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Is association of labile enediyne chromophore a mutually assured protection for carrier protein?

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Abstract

Most conjugate proteins undergo both conformational and stability changes on ligand removal. When architecture remains unchanged in the protein holo and apo forms, it is uncertain whether the protein stability also remains unaltered in both of the forms. Neocarzinostatin (NCS), a chromoprotein possessing a potent enediyne chromophore stands for such an instance. Protein–chromophore interaction has not been thoroughly explored previously due to a lack of strategies to independently and simultaneously monitor changes in the NCS conjugates. Here we report a method by which one can detect the signal exclusively from only one of the NCS conjugates without the spectral interference from the other. Stability of the NCS protein is significantly correlated to the protein-bound chromophore, irrespective of denaturation by heat, pH, urea, or ethanol. Despite the similarity in protein backbone conformation, protein stability of the NCS holo form diminishes and equalizes to that of the apo form when the chromophore is released and degraded. Although the enediyne chromophore is highly unstable, it intriguingly protects the protein by which it is protected. Significant mutual reliance between the carrier protein and its naturally associated ligand unveils important information on the NCS drug stability.

Keywords

Enediyne; Neocarzinostatin; Chromophore; Ligand binding; Holoprotein; Protein stability

Recently, much attention has been focused on the protein–ligand conjugation. Most conjugate proteins undergo changes in their conformation and subsequently, in stability on ligand removal [1–3]. For example, binding of heme to cytochrome changes the protein conformation and consequently, increases the protein stability [4]. It is rather uncommon for conjugate proteins to adopt a like conformation with or without the ligand. This includes azurin (β protein with a copper ion), flavodoxin (α/β protein with a flavin mononucleotide) [5], neocarzinostatin (NCS, $^1\beta$ protein with an enediyne chromophore) [6,7] and so on. In spite of structural similarity, the holo form of azurin is more stable than its apo form [8]. The flavodoxin holo form, in contrast, has little changes in either conformation or stability on its cofactor removal

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¹*Abbreviations used:* NCS, neocarzinostatin; holoNCS, neocarzinostatin chromoprotein conjugate complex; apoNCS, apoprotein of neocarzinostatin; NCS-C, neocarzinostatin chromophore; NCS-C(b), protein-bound neocarzinostatin chromophore; CD, circular dichroism; HPLC, high-performance liquid chromatography; EtBr, ethidium bromide; NOESY, nuclear Overhauser effect spectroscopy; NMR, nuclear magnetic resonance; T_m , temperature at which half of the protein is unfolded; T_R , temperature at which half of the NCS-C(b) is released from NCS complex; C_m , concentration of denaturant at which half of the protein is unfolded; C_R , concentration of denaturant at which half of the NCS-C(b) is released from NCS complex.

[9]. Whether the protein of holoNCS can maintain its stability on chromophore removal has not been thoroughly investigated.

NCS, a naturally occurring bioconjugate, is a potent antitumor antibiotic secreted by *Streptomyces carzinostaticus* (for reviews, see Refs. [10–19]). NCS is a 1:1 chromoprotein complex consisting of an apoprotein (apoNCS) and an enediyne chromophore (NCS-C [free form] or NCS-C(b) [protein-bound form]) (MW 659). ApoNCS is an all β -sheet protein of 113 amino acid residues with an immunoglobulin-like folding pattern [20]. Fig. 1 illustrates the holoNCS model in a neutral aqueous environment [21]. NCS has a superimposable backbone three-dimensional structure in both its holo and apo forms [22,23]. NCS-C is highly sensitive to chemicals, basic pH, light, and heat [24]. Owing to the labile nature of NCS-C, the drug carrier protein apoNCS stabilizes NCS-C in its binding cleft through noncovalent bonding [19]. The protein stability that ensures structural rigidity of its binding cleft is, in turn crucial for stabilizing the protein-bound NCS-C(b). Although the NCS protein binds to NCS-C(b) very tightly ($K_d \leq 0.1$ nM) [25], backbone conformational changes are not required on NCS-C(b) removal [7]. This poses important questions as to whether the NCS protein maintains its stability in both its holo and apo forms and whether the labile NCS-C(b) contributes to stability of its carrier protein.

Ample information concerning the association of labile NCS-C with apoNCS can be acquired by NCS denaturation studies. Because of a lack of methods to independently and simultaneously monitor the changes of conjugates in the NCS complex, the effect of protein–chromophore interaction on stability has not been analyzed previously. Here we report a unique circular dichroism (CD) method, by which it is possible to detect signal exclusively from only one of the NCS conjugates without the spectral interference of the other. We systematically analyzed the NCS protein stability with and without NCS-C(b) and outcome of the investigation provides insight into the NCS drug stability.

