV synthesis in bacteria, were obtained as previously described [20].

LMG194 bacteria were grown overnight in LB medium supplemented with ampicillin, kanamycin, and 2% glucose to repress the synthesis of both proteins. The next morning, the bacterial cells were centrifuged and resuspended in RM medium containing 0.02% rhamnose to induce hemeoxygenase expression, and the cells were grown for an additional 6 hours at 37 °C. iRFP expression was induced by the addition of 0.002% arabinose. The cells were then grown for 8 hours at 37°C, followed by incubation at 18–20 °C for 24 hours. Recombinant iRFP in holoform was purified using Ni+-agarose packed in His-GraviTrap columns (GE Healthcare) according to the manufacturer’s protocol; however, the elution buffer contained 100 mM EDTA instead of 400 mM imidazole. Further purification was achieved using ion-exchange chromatography on a MonoQ column (GE Healthcare). The protocol described above was slightly modified to express iRFP in apoform. The overnight LMG194 culture was centrifuged, resuspended in RM medium, and grown for 2–3 hours at 37 °C; then, 0.002% arabinose was added to induce protein synthesis. The subsequent steps were the same as for the expression of iRFP in holoform.

The purity of the protein was analyzed by SDS-PAGE using 15% polyacrylamide gels [71]. Protein concentration was determined by a theoretical extinction coefficient ($\epsilon_{280}=25350 \text{ M}^{-1}\text{cm}^{-1}$). The protein was concentrated and stored in 20 mM Tris-HCl buffer, pH 8.0. The optical density of the protein samples did not exceed 0.2, and the measurements were performed in 20 mM Tris-HCl buffer, pH 8.0, containing 1 mM TCEP.

Spectrophotometric experiments

Absorption spectra were recorded using a U-3900H spectrophotometer (Hitachi). The experiments were performed in 101.016-QS microcells (5 × 5 mm, Hellma) with path length of 5 mm at room temperature.

Analysis of 3D protein structure

The X-ray data of the CBD of bacterial phytochrome RpBphP2 (4E04.ent file) [49] deposited in the PDB [72] were used as the input data for the analysis of the microenvironment peculiarities of the tryptophan residues localized in the structure of iRFP. The analysis was performed as previously described [50, 51, 73, 74]. Figure 10 shows the amino acid sequences of iRFP, its parental CBD of RpBphP2 and crystallized variant of CBD of RpBphP2 (RpBphP-Cr).

Fluorescence spectroscopy

The fluorescence experiments were conducted using a Cary Eclipse spectrofluorometer with FLR cells (10 × 10 × 4 mm; Agilent Technologies, Australia) with path length of 10 mm. Fluorescence anisotropy and fluorescence lifetime were measured using homebuilt spectrofluorometers with steady-state and time-resolved excitation [75] using 101.016-QS microcells (5 × 5 mm; Hellma).

The tryptophan fluorescence of the protein was excited at the long-wave absorption spectrum edge ($\lambda_{\text{ex}} = 297 \text{ nm}$), where the contribution of the tyrosine residues in the bulk protein fluorescence is negligible. The position and form of the fluorescence spectra were characterized by parameter $A = I_{320}/I_{365}$, where I_{320} and I_{365} are the fluorescence intensities at the emission wavelengths of 320 and 365 nm, respectively [76, 77]. The values of parameter A and the fluorescence spectrum were corrected for the instrument sensitivity. The anisotropy of tryptophan fluorescence was calculated using the equation

$r = (I_V^V - GI_H^V) / (I_V^V + 2GI_H^V)$, where I_V^V and I_H^V are the vertical and horizontal components of the fluorescence intensity when excited by vertically polarized light, respectively, and G is the relationship of the vertical and horizontal components of the fluorescence intensity when excited by horizontally polarized light; G was calculated using the equation ($G = I_V^H / I_H^H$), $\lambda_{\text{em}} = 365 \text{ nm}$ [75]. The specific near infrared fluorescence of iRFP was excited at 690 nm, and emission was detected at 715 nm.

