A Purple-blue Chromoprotein from Goniopora tenuidens Belongs to the DsRed Subfamily of GFP-like Proteins*

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A number of recently cloned chromoproteins homologous to the green fluorescent protein show a substantial bathochromic shift in absorption spectra. Compared with red fluorescent protein from Discosoma sp. (DsRed), mutants of these so-called far-red proteins exhibit a clear red shift in emission spectra as well. Here we report that a far-red chromoprotein from Goniopora tenuidens (gtCP) contains a chromophore of the same chemical structure as DsRed. Denaturation kinetics of both DsRed and gtCP under acidic conditions indicates that the red form of the chromophore (absorption maximum at 436 nm) converts to the GFP-like form (384 nm) by a one-stage reaction. Upon neutralization, the 436-nm form of gtCP, but not the 384-nm form, renaturates instantly, implying that the former includes a chromophore in its intact state. gtCP represents a single-chain protein and, upon harsh denaturing conditions, shows three major bands in SDS/PAGE, two of which apparently result from hydrolysis of an acylimine C=N bond. Instead of having absorption maxima at 384 nm and 450 nm, which are characteristic for a GFP-like chromophore, fragmented gtCP shows a different spectrum, which presumably corresponds to a 2-keto derivative of imidazolidinone. Mass spectra of the chromophore-containing peptide from gtCP reveal an additional loss of 2 Da relative to the GFP-like chromophore. Tandem mass spectrometry of the chromoprotein shows that an additional bond is dehydrogenated in gtCP at the same position as in DsRed. Altogether, these data suggest that gtCP belongs to the same subfamily as DsRed (in the classification of GFP-like proteins based on the chromophore structure type).

A variety of fluorescent proteins from Anthozoa species has been described recently (1–6). These proteins represent the GFP-like family according to their homology to the green fluorescent protein (GFP) and have been described (4); they are conventionally divided into two groups, proteins with inherent fluorescence (FP)1 and naturally nonfluorescent proteins (CP). Remarkably, all known CPs can be converted to fluorescent analogues by mutagenesis (3) or by irradiation with light of fixed wavelength (8, 9). Despite great diversity in the colors of coral proteins, this variability seems to be achieved in a fairly conservative way. Presently, few reactions have been described as sufficient to generate the chromophore formation in different FPs and CPs. All known fluorescent proteins contain an invariant XYG consensus in the chromophore-forming region and, upon maturation, include the chromophore core built up from p-hydroxybenzylidenemimidazolidinone as a structural unit. Nevertheless, based on imidazolidinone derivatives, different groups of colors of FPs and CPs are currently considered to stem from chemically distinct species (10).

According to the chromophore structure, GFP-like proteins can be classified into subfamilies. The two best studied are GFP and DsRed, the fine crystal structures of which have been determined recently (11–14). In this study, we selected gtCP because light absorption of this protein is considerably shifted to longer wavelengths (λmax = 580 nm), and therefore we tested whether different chromophore chemical structures provide a satisfactory explanation for the color diversity. Unexpectedly, our results demonstrate that the purple-blue chromoprotein from Goniopora tenuidens contains the same chromophore as DsRed. Therefore, according to the above criteria, gtCP should be attributed to the DsRed subfamily of GFP-like proteins.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—For heterologous expression, a full-length cDNA encoding gtCP was cloned into the pQE30 vector (Qiagen). The recombinant protein with the N-terminal 6-His tag was expressed in Escherichia coli (5) and purified from cell lysates by HPLC, high pressure liquid chromatography; MS/MS, tandem mass spectrometry; DsRed, red fluorescent protein from Discosoma sp.; GFP, green fluorescent protein; gtCP, Goniopora tenuidens chromoprotein.

1 The abbreviations used are: FP, fluorescent protein; CP, chromoprotein; HPLC, high pressure liquid chromatography; MS/MS, tandem mass spectrometry; DsRed, red fluorescent protein from Discosoma sp.; GFP, green fluorescent protein; gtCP, Goniopora tenuidens chromoprotein.
metal-affinity chromatography on Ni-NTA resin (Qiagen).

Spectroscopy—Absorption spectra were recorded on a Cary 50 Bio UV/NIR spectrophotometer (Varian).

