# Cold Instability of Aponeocarzinostatin and its Stabilization by Labile Chromophore

Kandaswamy Jayachithra,* Thallampuranam Krishnaswamy Suresh Kumar,† Ta-Jung Lu,* Chin Yu,† and Der-Hang Chin*

*Department of Chemistry, National Chung Hsing University, Taichung, Taiwan, Republic of China; and †Department of Chemistry & Biochemistry, University of Arkansas, Fayetteville, Arkansas

ABSTRACT The conformational stability of aponeocarzinostatin, an all-$\beta$-sheet protein with 113 amino-acid residues, is investigated by thermal-induced equilibrium unfolding between pH 2.0 and 10.0 with and without urea. At room temperature, the protein is stable in a pH range of 4.0–10.0, whereas the stability of the protein drastically decreases below pH 4.0. The thermal unfolding of aponeocarzinostatin is reversible and follows a two-state mechanism. By two-dimensional unfolding studies, the enthalpy change, heat capacity change, and free energy change for unfolding of the protein are estimated. Circular dichroism profiles suggest that this protein undergoes both heat- and cold-induced unfolding. The ellipticity changes at far- and near-UV circular dichroism suggest that the tertiary structure is disrupted but the secondary structure remains folded at low temperatures. Interestingly, the labile enediyne chromophore, which is highly stabilized by the protein, is able to protect the protein against cold-induced unfolding, but not the heat-induced unfolding.

## INTRODUCTION

Chromoprotein antibiotics are naturally occurring teams with unique features. The interesting combination involves a labile toxin, a small but highly functional molecule, associated with a specifically made stabilizer, a nontoxic carrier apoprotein. The toxin molecule is tightly bound to the protein but the biological activity can only be realized after it is released from the protein. A study on the subtle balance between stability and flexibility of the protein conformation is a necessary step to explore the mechanism of the chromoprotein.

The enediyne class of antibiotics belongs to one of the most potent antitumor categories. It is also one of the most extensively studied and characterized families of chromoprotein antibiotics (Shen et al., 2003). Neocarzinostatin (NCS) (Goldberg and Kappen, 1995; Xi and Goldberg, 1999), isolated from *Streptomyces carzinostaticus* (Ishida et al., 1965), is the first enediyne chromoprotein (Edo et al., 1985). It consists of a biologically active dienediyne chromophore (MW = 659) that is very potent in causing DNA damage, and a carrier protein, aponeocarzinostatin (apoNCS, with 113 amino acids; see Goldberg, 1991). The x-ray crystallographic studies show that apoNCS is an all-$\beta$-sheet protein with a seven-stranded antiparallel $\beta$-barrel and two twisted antiparallel $\beta$-sheets arranged perpendicular to each other (Kim et al., 1993; Teplyakov et al., 1993). Fig. 1 shows the native conformation of NCS in a simulated aqueous environment (Chin, 1999). There is no evidence showing that apoNCS binds to the target DNA under physiological conditions (Jung and Kohnlein, 1981). The function of apoNCS is to store the biologically active chromophore and release it in a controlled manner. The biologically active chromophore has a strong affinity for its apoNCS ($K_D \sim 10^{-10}$ M; see Goldberg, 1991). The chromophore is very labile and is highly stabilized by apoNCS (Kappen and Goldberg, 1980; Povirk and Goldberg, 1980), but the mechanism by which NCS protein interacts with its chromophore is not fully clear.

Understanding the mechanism by which a protein changes its conformation is still a challenging task in modern biology. Studying thermal stability through measurement of conformational changes of proteins with temperature can provide useful fundamental information. Evaluation of the thermodynamic stability of apoNCS is important to understand the energy difference between the native and unfolded states. By performing two-dimensional unfolding studies, we show that apoNCS undergoes both heat- and cold-induced unfolding. More significantly, we find that the chromophore, despite itself being labile, is able to stabilize the protein against cold-induced unfolding. To our knowledge, this is the first study of cold-induced unfolding among the members of enediyne chromoprotein family.

## MATERIALS AND METHODS

### Protein purification

NCS powder was obtained from Kayaku Laboratories (Tokyo, Japan). The stock solutions in water (1.44 mM) were stored in aliquots at 193 K. The apoNCS used for this study was obtained by the removal of chromophore from the holoNCS using the method reported by Napier et al. (1979). Impure

*Submitted August 20, 2004, and accepted for publication March 22, 2005.*

Address reprint requests to Der-Hang Chin, Dept. of Chemistry, National Chung Hsing University, 250 Kuo-Kuang Road., Taichung 40227, Taiwan, R.O.C. Tel.: 886-4-22-840-411 ext. 304; Fax: 886-4-22-862-547; E-mail: chdhchin@dragon.nchu.edu.tw.

*Abbreviations used*: apoNCS, the apoprotein component of neocarzinostatin or aponeocarzinostatin; holoNCS, the chromoprotein of neocarzinostatin or holoneocarzinostatin; mdeg, millidegree; NCS, neocarzinostatin.



0006-3495/05/06/4252/10 $2.00

doi: 10.1529/biophysj.104.051722

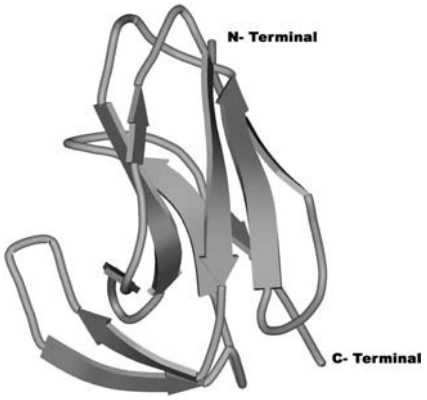


FIGURE 1 View of the backbone folding of apoNCS in an aqueous environment under neutral pH. The model is simulated by simple energy minimization with three layers of water molecules, modified from the known x-ray structure obtained in solid state (Brookhaven pdb.1nco.ent file).

apoNCS with minor amounts of chromophore was purified through the hydrophobic XAD-7 resin column (Napier et al., 1980). The concentration of the protein was 30–53 $\mu$M for the experiments throughout the study. The extinction coefficient at 278 nm (14,400 $M^{-1}$) is employed to determine the concentration of the protein (Napier et al., 1979; Povirk et al., 1981).

## Thermal-induced unfolding

Thermal-induced unfolding experiments were conducted in the temperature range of 273–363 K and monitored by circular dichroism (CD) using JASCO J-715 spectropolarimeter (Tokyo, Japan) equipped with a circulating water bath (Neslab, model RTE-140, Portsmouth, NH). All experiments were performed using a 0.1-cm pathlength water-jacketed quartz cell. The temperature of the water bath is controlled by a microprocessor and a temperature sensor. The details of the CD measuring experiments are as follows: resolution, 1 s; bandwidth, 1.0 nm; response time, 1 s; and ramp time, 15 min for each increment of temperature. The stabilization of the observed ellipticity followed by temperature setting is checked to ensure the equilibrium. For most measurements, the equilibrium can be reached within 10 min.