The unfolding (and refolding) of the protein was initiated by manual mixing 50 μl of the native protein (or pre-denatured protein for 8 h in 4 M GdnHCl) with 500 μl of a buffer solution containing a desired concentration of GdnHCl. GdnHCl (NacalaiTesque) was used without further purification. The concentration of the stock GdnHCl solution was determined by the refraction coefficient, which was determined using an Abbe refractometer (LOMO). The GdnHCl-dependent different fluorescent characteristics of iRFP were recorded following protein incubation in a solution of an appropriate denaturant concentration at 23 °C for 24 h. Further increases in the equilibration time did not result in noticeable changes in the detected characteristics. The spectrofluorometer was equipped with a thermostat that constantly maintained the temperature at 23°C.

The recorded fluorescence intensity was corrected to the total optical density of the solution (D_{Σ}). The corrected fluorescence intensity was defined as I/W , where $W = (1 - 10^{-D_{\Sigma}})/D_{\Sigma}$. For details on the correction and normalization of the fluorescence intensity, please refer to [52–54]. These studies show that $I/W \cong Dq$, where D and q are the absorption and fluorescence quantum yield of the fluorophore, respectively. Only the corrected fluorescence intensity can be used to evaluate the fraction of molecules that are in different structural states.

Circular dichroism measurements

CD spectra were obtained using a Jasco-810 spectropolarimeter (Jasco). The far-UV CD spectra were recorded in a 1 mm path length cell from 260 nm to 190 nm, with a step size of 0.1 nm. The near-UV CD spectra were recorded in a 10 mm path length cell from 320 nm to 250 nm, with a step size of 0.1 nm. The visible CD spectra were scanned from 800 to 320 nm, with a step size of 0.1 nm, using a 10 mm path length cell. An average of 3 scans was obtained for all of the spectra. The CD spectra of the appropriate buffer solution were recorded and subtracted from the protein spectra.

Equilibrium microdialysis

Equilibrium microdialysis was performed using a Harvard Apparatus/Amika device, which consists of two chambers (500 μ L each) separated by a membrane (MWCO 10,000) that is impermeable to particles larger than 10,000 Da. Equilibrium microdialysis involves the allocation of two interacting agents, a ligand and its receptor, in two chambers (#2 and #1, respectively) divided by a membrane that will allow the ligand to pass but is impermeable to the receptor. In our experiment, iRFP solution was placed in chamber #1, and the BV solution was placed in chamber #2. The protein and BV were dissolved in 20 mM Tris-HCl buffer, pH 8.6, for the microdialysis experiment. The iRFP concentration was 8 μ M, and the BV concentration varied from 25 to 70 μ M. BV hydrochloride (Frontier Scientific) was used without further purification. The BV concentration was determined by an extinction coefficient of $\epsilon_{376} = 45500 \text{ M}^{-1} \text{ cm}^{-1}$ [78].

The microdialysis device was left on a rocker for 5 days at 4°C to allow the equilibration of the free BV concentration in both chambers. The equilibration time was estimated from test experiments in which one chamber contained BV and the second chamber contained a buffer solution. After equilibration, the free BV concentration (C_f) in both chambers was equal, while the total BV concentration in chamber #1 was greater than that in chamber #2, due to the concentration of the bound chromophore (C_b), which was determined using the following equation: $C_b = C_0 - 2C_f$, where C_0 is the initial BV concentration in chamber #2.

The absorption spectrum of the solution in chamber #1 represents the superposition of the absorption spectra of free BV in the concentration C_f and of the iRFP/BV complex. The absorption spectrum of the latter was calculated by subtracting the absorption spectra in chamber #2 from the absorption spectra in chamber #1.

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Abbreviations

BphPs	bacterial phytochrome photoreceptors
BV	biliverdin IX α
CBD	chromophore-binding domain of BphPs, iRFP (or iRFP713), the near-infrared fluorescent protein from bacterial phytochrome RpBphP2 of the <i>R. palustris</i>
GdnHCl	the guanidine hydrochloride

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Figure 1.

The structure of iRFP dimer. (A) It is shown that iRFP is engineered from *R. palustris* bacterial phytochrome RpBphP2 by truncation of PHY and His kinase domains and consists only chromophore-binding domains (CBD) namely PAS and GAF domains which differ from wild type CBD of RpBphP2 by 13 amino acid substitutions (one in the N termini, three in the PAS domain and the others in the GAF domain six of which are located in the direct contact with biliverdin). (B) The 3D structure of CBD of bacterial phytochrome RpBphP2. The location of Cys 15, biliverdin (BV) and knotted loop are shown. PAS and GAF domains are shown in pink and green, respectively. (C) A figure-of-eight topology of the knot in RpBphP2. A trace of polypeptide chain of CBD of RpBphP2 is shown in different colors to make a comparison with schematic representation of the

knot easier. The chain crossovers in the protein are highlighted in circles. The drawing was made on the basis of X-ray data of the 4E04 file [49] deposited in the PDB [72].