Materials and methods

NCS, apoNCS, NCS-C and reconstituted holoNCS

Clinical NCS was a gift from Kayaku Company (Tokyo, Japan). ApoNCS was obtained by purifying the remaining NCS protein pellet after methanol extraction of NCS-C by using hydrophobic XAD-7 resin column [26]. Purity and concentration of holoNCS, apoNCS, and extracted NCS-C were examined by UV spectroscopy, mass spectrometry and high-performance liquid chromatography (HPLC). Reconstituted holoNCS was prepared by adding equimolar extracted NCS-C into an aqueous solution of apoNCS (pH 5.0) in an ice bath [27]. Integrity of NCS-C(b) in the reconstituted holoNCS was examined by HPLC [28].

CD spectroscopy

CD spectra were recorded on a Jasco J-715 spectropolarimeter (Tokyo, Japan) using a 0.1-cm path-length water-jacketed quartz cuvette. Temperature was regulated with a Neslab RTE-140 circulating water bath (Portsmouth, NH, USA) equipped with a microprocessor and a temperature sensor. Optical resolution, bandwidth, and response time were 1 s, 1 nm and 1 s, respectively. A protein concentration of 10–50 μ M in a final volume of 200 μ l of 15 mM sodium phosphate at specified pH was used. For each spectrum, buffer background was subtracted and the final spectrum of each sample was the average of five consecutive scans. Ellipticity changes were monitored in far- and near-UV wavelengths. For spectral comparison between chromophore and protein, CD data were expressed in millidegrees (mdeg) per micromolar (μ M) protein instead of conventional mean residue ellipticity.

Thermal-induced unfolding at different pH levels

Unfolding of apoNCS and holoNCS in 15 mM sodium phosphate buffer was monitored in a broad range of pH (2.0–10.0) and temperature (0–87°C). Ellipticity changes of apoNCS and holoNCS were monitored in far- and near-UV wavelengths. Ramp time (optimal time to reach temperature equilibrium was measured as being <10 min) was set at 15 min for each temperature increment.

Thermal denaturation involving ethidium bromide as a ligand

Ethidium bromide (EtBr)-bound NCS complex, NCS–EtBr, was prepared by mixing equimolar amounts of apoNCS and EtBr in 20 mM phosphate at pH 2.0 and 7.0. Thermal unfolding of 50 μ M NCS–EtBr was performed side by side with that of apoNCS under the same conditions in a temperature range of 3–90 °C. The protein conformational changes were followed by far-UV CD signals at 224 nm.

Urea-induced unfolding

Urea-induced unfolding was performed at 25 °C in a pH range of 2.0–4.0. Urea (Sigma–Aldrich, St. Louis, MO, USA) was recrystallized in ethanol and dried to evaporate traces of ethanol. Urea (0–9 M) in 15 mM phosphate with 0.2 M HCl and 0.05 M KCl was prepared, and the pH was adjusted to 2.0–4.0 using 5 N NaOH. ApoNCS or holoNCS was added to the urea solution at a final concentration of 15 μ M. A maximum variation of ± 0.05 pH was allowed in all preparations. Samples were equilibrated at 25°C in dark for at least 30 min before acquiring CD signals. Protein denaturation was monitored by measuring the ellipticity changes in far- and near-UV regions as a function of urea concentration. Urea-induced holoNCS unfolding studies at pH 4.0 and 25 °C showed an incomplete denaturation profile without a postunfolding arm even at the maximum urea concentration. In that case, unfolded fraction was estimated by adopting the posttransitional ellipticity value of complete denaturation of holoNCS at pH 2.0 under similar conditions.

Ethanol-involved thermal unfolding

ApoNCS or holoNCS at a concentration of 35 μ M in a final volume of 200 μ l of 15 mM phosphate (pH 2.0) was prepared in 0–40% (v/v) ethanol. Samples were equilibrated at 25 °C for 15 min in dark prior to thermal unfolding performed at 0–87 °C. Unfolding was followed by monitoring the ellipticity changes in far- and near-UV as a function of ethanol concentration and temperature.

Nuclear magnetic resonance spectroscopy

Two-dimensional nuclear Overhauser effect spectroscopy (NOESY) of apoNCS and holoNCS was acquired in a Bruker DMX 600-MHz nuclear magnetic resonance (NMR) spectrometer (Rheinstetten, Germany). Approximate 6 mg of apoNCS or holo-NCS in 15 mM phosphate (pH 2.0) was lyophilized and dissolved in an aqueous solvent containing 90% H₂O and 10% D₂O prior to analysis. Spectra were acquired in a temperature range from 25 to 48 °C.

HPLC analysis of NCS-C(b)

The remaining NCS-C(b) in urea-treated holoNCS at pH 2.0 was analyzed in a Waters Millennium HPLC (Milford, MA, USA) equipped with a 600E solvent delivery system, a 996 photodiode array detector, and a Waters 474 or Jasco FP-1520 fluorescence detector as described previously [7,28].