Chymotrypsin Digestion and Chromopeptide Purification—Histagged gtCP was denatured by the addition of NaOH solution to the final concentration of 0.1 M. The protein solution was further complemented with Tris (10 mM) and titrated with hydrochloric acid to pH 7.8. Chymotrypsin (Sigma) digestion (enzyme to protein ratio, 1/50 (w/w)) was performed at 37 °C for 5 h and finally quenched by acetic acid (to pH 4.0). The digest was applied to a reverse-phase HPLC column (UltraspHERE ODS, BD Biosciences) equilibrated with 10 mM sodium phosphate buffer, pH 4.0, and peptides were eluted by a linear gradient of acetonitrile in the same buffer. The effluent was monitored at 210 nm and 380 nm. The peptide fractions with absorption at 380 nm were collected and sequenced with a model 491 Procise sequenator (Applied Biosystems).

Mass Spectrometry of gtCP Chromopeptide—The mass spectral analysis was performed with Thermo Finnigan LCQ deca XP fitted to electrospray ionization source and ion trap mass analyzer. The peptide was injected at the concentration of 5 pm in 0.1% formic acid in 50/50 (v/v) water-methanol solution at the flow rate of 3 μl/min. The heated capillary was operated at 210 °C, sheath gas was set to 7 units, and the needle voltage was +3.2 kV. A relative collision energy of 29–35% was used in MS/MS experiments with an isolation width of 1.2 units.

RESULTS

"Red-to-Green" Conversion of gtCP Chromophore—The spectral diversity of GFP-like proteins is thought to derive from different types of chromophores involved in a protein structure and the way by which a chromophore interacts with a protein environment (12). In the denatured state, interactions with amino acid side chains do not contribute significantly to the overall absorption spectra, so that upon denaturation, the visible spectrum of the protein reflects the type of implicated chromophore structure. Furthermore, we anticipated that unfolding of the protein protective shell upon denaturation would allow spectral monitoring of the chromophore transformations after exposure to the surrounding solvents. Upon heat-induced (100 °C) or alkaline denaturation (0.1 M NaOH), the chromophore of gtCP converted instantly to a green form, i.e. absorption spectra of gtCP were very similar to the denatured GFP with the maxima at 384 nm (pH 3.0) and 450 nm (pH 14.0). Spectrophotometric titration of the green form of gtCP (not shown) indicated the presence of an ionizable group with pKₐ = 7.9, which correlated well with the value previously reported for the GFP chromophore phenolic group (15). Together, these data suggested that the gtCP chromophore shares common features with GFP and contains p-hydroxybenzylideneimidaizolidinone as a core structure.

The acid-stimulated denaturation of gtCP resulted in more complex spectral changes. Under native conditions, gtCP exhibited maximal absorption at 580 nm with a shoulder at 545 nm (3). Initially at pH 1.8, gtCP showed an absorption maximum at 436 nm (the red form of the chromophore of the denatured gtCP), which further gradually transformed to a 384-nm peak upon incubation (Fig. 1A). A single isosbestic point at 406 nm suggested that there are only two species that exist in a red-to-green pathway of the gtCP chromophore conversion and that these two forms interconvert directly without any detectable intermediates. When, in the course of a red-to-green spectral transition, pH was adjusted to 4.0, the 436-nm form of gtCP immediately renatured, as judged by the reappearance of the 580-nm peak, which is characteristic of the native form of gtCP, and by the disappearance of the 436-nm absorbance (Fig. 1B). Conversely, the 384-nm form retained both the net absorbance and peak position of the absorption band. Accordingly, the extent of renaturation correlated with the ratio of 436-nm to 384-nm forms, which reduced gradually upon incubation. These results implied that the 436-nm form includes a red-shifting modification, which extends a GFP-like chromophore-conjugated system. Remarkably, the red form of the denatured gtCP contains a chromophore in its intact state, as judged by immediate renaturation of this form. By contrast, conversion to the green state is irreversible in this case, suggesting that spectral transition from 436 to 384 nm coincides with the irreversible loss of bathochromic modification by the gtCP chromophore.