## Unfolding studies at different pH conditions

The thermal- and urea-induced unfolding at different pH conditions were monitored by far-UV CD at 224 nm and near-UV CD at 271 nm. Phosphate buffer (15 mM) was used throughout the pH range (2.0–10.0). The pH of the prepared buffer was checked repeatedly after the addition of urea to ensure the accuracy of the measured pH. A maximum variation of $\pm 0.05$ pH units was allowed in all of the buffer preparations.

## Unfolding with urea

Urea-assisted thermal unfolding and urea-induced unfolding at all conditions were monitored using far-UV and near-UV CD. Appropriate concentrations of urea were prepared in 15 mM phosphate buffer at desired pH for the unfolding experiments. For thermal-induced unfolding experiments, the sample exposure to high temperature was kept short to minimize chemical modification of the protein by the decomposition products of urea.

## Data analysis

Data analysis was performed using the general curve fit option in the Kaleidagraph program, Ver. 3.5 (Synergy Software, Reading, PA). The equilibrium unfolding data were analyzed using the two-state, i.e., *Native* ($N$) $\leftrightarrow$ *Denatured* ($D$) states model of unfolding (Santoro and Bolen, 1992; Schellman, 1978; Tanford, 1970). The raw data were converted to the fraction of the protein in unfolded state, $f_u$, as a function of urea concentration using the equations

$$f_u = \{Y_I - (Y_f + m_f[D])\}/\{(Y_u + m_u[D]) - (Y_f + m_f[D])\}, \quad (1)$$

where $Y_I$ is the observed spectroscopic property, $Y_f$ and $m_f$ are the intercepts and slope of the folded state baseline, $Y_u$ and $m_u$ represent the respective intercept and slope of the unfolded baseline, and [D] is the molar denaturant concentration;

$$\Delta G_u = -RT \ \ln \ K_{eq}, \quad (2)$$

where $K_{eq}$ is the equilibrium constant, $\Delta G_u$ is the change in free energy of unfolding in the presence of denaturant, and $R$ is the gas constant; and

$$K_{eq} = f_u/f_n, \quad (3)$$

where $f_n$ is the fraction of the protein in the folded state and $f_n = 1 - f_u$.

The raw thermal-induced unfolding data were converted to the fraction of the unfolded species ($f_u$) as a function of temperature using the equation

$$K_{eq} = f_u/(1 - f_u) = \{Y_I - (Y_f + m_f T)\}/\{(Y_u + m_u T) - Y_I\}. \quad (4)$$

A two-state $N \leftrightarrow U$ unfolding reaction is characterized by a change in heat capacity $\Delta C_p$, which is considered to be independent of temperature in the range of measurements used here (Pace et al., 1999; Schellman, 1987). The temperature-dependent values for the change in free energy ($\Delta G$), change in entropy ($\Delta S$), and change in enthalpy ($\Delta H$) are computed as

$$\Delta G = \Delta H - T\Delta S \quad (5)$$

$$\Delta H(T) = \Delta H_m + \Delta C_p(T - T_m) \quad (6)$$

$$\Delta S(T) = \Delta S_m + \Delta C_p \ \ln (T/T_m) \quad (7)$$

$$\Delta G(T) = \Delta H_m\{1 - (T/T_m)\} - \Delta C_p\{(T_m - T) + T \ \ln(T/T_m)\}, \quad (8)$$

where $T_m$ corresponds to the midpoint of the thermal transition, $\Delta G(T) = 0$, and $\Delta H_m$ and $\Delta S_m$ are the values of $\Delta H$ and $\Delta S$ at $T_m$.

According to the linear free energy model, all of the changes in $\Delta G'$, $\Delta H'$, $\Delta S'$, and $\Delta C_p'$ that occur during protein unfolding have linear dependences on the molar concentration of the denaturant, and their relationship is given by the equations

$$\Delta G' = \Delta G + m[D] \quad (9)$$

$$\Delta H' = \Delta H + h[D] \quad (10)$$

$$\Delta S' = \Delta S + s[D] \quad (11)$$

$$\Delta C_p' = \Delta C_p + c[D], \quad (12)$$

where the primes on the above parameters are the values determined in the presence of urea, and $m$, $h$, $s$, and $c$ are slopes of the measure of the protein-denaturant interaction. Alternatively, above equations are combined (Santoro and Bolen, 1988) to provide a direct conversion from the measured spectroscopic property.

# RESULTS

## Two-state unfolding of apoNCS

The far-UV CD spectrum of the purified apoNCS exhibits a positive maximum at 224 nm and a small negative

minimum located at ~212 nm (Fig. 2, *inset A*). The near-UV CD spectrum of apoNCS shows a negative band located at ~271 nm (Fig. 2, *inset B*). Both spectra are consistent with those in earlier reports (Heyd et al., 2000; Napier et al., 1981, 1980). The secondary and tertiary conformational changes occurring during unfolding of apoNCS were followed by far-UV CD and near-UV CD spectroscopy, respectively. Fig. 2 shows that the thermal unfolding profiles of apoNCS at neutral pH monitored by far-UV CD (224 nm) and near-UV CD (271 nm) are almost superimposable, indicating the two-state unfolding mechanism of apoNCS. The thermal unfolding profiles in the pH range of 4.0–10.0 monitored by far- and near-UV CD were all nearly superimposable (data not shown), suggesting that over this pH range the unfolding ↔ folding of the protein follows a two-state (native ↔ denatured) mechanism. In the urea-induced unfolding at room temperature, apoNCS starts to unfold when the urea concentration is beyond 6 M, but does not fully denature even at the maximum urea concentration (9 M) at neutral pH (Fig. 3). The incomplete far-UV CD (224 nm) and near-UV CD (271 nm) profiles are superimposable, suggesting the urea denaturation follows a two-state mechanism. The incomplete unfolding profiles monitored between pH 4.0 and 10.0 by far- and near-UV CD are all superimposable (data not shown), suggesting the urea-induced unfolding of the protein is likely to be reversible in the pH range (pH 4.0–10.0) investigated.

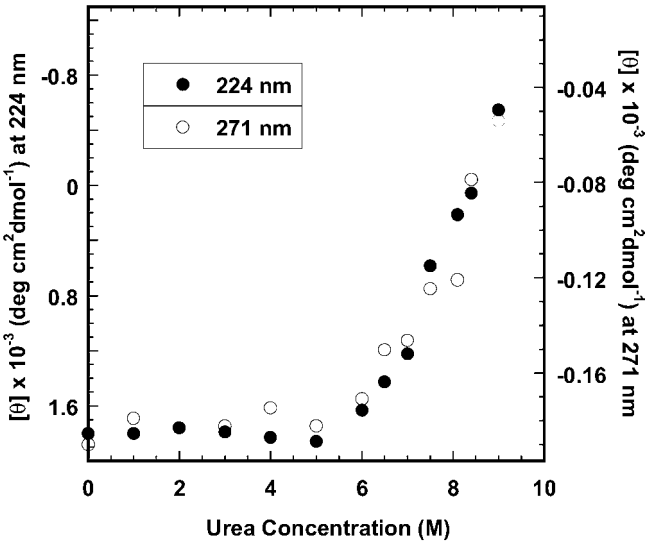


FIGURE 3 Urea-induced unfolding profiles of apoNCS at pH 7.0 monitored at room temperature by far-UV CD (224 nm, *solid circles*) and near-UV CD (271 nm, *open circles*) changes.