Results

Independent monitoring of NCS conjugates by CD spectroscopy

Ellipticity of isolated NCS components and NCS complex at the same concentration was recorded in far- and near-UV regions (200–320 nm), and the overlapping CD spectra are shown in Fig. 2. To avoid degradation of labile NCS-C, the spectrum of isolated NCS-C was acquired in acidic methanol at 0 °C, whereas the spectra of purified native apoNCS and holoNCS were acquired in aqueous solution at the same temperature. The spectra of apoNCS and holoNCS acquired at 0 °C coincided with the CD curves recorded at the ambient temperature [26,27, 29–31]. When holoNCS undergoes denaturation, NCS-C(b) is released and irreversibly degraded in an aqueous environment [25]. Ultimately, the solution contains degraded NCS-C and unfolded NCS protein molecules. Considering this, the CD spectra of unfolded apoNCS and holoNCS and degraded NCS-C (all obtained at high temperatures and pH 7.0) were compared (Fig. 2, inset). In the far-UV region of CD spectra, only native apoNCS and holoNCS exhibit intense positive ellipticity at 224 nm, whereas both intact and degraded NCS-C show negligible CD signals at this wavelength. Unfolded apoNCS and holoNCS, however, show significant negative ellipticity at 224 nm. This facilitates monitoring of secondary structural changes of NCS protein at 224 nm without the optical interference of the chromophore.

In the near-UV region, CD spectra of the extracted NCS-C and NCS-C(b) show an obvious negative ellipticity minimum at 255 nm, thereby making a distinction in the spectral properties from the protein. Ellipticity of NCS-C(b) is approximately three times more intense than that of NCS-C, suggesting a large spectroscopic effect of the protein binding. The near-UV CD spectrum of apoNCS shows a weak negative signal at 271 nm, which is a characteristic feature of the tertiary structure of apoNCS [31]. Further comparison of the dichroic signals at 271 nm in Fig. 2 reveals that the apoNCS signal is much weaker than that of the NCS-C or NCS-C(b) signal. The residual CD signal at 271 nm from NCS-C or NCS-C(b) is sufficient to interfere with the weak signal from apoNCS. As a consequence, changes in the tertiary structure of NCS protein in the presence of chromophore could not be measured accurately. On the other hand, the CD signal of NCS-C(b) is very significant at 255 nm. Degraded NCS-C does not display detectable ellipticity in a broad UV wavelength range (Fig. 2, inset). At 255 nm, the NCS protein in all forms, including native apoNCS and both thermally unfolded apoNCS and holoNCS display only negligible CD signals (Fig. 2). It appears that the prominent negative signal of holoNCS at 255 nm arises only from the contribution of the enediyne chromophore. The holoNCS ellipticity at 255 nm, therefore, could serve as a promising and valuable indicator of quantitative changes of NCS-C(b). Besides the above-mentioned thermal denaturation studied at pH 7.0 (Fig. 2, inset), denaturation under combinatorial conditions, such as pH 2.0 and 65 °C (apoNCS); pH 2.0 and 90 °C (holoNCS); 6.4 M urea, pH 2.0, and 25 °C (apoNCS); 6.8 M urea, pH 2.0, and 25 °C (holoNCS); 35% ethanol, pH 2.0, and 50 °C (apoNCS); and 35% ethanol, pH 2.0, and 60 °C (holoNCS), was also examined. Irrespective of the conditions, all spectra of the unfolded apoNCS and holoNCS (data not shown) were similar to those of the respective profiles shown in the Fig. 2 inset. The results indicate that the CD signal contribution of the denatured NCS protein component at 255 nm is negligible and independent of denaturants.

To further characterize whether the negative CD signal at 255 nm implicates the amount of NCS-C(b), ellipticity changes were monitored during a titration of extracted NCS-C to apoNCS in aqueous solution at room temperature. With an increased amount of NCS-C(b), negative ellipticity at 255 nm increased proportionately and the final spectrum (1:1 molar ratio) was essentially the same as that of the native holoNCS shown in Fig. 2.

Signal interference from possible binding between the degraded NCS-C and apoNCS cannot be overlooked. If degraded NCS-C has strong binding affinity to apoNCS, it may contribute

to the CD signal of apoNCS at 255 nm. To examine this, CD spectrum of apoNCS was critically evaluated before and after titration of thermally degraded NCS-C (1:1 molar ratio) at pH 2.0–10.0 and room temperature (data not shown). No remarkable changes were observed in the apoNCS CD signals at all conditions. All of the spectra were similar to the spectrum of the native apoNCS after mixing with the degraded NCS-C. The results show a negligible binding effect of the degraded NCS-C to apoNCS on the CD spectrum in a wide pH range.