Figure 2.

Spectral features of iRFP in holoform (dark cyan solid line) and in apoform (black dotted line). (A) Absorption spectra in the UV- and visible spectra regions; the absorption spectrum of free-BV is also shown (dark pink solid line). (B) CD in the far-UV region. (C) Intrinsic fluorescence spectra at the excitation wavelength of 295 nm, normalized to 1, and at the emission wavelength of 365 nm. (D) Intrinsic fluorescence spectra at the excitation wavelength of 295 nm taking into account the difference in the emission of the holoprotein and apoprotein. (E,F) CD in the near-UV and visible regions, respectively.

Figure 3.

Spectra of absorption (black line), near infrared fluorescence at the excitation wavelength of 690 nm (dark cyan line) and excitation at the emission wavelength of 715 nm (red line) of iRFP in holoform.

Figure 4.

Denaturation curves of GdnHCl-induced unfolding and refolding of iRFP in holoform. (A) The changes in tryptophan fluorescence intensity at the excitation wavelength of 295 nm and at the emission wavelength of 320 nm. The data were normalized to the fluorescence signal of the protein in its unfolded state. (B) The change in chromophore fluorescence intensity at the excitation wavelength of 690 nm. As the visible absorption spectrum of iRFP changed significantly over the range of GdnHCl concentrations, the chromophore fluorescence was corrected to the total optical density of the solution at the excitation wavelength, as described in the “Materials and methods” section. (C) The change in parameter $A = I_{320}/I_{365}$ at the excitation wavelength of 295 nm. (D) The change in fluorescence anisotropy at the emission wavelength of 365 nm and at the excitation wavelength of 297 nm. (E, F) The changes in ellipticity at 222 nm and light-scattering, respectively. Measurements were performed after 24 h incubation of the native or denatured protein in the presence of GdnHCl. Red symbols indicate unfolding, and blue symbols represent the refolding of iRFP.

Figure 5.

The change in visible absorption of iRFP in holoform during GdnHCl-induced unfolding (panel A) and refolding (panel B). The numbers on the curves indicate the final denaturant concentration in the protein sample. The black curve in panel B is the absorption spectrum of the holoprotein in buffer solution.

Figure 6.

Interaction of BV with iRFP in apoform. The UV-visible absorption (panel A) and near infrared fluorescence at the excitation wavelength of 690 nm (panel B) of iRFP in apoform (black dotted line) and of the apo-iRFP in complex with BV (green solid line). These spectra were recorded for the solutions obtained by equilibrium microdialysis (see “Materials and methods” section). This approach allowed us to evaluate the completeness of BV binding to iRFP in apoform. A protein concentration of 8 μM was used. According the experimental data, the concentration of bound BV was nearly 8 μM . The absorption and fluorescence spectra of free BV (dark pink solid line) at the concentration identical to the bound chromophore are also shown.

Panel C represents the CD in the near-UV and visible regions of iRFP in apoform in complex with BV.

Figure 7.

Denaturation curves of the GdnHCl-induced unfolding and refolding of iRFP in apoform. (A) The change in tryptophan fluorescence intensity at the excitation wavelength of 295 nm and at the emission wavelength of 320 nm. The data were normalized to the fluorescence signal of the protein in its unfolded state. (B) The change in ellipticity at 222 nm. (C) The change in parameter $A = I_{320}/I_{365}$ at the excitation wavelength of 295 nm. (D) The change in fluorescence anisotropy at the emission wavelength of 365 nm and at the excitation wavelength of 297 nm. Measurements were performed after 24 h incubation of the native or denatured protein in the presence of GdnHCl. Red symbols indicate unfolding, and blue symbols represent the refolding of the apoprotein.

Figure 8.

The stability of iRFP in holoform (black circles) and in apoform (dark cyan squares) against denaturation by GdnHCl.

Figure 9.