## pH stability of apoNCS

The conformational stability of the protein is investigated in the pH range of 2.0–10.0. At each fixed pH value, the stability is evaluated either by thermal-induced unfolding studies without urea (in the range of 273–368 K), or by urea-induced unfolding studies (in the range of 0.0–7.2 M) at a fixed temperature (308 K or greater). Without urea, the $T_m$ of the protein is almost unchanged in the pH range of 4.0–1.0 (Fig. 4, *inset*). The unfolding profiles monitored by the ellipticity changes at 224 nm at this pH range suggest that apoNCS is heat-stable until ~325 K (Fig. 4). However, the protein is destabilized at pH values <4.0, as shown by the large decrease in the $T_m$ at pH values <4.0 (Fig. 4, *inset*). In

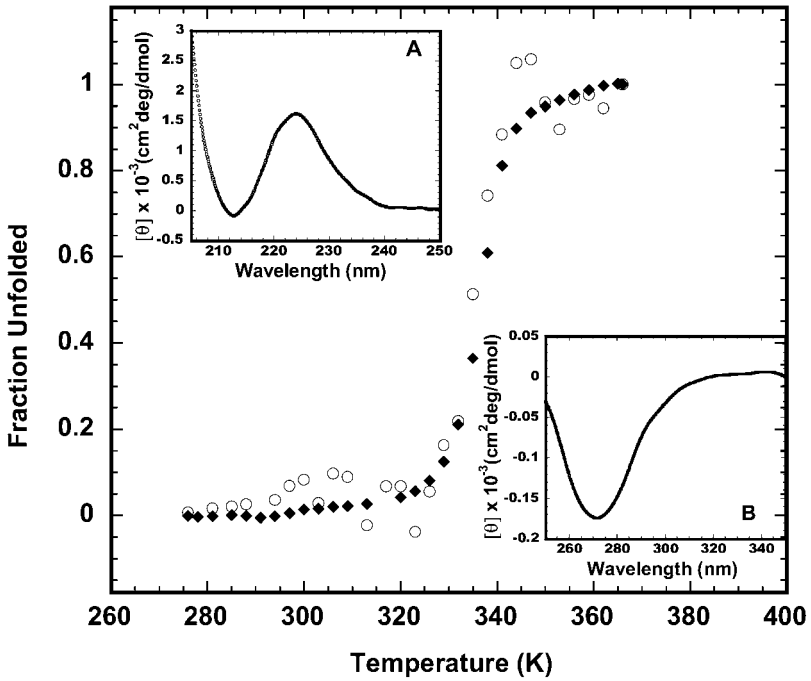


FIGURE 2 Thermal-induced unfolding of apoNCS at pH 7.0 monitored by far-UV CD (224 nm, *solid diamonds*) and near-UV CD (271 nm, *open circles*) changes. The far-UV and near-UV CD spectra (at pH 7.0) of apoNCS are shown in insets *A* and *B*, respectively.

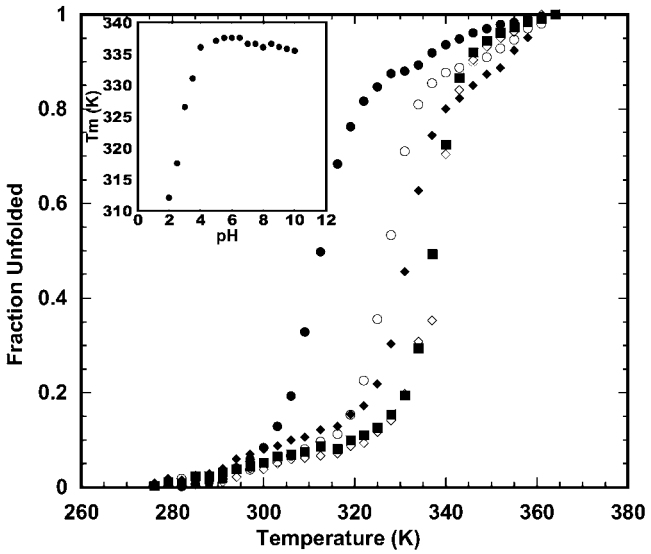


FIGURE 4 Thermal-induced unfolding profiles of apoNCS at various pH, ●, pH 2.0; ○, pH 3.0; ♦, pH 3.5; ◇, pH 4.0; and ■, pH 7.5, monitored by far-UV CD (at 224 nm). The inset figure shows the changes in $T_m$ as a function of the pH.

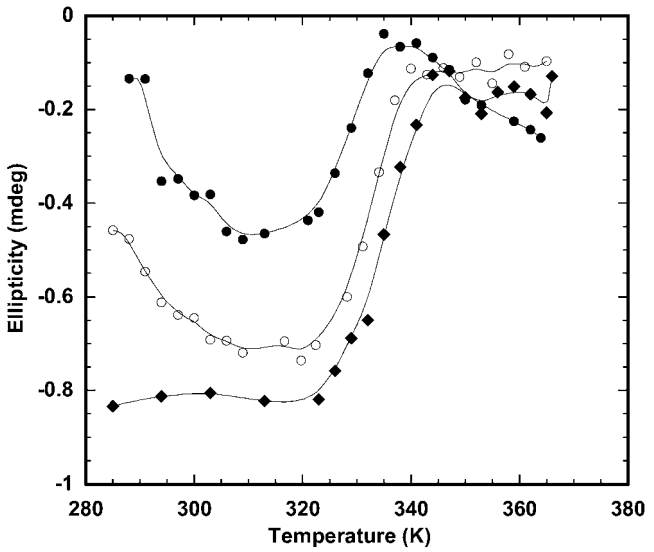


FIGURE 5 Thermal-induced unfolding of apoNCS at pH 7.0 under various concentrations of urea, ♦, 0.0 M; ○, 0.8 M; and ●, 2.0 M, monitored by ellipticity changes at 271 nm.

the urea-induced unfolding studies at a pH value >4.0, most profiles show that the unfolding process is incomplete at the maximum urea concentration, suggesting that apoNCS is highly resistant to urea denaturation at those pH conditions. When pH values are <4.0, where unfolding is complete at 308 K, the estimated $C_m$ (concentration of the denaturant at which 50% of the protein molecules exists in the unfolded state) decreases significantly with decrease in pH (data not shown). Both thermal- and urea-induced unfolding studies show consistent results and suggest that apoNCS is stable in the pH range of 4.0–10.0, where the apoNCS follows a two-state unfolding mechanism. We therefore chose pH 7.0 as the proper pH condition for the two-dimensional unfolding studies of apoNCS.

## Observation of the cold-induced unfolding in apoNCS

We performed the thermal unfolding studies of apoNCS and found a significant effect of urea on the tendency to undergo cold-induced unfolding. Two-dimensional thermal-induced unfolding experiments were done at pH 7.0, under 13 different urea concentrations from 0.0 M to 5.2 M, in the temperature range of 279–366 K, where the protein unfolds reversibly by a two-state mechanism. Conformational changes were monitored by using both near- and far-UV CD. Fig. 5 shows the near-UV CD thermal-induced unfolding profiles of apoNCS in the presence of urea at various concentration values. A prominent curvature is observed in the thermal unfolding profiles when the urea concentration is >0.8 M, indicating that the protein undergoes cold-induced unfolding. Urea decreases the apparent enthalpy of unfolding and shifts the unfolding zone to lower temperatures in the heat-induced transition and to higher temperatures in the cold-induced transition. This facilitates observation of cold-induced unfolding at temperatures well above the freezing point of the solution.