From the CD analysis of all possible combinations of NCS conjugates that could occur during the holoNCS denaturation process, it is evident that the ellipticity changes at 224 nm fairly represent the secondary conformational changes of the protein, whereas the signal at 255 nm evenly represents the quantitative measure of NCS-C(b). Thus, the possibility of obtaining unique information at two separate wavelength maxima enables us to analyze and discriminate the changes in each of the NCS conjugates. This technique extends the possibility of observing the interactions between each component in an NCS complex in a sensitive and simultaneous manner.

Thermal stability of NCS protein with and without NCS-C(b)

The stability of NCS-C is pH dependent [25], and so is the stability of apoNCS [31]. To evaluate the conformational stability of apo-NCS and holoNCS, two-dimensional unfolding was carried out in a wide range of temperature (0–90 °C) and pH level (2.0–10.0), followed by monitoring the ellipticity changes at 224 nm. The remaining NCS-C(b) at each equilibrated temperature was simultaneously determined from the ellipticity of holoNCS recorded at 255 nm. Figs. 3A and 3B show examples of thermal unfolding profiles at pH 2.5 and 7.5, respectively. At pH 2.5, apoNCS unfolding profile exhibits a transition slope of 0.048 mole fraction changes/°C, whereas that of holoNCS reduces to nearly half (0.026 mole fraction changes/°C) (Fig. 3A), indicating greater structural sustainability of holoNCS over that of apoNCS under acidic conditions. The large difference in the transition slopes corresponds well with the large difference in their T_m (temperature at which half of the protein is unfolded) values between apoNCS and holoNCS at pH 2.5. The average T_m of holoNCS at pH 2.5 is 55 ± 1 °C, whereas that of apoNCS is only 45 ± 1 °C. The thermal unfolding of holoNCS appears to be lagging significantly behind that of apoNCS under acidic conditions, where the NCS chromophore is known to be comparatively stable [25]. Under neutral and basic conditions where the chromophore is unstable, both holoNCS and apoNCS share an overlying unfolding pattern with similar steep transition slopes (both are ~ 0.059 mole fraction changes/°C at pH 7.5) (Fig. 3B). Also, there is no significant difference in the T_m (64 ± 1 °C) between the two forms of NCS protein at pH 7.5. Unlike in acidic conditions, no major differences in the conformational stability of NCS protein between the apo and holo forms were observed in neutral and basic conditions.

Correlation between the protein stability of holoNCS and remaining NCS-C(b) is demonstrated by comparing the T_r (temperature at which half of the NCS-C(b) is released from holoNCS) with the T_m of holoNCS. Fig. 4 shows the pH dependence of T_r (measured from CD signals at 255 nm) and T_m (measured from CD signals at 224 nm) of apoNCS and holoNCS. Judged by the T_m values, holoNCS in acidic pH displays an enhanced stability that subsequently decreases to nearly the same level as that of apoNCS at neutral and basic pH levels. At pH < 5.0, the T_r of NCS-C(b) is close to the T_m of holoNCS, indicating that release of NCS-C(b) is nearly concomitant with unfolding of holoNCS. However, at pH > 5.0, the T_r of NCS-C(b) drops significantly below the T_m of holoNCS, demonstrating the instability of NCS-C(b) at higher pH levels. Together, the results imply that the stability of holoNCS is enhanced only when stable NCS-C(b) binds with the NCS protein.

Thermal stability of NCS protein with and without EtBr

There has been recent interest in exploring the possibility of docking small synthetic molecules such as EtBr [32], naphthoate ester derivatives [33–35], and flavone-based ligand [36] in place of natural chromophore. Among the synthetic ligands, EtBr exhibits a relatively high binding constant (8.57×10^{-7} M) [32] and has been frequently used to examine the binding properties of NCS mutants [37,38]. NMR study also suggests that the EtBr binds to the NCS protein in the same cleft where the natural enediene chromophore binds [32]. Because the stability of NCS protein can be substantially enhanced by binding with its natural chromophore, this leads to whether the NCS protein can be stabilized by other synthetic ligand such as EtBr. In this context, we studied the effect of EtBr binding on the stability of NCS protein. The spectral properties of EtBr, apoNCS, and the 1:1 NCS–EtBr complex were compared by the CD spectrum acquired at 25 °C and pH 5.0 in a broad wavelength region (200–350 nm). Under the same concentration (50 μ M each), no significant signal from EtBr could be identified and the spectrum of NCS–EtBr essentially overlaps with that of the native apoNCS (data not shown). To evaluate the conformational stability difference between apoNCS and NCS–EtBr, thermal unfolding was carried out for both samples side by side at pH 2.0 and 7.0 in a wide range of temperature (3–90 °C), followed by monitoring the ellipticity changes at 224 nm (Fig. 5). Irrespective of pH levels, T_m of NCS–EtBr complex is essentially the same as that of apoNCS. The results show no stability difference in the NCS protein secondary structure regardless of EtBr binding.