A similar situation was observed for the chromophore of DsRed, another protein from the GFP family (14). A GFP-like imidaizolidinone-conjugated system in DsRed is extended by an additional acylimine substituent (Fig. 2B, I). In the case of DsRed, the abolition of a red-shifting modification upon denaturation was shown to be due to hydration of an acylimine (Fig. 2B, II), upon which the spectra of DsRed immediately reverted back to a GFP-like absorbance (14). To test whether a red-to-
green conversion is described by the same spectral transition, similar experiments were carried out with DsRed. As compared with gtCP, DsRed proved to be more sensitive to acid-induced denaturation; therefore, spectral transition in this case was monitored upon incubation of the protein at pH 2.8. As shown in Fig. 1C, DsRed exhibits a red-to-green spectral transition with the same maximum of the red form (436 nm) and an isosbestic point at 406 nm.

Fragmentation of gtCP upon Denaturation—In earlier reports (15), GFP was shown to migrate as a single band in SDS/PAGE, with an Mr of 28 kDa. It has been demonstrated (14) that DsRed partially splits into fragments, the extent of fragmentation depending on the conditions of denaturation. The purple chromoprotein from Anemonia sulcata, asFP595, has been shown to exist predominantly in a fragmented form regardless of denaturing conditions (16). These data suggest that the conditions and the extent of fragmentation are characteristic features for a chromophore type of GFP-like proteins. The fragmentation of DsRed was shown to occur after the complete hydrolysis of an acylimine and does not exceed 50% of the total protein, because mature DsRed contains red and green species in an approximately equal ratio (14, 17). As expected, DsRed, boiled in the electrophoresis buffer, showed two fragment bands in SDS/PAGE, with apparent masses of 10 kDa and 18 kDa and a major band of a full-length protein, with an Mr of 28 kDa (Fig. 2A, lane a1). The extent of DsRed fragmentation increased considerably when the protein was preboiled in 0.1 M HCl (Fig. 2A, lane a2). Likewise, gtCP, boiled in a sample buffer, migrated predominantly as a single band, indicating that the mature protein originally exists as a single-chain polypeptide (Fig. 2A, lane b1). However, the situation changed when gtCP was preincubated with 0.1 M HCl (Fig. 2A, lane b2). In this case, gtCP showed a remarkable increase in the extent of fragmentation (80% of the overall protein), indicating that a certain bond near the chromophore center undergoes acid-stimulated hydrolysis. Assuming that this bond corresponds to the red-shifting modification of the GFP-like chromophore, the mature gtCP should include no less than 80% of the red chromophore.

Although both DsRed and gtCP split into common fragments after acid-induced hydrolysis of a red-shifting bond, there was no direct evidence that fragmentation of gtCP occurs by means of the DsRed pathway; i.e. it is hydrolysis of an acylimine that leads to the fragmentation of gtCP. One possible way to obtain the information concerning the route of fragmentation is by analysis of the end products of hydrolysis of acylimine-substituted imidazolidiones. By carefully inspecting the structure of the product of complete hydrolysis of acylimine as published by Gross et al. (14), one can find an additional C=O electron-withdrawing substituent. Therefore, it could be expected that hydration of acylimine (Fig. 2B, II) would shift spectra back to a GFP-like absorbance, and hydrolysis by means of the DsRed pathway would further lead to a spectrally distinct form corresponding to a 2-keto derivative of imidazolidinone (Fig. 2B, III). To check the last assumption, DsRed and gtCP were boiled in 0.1 M HCl, containing 6 M guanidine HCl, and the absorption spectra of both samples were measured. At acidic conditions, hydrolyzed DsRed displayed an absorption maximum at 380 nm and a shoulder at 425 nm. When adjusted to pH 9.0, the protein showed maxima at 458 nm, 377 nm, and a shoulder at 516 nm (not shown). We interpret these data as a superposition of spectra of nonhydrolyzed and hydrolyzed forms, because DsRed contains both red and green species (14). The situation was much clearer with gtCP, because this protein apparently includes a chromophore in a predominantly red form. At pH 1.8, hydrolyzed gtCP showed a single peak at 425 nm, which became double with maxima at 516 and 377 nm when pH was adjusted to 9.0 (Fig. 2C). Comparative analysis of the above data implies that an absorption maximum at 425 nm (pH 1.8) and peaks at 377 and 516 nm (pH 9.0) correspond to the structure III (Fig. 2B) of both fragmented proteins.