Fig. 6 shows the thermal-induced unfolding profiles of apoNCS monitored by far-UV CD at different urea concentration values. The measured $T_m$ in the heat-induced unfolding is consistent with that obtained from near-UV CD

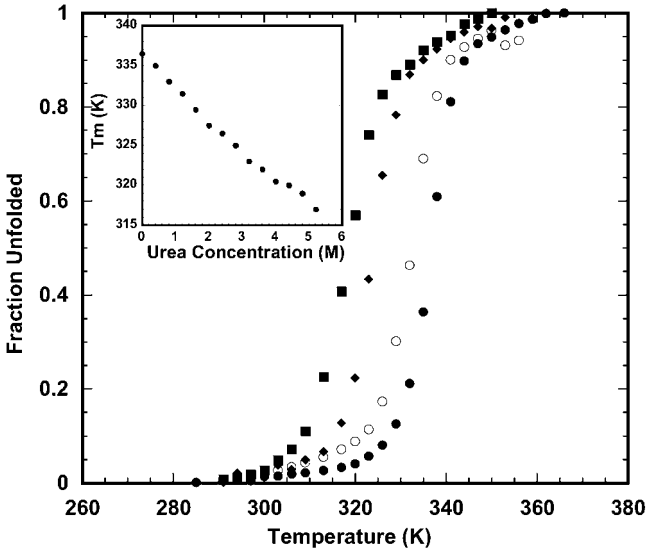


FIGURE 6 Thermal-induced unfolding of apoNCS at pH 7.0 under various concentrations of urea, ●, 0.0 M; ○, 1.2 M; ♦, 2.8 M; and ■, 5.2 M, monitored by far-UV CD changes at 224 nm. The inset figure shows marked decrease of $T_m$ with increase in urea concentration.

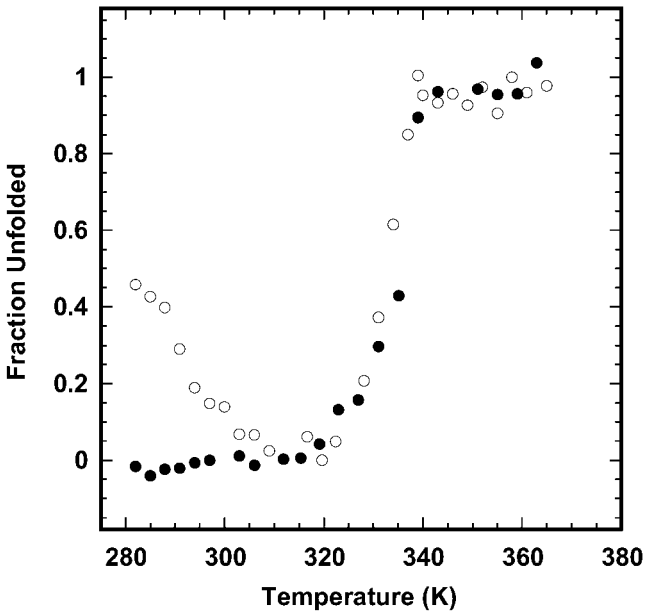


FIGURE 7 Thermal-induced unfolding of apoNCS (*open circles*) and holoNCS (*solid circles*) in 0.8 M urea (pH 7.0) monitored by ellipticity changes at 271 nm. Because mean residue ellipticity at 271 nm from apoNCS is ~3.5–4.0-folds less intense than that from holoNCS, fraction of unfold is used here to replace ellipticity changes for a purpose of close comparison. The fraction of unfold in the incomplete cold denaturation limb is estimated based on the heat denaturation process.

under the same urea concentration. It is interesting to observe that the far-UV CD profiles in Fig. 6 do not show the perturbation of the secondary structure at low temperatures, whereas the near-UV CD profiles (Fig. 5) indicate that the tertiary structure is disrupted. The results suggest that the cold-induced unfolded protein exists possibly in a partially unfolded state, in that the secondary structure remains folded. The inset of Fig. 6 shows that the $T_m$ of the heat-induced transitions is linearly proportional to the urea concentration. Urea reduces the $T_m$ of apoNCS under neutral pH at an average of 3.7 K per additional molar concentration of urea. A strong correlation ($R = 0.995$) between the two parameters is apparent, suggesting that urea is a proper denaturant for the two-dimensional thermal unfolding studies of apoNCS.

## Comparison of the thermal-induced unfolding of apo- and holoNCS

Although the CD spectrum of holoNCS containing the chromophore is distinctively different from that of apoNCS (Napier et al., 1979, 1980, 1981), a number of structural studies by NMR (Adjadj et al., 1990, 1992a,b; Gao, 1992; Gao and Burkhart, 1991; Remerowski et al., 1990; Tanaka et al., 1993) and x-ray crystallography (Kim et al., 1993; Sieker et al., 1976; Teplyakov et al., 1993) suggest that the protein conformation of apo- and holoNCS are almost superimposable. The stabilities of apo- and holoNCS are compared here, to assess the contribution of the bound enediyne group to the conformational stability of the chromoprotein. The thermal unfolding profile of apoNCS (monitored at 271 nm in the temperature range of 280–365 K at pH 7.0 with 0.8 M urea) shows that, below 303 K, the negative ellipticity at 271 nm decreases with the decrease in temperature (Fig. 7). A prominent curvature below 303 K is observed, suggesting that cold-induced unfolding of apoNCS occurs. In the temperature range of 303–323 K, the ellipticity value at 271 nm does not show significant change. Further increase in temperature beyond 323 K results in progressive unfolding of the protein. Above 343 K, apoNCS exists in an unfolded state.

Interestingly, thermal unfolding of holoNCS under the same condition monitored by the ellipticity change at 271 nm shows no observable change in the temperature range of 280–323 K (Fig. 7). However, an increase in temperature above 323 K results in a similar progressive loss in ellipticity at 271 nm. The heat-induced limb (in the temperature range of 323–363 K) of the thermal unfolding curves of apo- and holoNCS are superimposable, indicating that the enediyne group does not stabilize the protein against heat-induced unfolding. In contrast, the cold unfolding limb (from 278 K to 303 K) of apo- and holoNCS are not superimposable. A significant unfolding curvature is observed for apoNCS, but not for holoNCS, at low temperatures. Apparently, holoNCS does not show a tendency to undergo cold-induced unfolding as apoNCS does. These results suggest that binding of the enediyne group confers resistance to the protein against cold unfolding.