Conformational stability of NCS protein against chemical denaturant

Structural stability of apoNCS and holoNCS against chemical denaturants was characterized by urea-induced unfolding at 25 °C. We previously demonstrated the incomplete denaturation of apoNCS at the maximum concentration of 9 M urea at pH > 4.0 [31,39]. This led us to study the effect of urea on NCS conformational stability at pH $\leq$ 4.0. At pH 2.0, where the NCS protein is comparatively unstable [31], apoNCS and holoNCS completely denature at 4 and 6.5 M urea, respectively (Fig. 6A). However, at pH 4.0, apoNCS begins to unfold at 4 M urea, whereas holoNCS begins to unfold only at 6 M urea and does not completely unfold even at the maximum urea concentration (9 M) (Fig. 6B). Differential stability between apoNCS and holoNCS as a function of urea concentration is evaluated based on the C_m (concentration of urea required to unfold half of the protein). At pH 2.0, the C_m of holoNCS is 3.8 ± 0.1 M, which is nearly two-fold that of apoNCS (2.0 ± 0.1 M). At pH 4.0, because of incomplete unfolding, the C_m of holoNCS can only be estimated as >7 M, which is at least 30% higher than that of apoNCS (5.2 ± 0.1 M). Besides, the C_r (concentration of urea needed to release half of the bound ligand) of NCS–C(b) and C_m of holoNCS are close to each other at pH 2.0–4.0 (Fig. 6), suggesting that the process of NCS–C(b) release corresponds to that of holoNCS unfolding. Similar to the thermal stability of the NCS protein, urea-induced unfolding profiles demonstrate that, under acidic conditions where the protein-bound NCS–C(b) is stable, conformational endurance of holoNCS against chemical denaturants is greater than that of the apoNCS.

HPLC validation of independent spectral contribution of NCS–C(b) determined by CD spectroscopy

The remaining NCS–C(b) in holoNCS after various treatments can be efficiently determined by our reported HPLC method [28]. For a chromoprotein complex that undergoes equilibrium thermal unfolding, it is technically difficult to perform HPLC at each equilibrated temperature. However, in urea-induced unfolding study, HPLC analysis becomes accessible because unfolding of holoNCS and release of NCS–C(b) are followed at room temperature. To ascertain whether the CD signal at 255 nm effectively represents the quantitative changes of NCS–C(b) in the urea-treated sample, holoNCS in different urea concentrations (0–9 M) at pH 2.0 was

subjected to both CD (at 255 nm) and HPLC. Ellipticity intensity of the remaining NCS-C(b) determined by the CD measurements and the integrated peak area obtained from the HPLC profile are compared in Fig. 7. Both of the values correlate very well in the broad urea concentration range and confirm the validity of using the CD analysis at 255 nm for evaluating quantitative changes of NCS-C(b).

Effect of ethanol-induced NCS-C(b) release on protein stability toward thermal unfolding

Organic solvents facilitate NCS-C(b) release from holoNCS, by which the NCS-mediated DNA cleavage is enhanced in vitro [40]. To determine the effect of NCS-C(b) release on NCS protein stability, we applied ethanol to trigger the chromophore release and monitored the protein stability by denaturation. Ethanol is not an effective denaturant of apoNCS at pH 5.0 and room temperature [39]. Therefore, we performed two-dimensional unfolding of apo-NCS and holoNCS at pH 2.0 as a function of both temperature (0–90 °C) and ethanol concentration (0–40%, v/v). The T_m of apoNCS and holoNCS and the T_r of NCS-C(b) were estimated from the ellipticity changes at far-UV (224 nm) and near-UV (255 nm) and are compared as shown in Fig. 8. At low ethanol concentrations (0–5%), the difference in T_m between apoNCS and holoNCS is approximately 10 °C on average. The incremental increase in ethanol concentration from 5 to 30% resulted in a drastic effect on protein stability as evaluated from the T_m values of both proteins. Also, the T_r of NCS-C(b) and the T_m of holoNCS are close to each other in ethanol below 30%, suggesting that both the chromophore release and the protein unfolding are nearly concomitant. At ethanol concentrations above 30%, holoNCS and apoNCS share the same level of protein stability, whereas the T_r of NCS-C(b) is significantly lower than the T_m of holoNCS. A high correlation between the release of NCS-C(b) and the decrease in stability of holoNCS was observed unambiguously. Together, the results demonstrate that, when release of NCS-C(b) is triggered and enhanced by ethanol, holoNCS concomitantly loses its superiority and eventually equalizes to apoNCS in conformational stability against heat.