Denaturation curves of GdnHCl-induced unfolding and refolding of iRFP mutant with Cys15Ser substitution in the presence of BV. (A) The change in parameter $A = I_{320}/I_{365}$ at the excitation wavelength of 295 nm. (B) The change in chromophore fluorescence intensity at the excitation wavelength of 690 nm. The chromophore fluorescence was corrected to the total optical density of the solution at the excitation wavelength, as described in the “Materials and methods” section. Measurements were performed after 24 h incubation of the native or denatured protein in the presence of GdnHCl. Red symbols indicate unfolding, and blue symbols represent the refolding of iRFP.

	1	10	20	30	40	50	60
RpBphP2	MTEGSVARQPD	STCDDEPIHIPGAIQPHGLLLALAADMTIVAGSDNLPELTGLAIGALIGRSA					
iRFP	MTEGSVARQPD	STCDDEPIHIPGAIQPHGLLLALAADMTIVAGSDNLPELTGLAIGALIGRSA					
RpBphP2-Cr	MTEGSVARQPD	STCDDEPIHIPGAIQPHGLLLALAADMTIVAGSDNLPELTGLAIGALIGRSA					
	70	80	90	100	110	120	
RpBphP2	ADVFDSETHNRLTIALAEPGAAVGAPIAVGFTMRKDAGFV-GSWHRHDQLVFLELEPPQRDVAEPPQ						
iRFP	ADVFDSETHNRLTIALAEPGAAVGAPIAVGFTMRKDAGFV-GSWHRHDQLVFLELEPPQRDVAEPPQ						
RpBphP2-Cr	ADVFDSETHNRLTIALAEPGAAVGAPIAVGFTMRKDAGFV-GSWHRHDQLVFLELEPPQRDVAEPPQ						
	130	140	150	160	170	180	190
RpBphP2	AFFRRTNSAIRRLQAETLESACAAAQEVREITGFDRVMIYRFASDFSGEVIAEDRCAEVESYL						
iRFP	AFFRRTNSAIRRLQAETLESACAAAQEVREITGFDRVMIYRFASDFSGEVIAEDRCAEVESYL						
RpBphP2-Cr	AFFRRTNSAIRRLQAETLESACAAAQEVREITGFDRVMIYRFASDFSGEVIAEDRCAEVESYL						
	200	210	220	230	240	250	
RpBphP2	GLHF PASDIPAQARRLYTINPVRIIPDINYPVPVTPDLNPVTGRPIDLSFAILRSVSPVHLEYM						
iRFP	GLHF PASDIPAQARRLYTINPVRIIPDINYPVPVTPDLNPVTGRPIDLSFAILRSVSPVHLEYM						
RpBphP2-Cr	GLHF PASDIPAQARRLYTINPVRIIPDINYPVPVTPDLNPVTGRPIDLSFAILRSVSPVHLEYM						
	260	270	280	290	300	310	
RpBphP2	RNIGMHGTMSISILRGERLWGLIACHHRKPNYVDLDGRQACELVAQVLAWQIGVMEE						
iRFP	RNIGMHGTMSISILRGERLWGLIACHHRKPNYVDLDGRQACELVAQVLAWQIGVMEE						
RpBphP2-Cr	RNIGMHGTMSISILRGERLWGLIACHHRKPNYVDLDGRQACELVAQVLAWQIGVMEE						

Figure 10.

Alignment of the amino acid sequences of iRFP with RpBphP2 and its crystallized variant (denoted as RpBphP2-Cr). Mutations introduced into iRFP comparing with RpBphP2 are highlighted in green. Mutations introduced into RpBphP2 to crystallize it are shown in blue.

Table 1

Fluorescence characteristics of the iRFP holoform and apoform.

Parameter	iRFP in holoform	iRFP in apoform
<i>Intrinsic fluorescence</i>		
λ_{max} , nm ($\lambda_{\text{ex}}=295$ nm)	331	332
Parameter A ($\lambda_{\text{ex}}=295$ nm)	1.7	1.6
r ($\lambda_{\text{ex}}=297$ nm, $\lambda_{\text{em}}=365$ nm)	0.15	0.16
τ , ns ($\lambda_{\text{ex}}=297$ nm, $\lambda_{\text{em}}=330$ nm)	3.7	1.2
<i>Chromophore fluorescence</i>		
λ_{max} , nm ($\lambda_{\text{ex}}=690$ nm)	715	-
I_{max} , %	100	-