The enediyne chromophore is known to be very labile. The extracted NCS chromophore has a mean lifetime of only 12 s at pH 8 and 25°C (Povirk and Goldberg, 1980). The degradation of the labile enediyne group at high temperatures prohibits our performing thorough thermodynamic unfolding studies of holoNCS. The ineffectiveness of the enediyne group to stabilize the protein against heat instability is likely due to its degradation. However, the enediyne chromophore can be substantially stabilized by apoNCS (Povirk and Goldberg, 1980). Kappen and Goldberg (1980) reported that $<10\%$ activity was lost when holoNCS was preincubated at pH 5.0 and 37°C for 30 min. At the same pH and temperature, Edo et al. (1988) found that only 4% bound chromophore was lost in 24 h. The discrepancy in chromophore stability among reports is not surprising, because the lifetime of the bound chromophore in holoNCS appears to be concentration-dependent (Jung and Kohnlein, 1981). The concentration of holoNCS used in the present study was 53 $\mu$M. A solution of 85 $\mu$M holoNCS was estimated to have a long half-life of ~48 h at 330 K (Edo et al., 1988). At the same temperature, one-fourth fraction of the NCS protein becomes unfolded, as shown in the present study (Fig. 7). Thus, the ineffectiveness of the chromophore to protect protein against heat could not entirely contribute to its

degradation at high temperatures. The intrinsic ability of the chromophore to stabilize the protein and the difference in nature between cold- and heat-induced denaturing processes are the other possible factors.

## Estimation of $\Delta C_p$ from two-dimensional unfolding studies

A single thermal unfolding profile alone cannot provide reliable estimates of important thermodynamic parameters, including the change in heat capacity ($\Delta C_p$). A good value of $\Delta C_p$ is needed to construct a reliable stability curve for apoNCS. There are different methods of estimating $\Delta C_p$ of a protein. Accurate estimation of $\Delta C_p$ can be achieved from two-dimensional unfolding studies by varying $\Delta H_m$ and $T_m$ over a wide range. A value of $\Delta C_p$ can be calculated by the relation of $\Delta C_p = d\ \Delta H_m/T_m$ (Swint and Robertson, 1993). The enthalpy change $\Delta H$ for unfolding of apoNCS is estimated by making use of the van't Hoff equation, $\ln(K) = -\Delta H/RT + \Delta S/R$. If $\Delta C_p$ is zero, $\ln(K)$ is a linear function of $1/T$. The slope of the straight line obtained by plotting $\ln(K)$ versus $1/T$ is then $-\Delta H/R$. The open circles in Fig. 8 represent the experimental values of $\ln(K)$ versus $1/T$ in the thermal-induced unfolding transition in apoNCS at pH 7.0 without urea. The linear least-square fitting of the data yields a value $\Delta H = 65 \pm 1$ kcal/mol. The small curvature in the van't Hoff plot shows the effect of the non-zero value of $\Delta C_p$ (Chaires, 1997; Koblan and Ackers, 1992; Liu and Sturtevant, 1995). Values of $\Delta H_m$ under 13 different concentrations of urea are obtained analogically from the slope of $\ln(K)$ versus 1/T. The variation of $\Delta H_m$ versus $T_m$ with different urea concentrations is shown in Fig. 9. A value of $\Delta C_p$, 1.04 ± 0.03 kcal mol$^{-1}$ K$^{-1}$, is calculated here from slope $[d\ \Delta H_m/T_m]$. Because of the intrinsic nature in the methodology we applied, the error might be greater than 0.03 kcal mol$^{-1}$ K$^{-1}$, which is estimated solely based on variation of the data from measurement. Using this value of $\Delta C_p$ in Eq. 7 and converting $\Delta G$ to $\ln(K)$ by Eq. 2, a theoretical curve is calculated as shown in the dotted line in the van't Hoff plot (Fig. 8). The experimental values agree well with the theoretical curve.

A consistent but less accurate value of $\Delta C_p$ (1.03 ± 0.06 kcal mol$^{-1}$ K$^{-1}$) is obtained from the variation of $T_m$ with five different pH values (pH 2.0–4.0, see Fig. 4, *inset*). The self-consistent value of $\Delta C_p$, from variation of urea concentration or pH, suggests that the $\Delta C_p$ is independent of the denaturant. We checked this by plots of $\Delta H_m$ or $T_m$ versus urea concentration. The slope of a linear plot of $\Delta H_m$ versus urea concentration (−3.7 kcal/mol/M, $R = 0.997$) is nearly identical to that from $T_m$ versus urea concentration (−3.7 K/M, $R = 0.997$). The $\Delta C_p$, which is derived from changes of $\Delta H_m$ with $T_m$, is thus independent of the urea concentration. The slope in Eq. 12 is near zero in the case of apoNCS. This phenomenon is not a rare one. For example, a nearly constant $\Delta C_p$ value for $\beta$-lactoglobulin over a wide range of urea concentration was reported in 1968 (Pace and Tanford, 1968). Some proteins do appear to show low denaturant sensitivity with regard to the heat capacity (Otzen and Oliveberg, 2004).

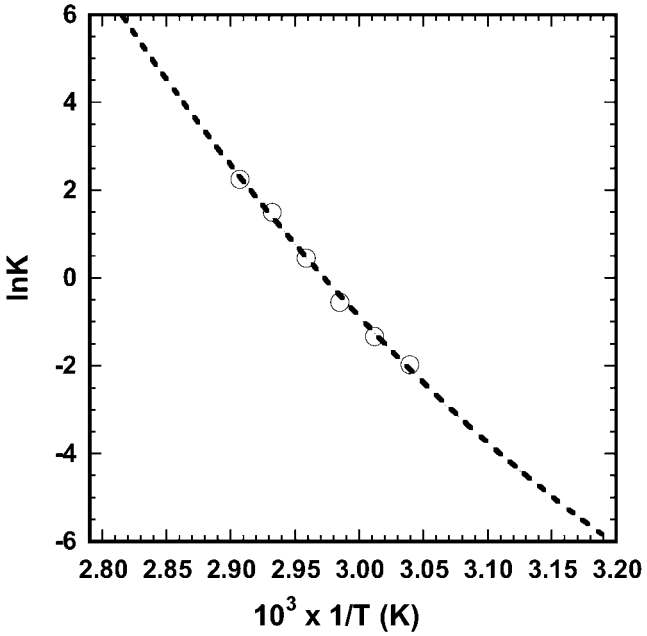


FIGURE 8 van't Hoff plot for the unfolding of apoNCS at pH 7.0 in 0.0 M urea. The $\Delta H$ from the slope is estimated to be 65 ± 1 kcal mol$^{-1}$ (*open circles*). The dotted line represents the theoretical $\Delta G$ calculated from Eq. 8. The theoretical $\ln(K)$ is converted from the theoretical $\Delta G$ using Eq. 2.