Differential thermal stability between apoNCS and holoNCS as observed by NMR

Multidimensional NMR techniques have been successfully employed to characterize the conformational state of proteins under various experimental conditions. In NMR analysis, we found that the presence of NCS-C(b) does not significantly change the dispersion level of protein resonances. This enabled us to examine the thermal-induced NCS protein conformational stability by performing multidimensional NMR. To substantiate the enhanced thermal stability of holoNCS over its apo form at pH 2.0 as described earlier (Fig. 4), two-dimensional homonuclear NOESY spectra of apoNCS and holoNCS at pH 2.0 were acquired at different temperatures (Fig. 9). At 25 °C, all of the cross-peaks are well dispersed for both apoNCS and holoNCS (Figs. 9A and C), suggesting that both proteins are in a native state. However, the apoNCS resonances recorded at 48 °C show drastic changes (Fig. 9B), where most of the cross-peaks occupy only a narrow region (7.5–8.0 ppm), indicating the denaturation of apoNCS. In contrast, holoNCS resonance signals at the same temperature remain well dispersed (Fig. 9D). The results confirmed the greater stability of holoNCS over its apo form against thermal unfolding under acidic conditions.

Discussion

Simultaneous monitoring of changes in each component of NCS complex

Protein stability is often studied by monitoring the spectral changes during unfolding process. When a protein complex such as NCS contains an optically active chromophore, legitimate spectroscopic monitoring becomes obscure. To search for an unambiguous spectroscopic analysis method, we examined CD spectra of all possible combinations of NCS conjugates that could form during the NCS denaturation. Those forms include isolated or combined, bound or

unbound, native or unfolded (protein component), and intact or degraded (chromophore component). Analysis manifests that the ellipticity changes at 224 nm correspond exclusively to the secondary conformational changes of the NCS protein and that the prominent negative ellipticity at 255 nm is only from the intact chromophore (Fig. 2). The established outcome provides a good foundation to simultaneously and independently follow the NCS protein conformational changes and the NCS-C(b) quantitative changes. This CD spectroscopic method was further validated by HPLC quantitative analysis. Intensity of the CD signal at 255 nm correlates well with the remaining amount of NCS-C(b) (Fig. 7).

Enhanced stability of holoNCS over its apo form

Previously, we demonstrated that the NCS-C(b) release does not require holoNCS backbone conformational changes [7]. Although the conformation remains unchanged, we compared the stability of NCS protein in the conversion of holo to apo form as induced by various modes of denaturation induced by heat, pH, urea, and ethanol. Enhanced stability of holoNCS over apoNCS under acidic conditions is evident as judged by its shallower transition slope and higher T_m and C_m values (Figs. 3, 4 and 6). At pH 2.0, the T_m of holoNCS against heat is approximately 10 °C higher than that of apoNCS (Fig. 4), whereas the C_m of urea-induced holoNCS denaturation is nearly double that of apoNCS (Fig. 6). Results of thermal unfolding determined by CD analysis in aqueous solution are complemented by NMR observation (Fig. 9). When 15% (v/v) ethanol was applied to facilitate NCS-C(b) release, the T_m of holoNCS is still 6 °C higher than that of apoNCS (Fig. 8). However, in $\geq 30\%$ ethanol (Fig. 8) or in aqueous solution at pH ≥ 7.0 (Figs. 3 and 4), where NCS-C(b) can be completely released and degraded, the thermal stability of holoNCS equalizes to that of apoNCS. Collectively, the data suggest the enhanced stability of holoNCS over its apo form, and the stability is correlated to the association of NCS protein with the stable NCS-C(b).

Thermodynamic parameters for unfolding proteins are useful for making conformational stability comparisons. For evaluating protein stabilities, linear extrapolation is a common approach to estimate free energy of unfolding in the absence of a denaturant, ΔG (H₂O) [41]. Application of this method to the urea denaturation profiles at pH 2.0 (Fig. 6) gave ΔG (H₂O) values of 8.4 ± 0.1 and 10.5 ± 0.1 KJ mol⁻¹ for apoNCS and holoNCS, respectively. The greater free energy of holoNCS unfolding implies its greater stability compared with apoNCS. The slope of free energy of unfolding versus denaturant concentration, also known as the m value, has been considered as the measure of the accessible surface of a protein on unfolding [42, 43]. The m values calculated for apoNCS and holoNCS in urea-induced unfolding at pH 2.0 are 4.5 and 2.8 KJ mol⁻¹ M⁻¹, respectively. The low m value of holoNCS indicates that the protein-bound chromophore reduces the exposed protein surface during unfolding. The binding of NCS-C(b) may make holoNCS protein less accessible for the denaturant urea interacting with nonpolar residues. Proteins with higher T_m quite often have lower m values [44], and so does the holoNCS protein. Overall, the thermodynamic parameter comparison between apoNCS and holoNCS reinforces the enhanced stability of holoNCS over its apo form.