Mass-spectral Analysis of gtCP Chromopeptide—Denatured gtCP was digested extensively by chymotrypsin, and a chromophore-bearing peptide was isolated by HPLC. Sequence
chromopeptide (parent mass = 1187.4 Da) allowed positioning of this additional dehydrogenation site between α-ammonium and α-carbon of Gln-65 (Fig. 3B). The most prominent peaks in the secondary MS resulted from the intramolecular cyclization reaction between α-carbon and δ-nitrogen of Gln-65, followed by splitting of the C= N bond (Table I, II and complement fragment I) (Fig. 3B). Similar fragmentation species were detected in MS/MS spectra of the DsRed chromopeptide (14). Many other daughter ions of the gtCP chromopeptide were observed in the secondary mass spectrum, the most distinctive originating from fragmentation in the vicinity of the acylimine C=N bond (Table I, V, III, and IV) (Fig. 3B).

**DISCUSSION**

Despite the recent shift in emphasis to the biotechnological utilization of GFP-like proteins, it seems that many fundamental questions should be solved as far as the chemistry of these proteins is concerned. Among these are the mechanisms of self-assembly of the chromophore moiety and the relationships between a chromophore chemical structure and emission maximum of FPs or color of CPs. After a DsRed chromophore was discovered to be an extended π-electron variant of GFP imidazolidinone structure (12–14), it seemed very likely that a further red shift of coral proteins would also inevitably accompany the chromophore structure changes.

Because gtCP readily lost a red-shifting modification upon denaturation, our strategy in the chromophore structure reconstruction initially was to monitor the pathway of the red-to-green conversion and to identify intermediate products that might have been related to the chromophore in its native state. This approach was facilitated by the fact that relevant data were obtained previously for two other GFP-like proteins with known chromophore structures (14, 18).

Upon different denaturing conditions, gtCP eventually showed absorption maxima at 384 nm (pH 3.0) and 450 nm (pH 14.0). Because these two forms proved to be interconvertible with $pK_a = 7.9$, these results suggested that the gtCP chromophore includes a GFP-like imidazolidinone core structure. The next set of experiments indicated that imidazolidinone structure of the gtCP chromophore is extended by a red-shifted modification. Upon denaturation at acidic conditions, the gtCP chromophore initially exists as a 436-nm red form, which further converts to the green GFP-like form ($\lambda_{max} = 384 \text{ nm}$). Remarkably, both gtCP and DsRed exhibited the same absorption maxima of the chromophore red forms and the same positions of the isosbestic point of the red-to-green conversion. The red form of denatured gtCP readily restored its native absorption spectra after adjusting pH to 4.0; the green form did not. Therefore, we interpret this phenomenon as the irreversible
loss of a red-shifting modification by the gtCP chromophore in the course of transition. Two additional points should be noted. First, gtCP naturally includes the same chromophore chemical structure as a red form of the denatured protein, as judged by instant renaturation of this form. Second, a red-to-green conversion of the gtCP chromophore is described by the single isosbestic point, suggesting that there are only two species participating in this reaction, and one converts into another directly by a one-stage reaction. Consequently, these results allowed rejecting any suspected intermediate products that might have been related to the chromophore structure in its native state.

Originally, gtCP represented a single polypeptide chain protein, as judged by a major band of $M_r = 28$ kDa in SDS/PAGE. Nevertheless, at harsher denaturing conditions, especially after boiling in 0.1 M HCl, the majority of protein molecules participated in this reaction, and one converts into another directly by a one-stage reaction. Consequently, these results allowed rejecting any suspected intermediate products that might have been related to the chromophore structure in its native state.

The recently published crystallographic structures of a blue CP pocilloporin and a far-red FP from the sea anemone Entacmaea quadricolor revealed that these GFP-homologous pigments include a conformational analogue of the DsRed chromophore (19, 20). Although we were unable to gain information relevant to the gtCP chromophore conformation by the methods we used in this study, both published data and our results suggest that a drastic chromophore structural reconstruction is not a prerequisite to the shift of spectra of GFP-like proteins to the far-red region. Thus, isomerization of the chromophore moiety and/or rearrangements in its interactions with surrounding amino acid side chains seems to be sufficient in some cases for the color manifold of the GFP-family proteins.

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