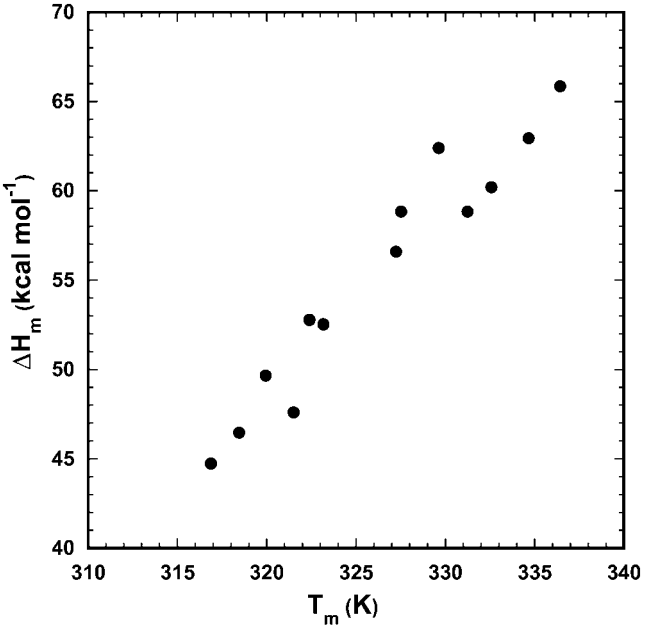


FIGURE 9 $\Delta C_p$ plot for the unfolding of apoNCS based on thermal-induced unfolding profiles at pH 7.0 under 13 different concentrations of urea (0.0–5.2 M). The $\Delta C_p$ from the slope is estimated to be 1.04 ± 0.03 kcal mol$^{-1}$ K$^{-1}$.

The heat capacity value $\Delta C_p$ of 1.04 ± 0.03 kcal mol$^{-1}$ K$^{-1}$ for the transition of apoNCS ranks approximately in the middle among known values of $\Delta C_p$ obtained for wild type proteins of similar size (MW 11,000–12,000), shown in the newest version of thermodynamic database for proteins and mutants (Bava et al., 2004) (URL http://gibk26.bse.kyutech.ac.jp/jouhou/Protherm/protherm.html). Heme chromoproteins such as myoglobin and cytochrome *c* have values of 1.23 kcal mol$^{-1}$ K$^{-1}$ and 0.945 kcal mol$^{-1}$ K$^{-1}$, respectively, averaged from the deposited data that were obtained under various experimental conditions and measuring methods. These deposited $\Delta C_p$ values translate into an average value of 8.05 cal mol$^{-1}$ K$^{-1}$ per residue for myoglobin, and of 9.08 cal mol$^{-1}$ K$^{-1}$ per residue for cytochrome *c*. The measured $\Delta C_p$ of apoNCS gives a value of 9.2 cal mol$^{-1}$ K$^{-1}$ per residue, which is compatible with that of myoglobin and cytochrome *c*. A more close comparison should be made with apocytochrome, which has a size similar to apoNCS. Apocytochrome $b_{562}$ has a value of $\Delta C_p$ 1.1 kcal mol$^{-1}$ K$^{-1}$ (Feng and Sligar, 1991), which is very close to $\Delta C_p$ for the transition of apoNCS. However, a much smaller value of $\Delta C_p$ for apocytochrome $b_{562}$, 0.56 kcal mol$^{-1}$ K$^{-1}$ at pH 7.4 (Robinson et al., 1998), was also reported later. The $\Delta C_p$ of apocytochrome $b_5$ was also reported to have a close (1.0 kcal mol$^{-1}$ K$^{-1}$) (Pfeil, 1993) or slightly smaller (0.86 kcal mol$^{-1}$ K$^{-1}$) (Manyusa and Whitford, 1999) value.

## DISCUSSION

NCS and other enediyne chromoproteins have been shown to exhibit very potent antitumor activity against a variety of tumors (Greish et al., 2003; Maeda, 2001). The enediyne group is highly stabilized by the tightly bound NCS protein (Povirk and Goldberg, 1980) until it reaches the target DNA. Earlier we found that charge repulsion, rather than size exclusion, plays an important role in apoNCS for the chromophore protection against thiols (Chin, 1999). We also studied the trifluoroethanol-induced release of the bioactive chromophore from NCS chromoprotein (Sudhahar et al., 2000). The enediyne chromophore can be released before major backbone conformational changes in the protein. Very little is known about the mechanism by which the enediyne group is regulated by the protein. Thermodynamic unfolding studies allow one to predict the stability and flexibility of the protein under various conditions. It becomes an important tool to understand the tendency of conformational changes of the protein and how such changes associate with its bound toxin molecule.

### Analysis of stability curve for apoNCS

The variation of the conformational free energy of unfolding with temperature represents the stability nature of the protein. The measured enthalpy change at the midpoint ($\Delta H_m$), the change in heat capacity ($\Delta C_p$), and the midpoint temperature ($T_m$) are used to construct a reliable stability curve of apoNCS. The apparent $\Delta G$ values are calculated based on Eq. 7 using constant $\Delta C_P$ (1.04 ± 0.03 kcal mol$^{-1}$ K$^{-1}$, calculated based on $\Delta H_m$ versus $T_m$). Fig. 10 depicts the stability curve of apoNCS at pH 7.0 under various concentrations of urea. The temperature of maximum stability in the heat-denatured regions shift to lower temperatures with increase in urea concentration. Most significantly, the free energy change associated with the unfolding process decreases at both high and low temperatures. The latter is an indication of cold instability of apoNCS and its tendency to undergo cold-induced unfolding.

The maximum conformational stability of apoNCS is estimated to be 5.8 kcal mol$^{-1}$, as inferred from Fig. 10. This value is higher than the reported value of $\Delta G$ for apocytochrome $b_{562}$ (3.2 kcal mol$^{-1}$, Feng and Sligar, 1991) and is two-to-threefold larger than the values for apocytochrome $b_5$ ($\Delta G$, 1.7 kcal mol$^{-1}$ , Pfeil, 1993; 2.8 kcal mol$^{-1}$, Manyusa and Whitford, 1999). In fact, the $\Delta G$ value of apoNCS is more comparable to the reported value of holocytochrome $b_5$ (6.0 kcal mol$^{-1}$, Pfeil, 1993). These results imply that the apoNCS is comparatively stable. Considering that the labile chromophore needs to be well preserved during the drug delivery time course, it is necessary having a very stable carrier protein to protect the chromophore.

### Cold-induced unfolded state in apoNCS

Many proteins have been shown to unfold under cold conditions (Privalov, 1990). In general, cold denaturation is

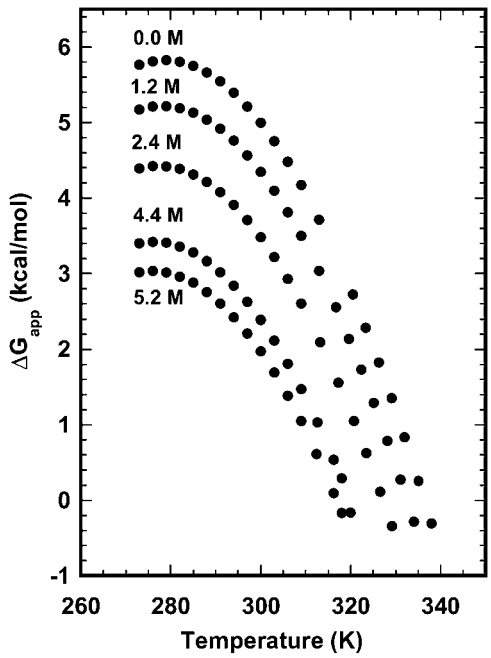


FIGURE 10 Stability curves of apoNCS at pH 7.0 as a function of temperature at various concentrations of urea.

caused by strongly temperature-dependent interactions of protein groups with water. Hydration of protein groups is favorable thermodynamically. As a result, the polypeptide chain, tightly packed in a compact native structure, unfolds at a sufficiently low temperature, exposing internal nonpolar groups to water. Most of these proteins studied belong either to all-$\alpha$ or $\alpha+\beta$-structural classes. Limited information exists on the cold-induced unfolded states of all-$\beta$-sheet proteins (Chi et al., 2001). ApoNCS is an all-$\beta$-sheet protein and the observation of cold-induced unfolding in apoNCS can help to fill this lacuna.