NCS-C(b) contributes to the enhanced stability of holoNCS

Because backbone conformation of apoNCS and holoNCS is superimposable [22,23], it is conceivable that conformational changes do not contribute to the enhanced stability of the holoNCS protein. Comparisons between the T_m or C_m of holoNCS and the T_r or C_r of NCS-C(b) (Figs. 3, 4, 6 and 8) reveal that holoNCS unfolding is nearly concomitant with NCS-C(b) release. Furthermore, the differential stability observed at approximately the same protein conformation with or without NCS-C(b) implies that chromophore conjugation is the key factor contributing to the enhanced stability of holoNCS.

Correlation between the existence of NCS-C(b) and the enhanced stability of holoNCS was further evaluated from the comparison of ΔT_m between the apo and holo forms of NCS protein ($\Delta T_m = T_m$ of holoNCS $- T_m$ of apoNCS) and the remaining amount of NCS-C(b) estimated at the T_m of holoNCS. Fig. 10 demonstrates that the ΔT_m in a wide pH range corresponds well with the remaining percentage of NCS-C(b). Between pH 3.0 and 7.0, ΔT_m and the remaining NCS-C(b) percentage decreases in nearly linear correspondence. Below pH 3.0 and above pH 7.0, both ΔT_m and NCS-C(b) percentage show a similar tendency with relatively unchanged values. The magnitude of ΔT_m may fairly represent the extent of enhanced holoNCS stability. A good correlation between ΔT_m and the remaining NCS-C(b) percentage indicates that the conjugation of labile NCS-C(b) is possibly the only factor that causes enhanced stability of holoNCS.

Protein and chromophore conjugation ensures functional mutualism in the NCS complex

Recently, we demonstrated that apoNCS is a superior drug carrier that is stable between pH 4.0 and 10.0 [31] and is highly resistant against chemical denaturants [39]. NCS-(b) binding with 1:1 apoNCS degrades at a rate of approximately 4000 times slower than the isolated NCS-C [25]. In the absence of apoNCS, mean lifetime of the active NCS-C is only 12 s at pH 8.0 and 25 °C [25]. It has long been known that apoNCS plays a key role in protecting the very labile NCS-C(b) from degradation [19]. The enediyne chromophore, being very unstable by itself, had not been thought to have a role in stabilizing NCS protein until we noted that only holoNCS, but not apoNCS is resistant against cold denaturation [31]. This prompted us to conduct a thorough and systematic investigation in comparing the stability between apoNCS and holoNCS under versatile conditions. Under all the experimental conditions, NCS-C(b) was found to have significant contributions to the enhanced stability of NCS protein. Interestingly, EtBr, which binds to the same binding site as the natural enediyne chromophore, provides no contribution to the protein stability (Fig. 5). Although the binding constant of EtBr to apoNCS is quite large ($K_d = 8.57 \times 10^{-7}$ M) [32], the binding affinity of EtBr is still more than 10,000-fold smaller than that of NCS-C ($K_d \leq 0.1$ nM) [25]. The binding interactions between the naturally occurring enediyne ligand and the NCS protein could be very specific. Lacking such specific interactions, EtBr fails to contribute stability of the NCS protein. Interaction between NCS protein and NCS-C(b) is suggested to be hydrophobic by structural simulation [45]. The specific NCS-C(b) binding that reduces the solvent exposure near the binding cleft and protects the internal hydrophobic core of the protein might be crucial for the protein stability [46]. Chemically, it remains obscure why the NCS-C that bears an epoxide moiety is more stable in acids than in bases. The exact biochemical mechanism that correlates chemical properties of NCS-C(b) to the stability of NCS protein needs to be defined. In the current investigation, it is evident that when NCS protein protects NCS-C(b) from degradation, NCS-C(b), in retrospect and for self-protection provides conformational stability against protein unfolding. As an antitumor drug, NCS is effectively used against different types of tumors including gastric cancer [47]. The pH of the stomach is approximately 2.0. Most proteins, including NCS, are unstable at such a low pH because of losing charge-charge interaction by protonation of acidic residues. The functional mutualism between the labile enediyne chromophore and the protein in the NCS complex, therefore, seems to be an elegant molecular adaptation of the antitumor antibiotic to endure adverse conditions in vivo.

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Fig. 1.