A wide range of unfolded non-native states has been found in several proteins (Russell and Bren, 2002; Shortle, 1996). These non-native states can vary from a partially structured state to unstructured random coils. The present studies suggest that apoNCS under cold-induced unfolding exists in a partially structured state. The transition profile monitored by CD suggests that the tertiary structure of apoNCS is disrupted, but the secondary structure remains folded. The partial loss of the highly compacted structure in the cold-induced unfolding is not a rare phenomenon for $\alpha$-helical-based proteins. Similar observation has been reported on the molten globule state of cytochrome *c*, in which the secondary structure is formed, but the tertiary structure fluctuates considerably (Kuroda et al., 1992). The cold-induced unfolding of myoglobin also yields a partially unfolded state characterized by having a persistent amount of secondary structure, whereas rigid tertiary structure is lost (Meersman et al., 2002; Privalov et al., 1986). Whether a $\beta$-sheet protein can form a molten globule folding intermediate, which has native-like secondary structure (by far-UV CD) but does not have the native tertiary structure, has been a controversial question. Several reports show observation of molten globule-like intermediate from an $\beta$-sheet protein (Kumar et al., 1995; Sivaraman et al., 1997; Dalessio and Ropson, 2000; Samuel et al., 2000). More recent computational theories on CD of protein-folding intermediates suggest that it is possible for an all-$\beta$ protein in a molten globule state to retain all or most of the individual units of secondary structure—$\beta$-sheets and probably $\beta$-hairpins (Sreerama and Woody, 2004; Woody, 2004; R.W. Woody, personal communication, 2004). These secondary structure units fluctuate with respect to each other. It is these fluctuations in packing of the secondary structure elements that lead to variations in the environment of the aromatic side chains, causing their near-UV CD bands to be diminished through extensive averaging over different environments. In most cases, the $\beta$-sheets are still rather local and not assembled from very remote segments in the sequence. The common types of sheets are likely to maintain their integrity under the mild conditions leading to molten globule formation. It is therefore reasonable to assume that the $\beta$-sheet apoNCS forms an unfolding intermediate by losing its near-UV CD signal without losing much of its signal in the far-UV CD. Whether this partially structured state of apoNCS could lead to the regulation or other important functions of NCS chromoprotein is not yet known. Further characterization is likely to throw light on studying such a possibility.

## Cold instability of apoNCS and its stabilization by chromophore

Lately, a re-evaluation of the hydration effect and revision of the conventional concept on the hydrophobic effect in protein folding leads to new propositions to explain the cold- and heat-induced unfolding on a molecular basis (Privalov, 1990; Privalov and Makhatadze, 1993; Tsai et al., 2002). Cold-induced unfolding is mainly caused by interaction of protein groups with water. The exposure of internal surface with water reduces the enthalpy of the system and overcomes the unfavorable reduction in entropy at low temperatures. The tightly bound enediyne chromophore, which occupies the central cavity in the compact native structure of NCS protein, is expected to diminish the chances of exposing internal protein groups to water. Thus the chromophore, though it is very labile by itself, can stabilize the bound protein against cold-induced unfolding by efficiently reducing the hydration effect. On the other hand, the heat-induced unfolding is mainly initiated by a large increase in entropy gain at high temperatures. The size of the chromophore is relatively small compared to that of the protein and there is no covalent bonding involved in between. Unlike heme in cytochromes (Feng and Sligar, 1991; Manyusa and Whitford, 1999; Robinson et al., 1998), NCS chromophore is not a steady prosthetic group. It would be hard to expect the chromophore to act as an effective stabilizer for a bound protein that is being disrupted by the heat-induced molecular motions.

Most enediyne chromoproteins are well-characterized structurally, yet the mechanism of self-resistance of the source organism to these toxins has remained unclear. The intriguing interaction and coordination between the chromophore and its bound protein needs to be explored to understand such a mechanism. Cold-induced unfolding has been suggested as a general phenomenon (Privalov, 1990). Our results indicate that apoNCS exhibits a cold instability nature, suggesting that it does not escape from such a general prediction for proteins. NCS is a very potent antibiotic. Minimum inhibitory concentration against Gram-positive bacteria can be as low as 0.5 $\mu$g/ml (Ishida et al., 1965). When apoNCS conformation is unstable or unfolded under cold, the binding equilibrium can shift to raise the rate of dissociating the toxic chromophore from the chromoprotein. A needlessly released toxin could then create a local fatal environment to the organism that produces NCS. In most places, the temperature of soil, even in the hot seasons, is well below the $T_m$ of NCS. Heat instability of the protein component of NCS should not be a threat of self-destruction to the bacteria that produces antibiotic. Considering the survival of the bacteria in soil during cold seasons,

the stability of the protein under cold becomes vital. If temperature is low enough, the cold sensitivity of apoNCS could pose a potential danger in launching a suicidal action. It is interesting to observe that the NCS chromophore, which is labile by itself, is able to stabilize the protein against cold instability. This finding could provide some logical clues in understanding the mechanisms of cellular self-protection under cold. The stabilization of neocarzinostatin by the biologically active chromophore might have a significant survival value for its own producer, *Streptomyces carzinostaticus*.

We thank the Kayaku Co. for the supply of NCS powder. We thank Dr. Robert L. Baldwin, Stanford University, for his many valuable suggestions on the manuscript. We also thank Dr. Robert W. Woody, Colorado State University, for his kind help on CD theories.

This work was supported by a laboratory grant to D.-H.C. (No. NHRI-EX90-8807BL) from National Health Research Institutes, The Executive Yuan, Republic of China, and individual grants to D.-H.C. (Nos. NSC 91-2320-B-005-007 and 91-2113-M-005-031) from National Science Council, The Executive Yuan, Republic of China. This work was also supported in part by grants from the National Institutes of Health (NIH NCRR COBRE grant No. 1 P20 RR 15569) to C.Y. and the Arkansas Bioscience Institute to C.Y. and T.K.S.K.

## REFERENCES

Adjadj, E., J. Mispelter, E. Quiniou, J. L. Dimicoli, V. Favaudon, and J. M. Lhoste. 1990. Proton NMR-studies of apo-neocarzinostatin from *Streptomyces carzinostaticus*: sequence-specific assignment and secondary structure. *Eur. J. Biochem.* 190:263–271.

Adjadj, E., E. Quiniou, J. Mispelter, V. Favaudon, and J. M. Lhoste. 1992a. Three-dimensional solution structure of apo-neocarzinostatin from *Streptomyces carzinostaticus* determined by NMR-spectroscopy. *Eur. J. Biochem.* 203:505–511.