View of holoNCS backbone folding in aqueous environment at pH 7.0. Protein (cyan) is represented by ribbon and chromophore (red) by ball-and-stick model. This model [21] is simulated by simple energy minimization with three layers of water molecules, modified from the known X-ray structure obtained in solid state under acidic conditions. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Fig. 2. CD spectra of holoNCS (—), apoNCS (-----), and NCS-C (·····). To avoid degradation, CD spectra (measured in mdeg/μM) of samples (50 μM) were recorded at 0 °C. HoloNCS and apoNCS were prepared in 15 mM phosphate (pH 5.0). Extracted NCS-C was prepared in 92% (v/v) methanol containing 20 mM sodium citrate (pH 4.0). The inset shows CD spectra of 50 μM thermally denatured proteins and degraded NCS-C in 15 mM phosphate buffer (pH 7.0): holoNCS, 79 °C (—); apoNCS, 77 °C (-----); NCS-C, 55 °C (·····).

Fig. 3.

Comparison of heat-induced changes of apoNCS, holoNCS and NCS-C(b). (A) Samples (50 μ M) in 15 mM phosphate (pH 2.5). ApoNCS ($\circ$) and holoNCS ($\bullet$) were monitored by far-UV CD at 224 nm, and NCS-C(b) ($\diamond$) was monitored by near-UV CD at 255 nm. (B) Samples were prepared and monitored as in panel A except that the pH was 7.5.

Fig. 4.

Changes in T_m and T_r of NCS conjugates at pH 2.0–10.0. T_m of apoNCS (●) and holoNCS (○) was estimated from thermal unfolding curves acquired in far-UV CD at 224 nm at specified pH values. T_r of NCS-C(b) in holoNCS (▲) was determined from the profile monitored by near-UV CD at 255 nm. ApoNCS and holoNCS (50 μ M) were prepared in 15 mM phosphate (pH 2.0–10.0).

Fig. 5.

Comparison of thermal unfolding of apoNCS and NCS-EtBr at pH 2.0 and 7.0. Ellipticity changes of 50 μ M apoNCS and NCS-EtBr (1:1 molar ratio of apoNCS and EtBr) in 20 mM phosphate (pH 2.0 or 7.0) were monitored by far-UV CD at 224 nm: apoNCS (□) and NCS-EtBr (◆) at pH 2.0; apoNCS (○) and NCS-EtBr (▲) at pH 7.0.

Fig. 6.

Comparison of urea-induced changes of apoNCS, holoNCS, and NCS-C(b) at 25 °C. (A) NCS samples (15 μ M) were prepared in 15 mM phosphate (pH 2.0) with 0.2 M HCl and 0.05 M KCl containing urea (0.0–9.0 M). After the addition of urea, pH was readjusted by titration with 5 N sodium hydroxide. ApoNCS ($\circ$) and holoNCS ($\bullet$) were monitored by far-UV CD at 224 nm, and NCS-C(b) ($\diamond$) was monitored by near-UV CD at 255 nm. (B) Samples were prepared and monitored as in panel A except that the pH was 4.0.

Fig. 7.

Correlation between HPLC-analyzed NCS-C(b) ($\circ$) and ellipticity (mdeg) monitored at 255 nm ($\bullet$) in urea-treated holoNCS samples. The remaining NCS-C(b) was determined by HPLC integrated peak area ($\mu\text{V s } 10^{-6}$) recorded at 226 nm. HoloNCS (15 μM) in 15 mM phosphate (pH 2.0) with 0.2 M HCl, 0.05 M KCl, and 0.0–9.0 M urea was incubated in dark at 25 $^{\circ}\text{C}$ for 30 min prior to HPLC and CD analyses. After adding urea, pH was readjusted by using 5 N sodium hydroxide.

Fig. 8.

T_m values of apoNCS (●) and holoNCS (○), and T_r values of NCS-C(b) (▲) in the presence of ethanol. T_m was measured from thermal unfolding curves monitored by far-UV CD at 224 nm, and T_r was measured by near-UV CD at 255 nm. NCS samples (35 μ M in 15 mM phosphate, pH 2.0) were prepared in aqueous solutions containing 0–40% (v/v) ethanol and incubated in dark at 25 °C for 15 min prior to thermal denaturation analysis.

Fig. 9.

Two-dimensional NOESY NMR spectra of apoNCS and holoNCS (6 mg each in 15 mM phosphate, pH 2.0) acquired at different temperatures: (A) apoNCS at 25 °C; (B) apoNCS at 48 °C; (C) holoNCS at 25 °C; (D) holoNCS at 48 °C.

Fig. 10.

Correlation between ΔT_m (○) (holoNCS T_m value that exceeds that of apoNCS at each specified pH) and remaining NCS-C(b) percentage (●) (estimated at T_m of holoNCS at same pH). T_m was evaluated from thermal unfolding profiles at pH 2.0–10.0 and monitored by far-UV CD changes at 224 nm. Remaining NCS-C(b) percentage in holoNCS was estimated from profiles monitored by near-UV CD at 255 nm.