Adjadj, E., E. Quiniou, J. Mispelter, V. Favaudon, and J. M. Lhoste. 1992b. The seven-stranded $\beta$-barrel structure of apo-neocarzinostatin as compared to the immunoglobulin domain. *Biochimie.* 74:853–858.

Bava, K. A., M. M. Gromiha, H. Uedaira, K. Kitajima, and A. Sarai. 2004. ProTherm, version 4.0: thermodynamic database for proteins and mutants. *Nucleic Acids Res.* 32:D120–D121.

Chaires, J. B. 1997. Possible origin of differences between van't Hoff and calorimetric enthalpy estimates. *Biophys. Chem.* 64:15–23.

Chi, Y., T. K. Kumar, H. M. Wang, M. C. Ho, I. M. Chiu, and C. Yu. 2001. Thermodynamic characterization of the human acidic fibroblast growth factor: evidence for cold denaturation. *Biochemistry.* 40:7746–7753.

Chin, D.-H. 1999. Rejection by neocarzinostatin protein through charges rather than sizes. *Eur. J. Chem.* 5:1084–1090.

Dalessio, P. M., and I. J. Ropson. 2000. Beta-sheet proteins with nearly identical structures have different folding intermediates. *Biochemistry.* 39:860–871.

Edo, K., M. Mizugaki, Y. Koide, H. Seto, K. Furihata, N. Otake, and N. Ishida. 1985. The structure of neocarzinostatin chromophore possessing a novel bicyclo[7, 3, 0]dodecadiyne system. *Tetrahedron Lett.* 26:331–334.

Edo, K., K. Saito, Y. Akiyama-Murai, M. Mizugaki, Y. Koide, and N. Ishida. 1988. An antitumor polypeptide antibiotic neocarzinostatin: the mode of apoprotein-chromophore interaction. *J. Antibiot. (Tokyo).* 41: 554–562.

Feng, Y. Q., and S. G. Sligar. 1991. Effect of heme binding on the structure and stability of *Escherichia coli* apocytochrome $b_{562}$. *Biochemistry.* 30:10150–10155.

Gao, X. L. 1992. Three-dimensional solution structure of apo-neocarzinostatin. *J. Mol. Biol.* 225:125–135.

Gao, X. L., and W. Burkhart. 1991. Two-dimensional and three-dimensional proton NMR-studies of apo-neocarzinostatin. *Biochemistry.* 30:7730–7739.

Goldberg, I. H. 1991. Mechanism of neocarzinostatin action: role of DNA microstructure in determination of chemistry of bistranded oxidative damage. *Acc. Chem. Res.* 24:191–198.

Goldberg, I. H., and L. S. Kappen. 1995. Neocarzinostatin: chemical and biological basis of oxidative DNA damage. *In* Enediyne Antibiotics as Antitumor Agents. D.B. Borders and T.W. Doyle, editors. Marcel Dekker, Hong Kong.

Greish, K., J. Fang, T. Inutsuka, A. Nagamitsu, and H. Maeda. 2003. Macromolecular therapeutics: advantages and prospects with special emphasis on solid tumour targeting. *Clin. Pharmacokinet.* 42:1089–1105.

Heyd, B., G. Lerat, E. Adjadj, P. Minard, and M. Desmadril. 2000. Reinvestigation of the proteolytic activity of neocarzinostatin. *J. Bacteriol.* 182:1812–1818.

Ishida, N., K. Miyazaki, K. Kumagai, and M. Rikimaru. 1965. Neocarzinostatin an antitumor antibiotic of high molecular weight—isolation physicochemical properties and biological activities. *Antibiot. J.* 18: 68–76.

Jung, G., and W. Kohnlein. 1981. Neocarzinostatin: controlled release of chromophore and its interaction with DNA. *Biochem. Biophys. Res. Commun.* 98:176–183.

Kappen, L. S., and I. H. Goldberg. 1980. Stabilization of neocarzinostatin nonprotein chromophore activity by interaction with apoprotein and with HeLa cells. *Biochemistry.* 19:4786–4790.

Kim, K. H., B. M. Kwon, A. G. Myers, and D. C. Rees. 1993. Crystal structure of neocarzinostatin, an antitumor protein-chromophore complex. *Science.* 262:1042–1046.

Koblan, K. S., and G. K. Ackers. 1992. Site-specific enthalpic regulation of DNA transcription at bacteriophage $\lambda$ $O_R$. *Biochemistry.* 31:57–65.

Kumar, T. K. S., G. Jayaraman, C. S. Lee, T. Sivaraman, W. Y. Lin, and C. Yu. 1995. Identification of ''molten globule''-like state in all $\beta$-sheet protein. *Biochem. Biophys. Res. Commun.* 207:536–543.

Kuroda, Y., S. Kidokoro, and A. Wada. 1992. Thermodynamic characterization of cytochrome *c* at low pH. Observation of the molten globule state and of the cold denaturation process. *J. Mol. Biol.* 223: 1139–1153.

Liu, Y., and J. M. Sturtevant. 1995. Significant discrepancies between van't Hoff and calorimetric enthalpies. II. *Protein Sci.* 4:2559–2561.

Maeda, H. 2001. SMANCS and polymer-conjugated macromolecular drugs: advantages in cancer chemotherapy. *Adv. Drug Deliv. Rev.* 46: 169–185.

Manyusa, S., and D. Whitford. 1999. Defining folding and unfolding reactions of apocytochrome $b_5$ using equilibrium and kinetic fluorescence measurements. *Biochemistry.* 38:9533–9540.

Meersman, F., L. Smeller, and K. Heremans. 2002. Comparative Fourier transform infrared spectroscopy study of cold-, pressure-, and heat-induced unfolding and aggregation of myoglobin. *Biophys. J.* 82:2635–2644.

Napier, M. A., B. Holmquist, D. J. Strydom, and I. H. Goldberg. 1979. Neocarzinostatin—spectral characterization and separation of a non-protein chromophore. *Biochem. Biophys. Res. Commun.* 89:635–642.

Napier, M. A., B. Holmquist, D. J. Strydom, and I. H. Goldberg. 1981. Neocarzinostatin chromophore: purification of the major active form and characterization of its spectral and biological properties. *Biochemistry.* 20:5602–5608.

Napier, M. A., L. S. Kappen, and I. H. Goldberg. 1980. Effect of nonprotein chromophore removal on neocarzinostatin action. *Biochemistry.* 19: 1767–1773.

Otzen, D. E., and M. Oliveberg. 2004. Correspondence between anomalous *m*- and $\Delta C_p$-values in protein folding. *Protein Sci.* 13:3253–3263.

Pace, C. N., G. R. Grimsley, S. T. Thomas, and G. I. Makhatadze. 1999. Heat capacity change for ribonuclease A folding. *Protein Sci.* 8:1500–1504.

Pace, C. N., and C. Tanford. 1968. Thermodynamics of the unfolding of $\beta$-lactoglobulin A in aqueous urea solutions between 5 and 55°. *Biochemistry.* 7:198–208.

Pfeil, W. 1993. Thermodynamics of apocytochrome $b_5$ unfolding. *Protein Sci.* 2:1497–1501.

Povirk, L. F., N. Dattagupta, B. C. Warf, and I. H. Goldberg. 1981. Neocarzinostatin chromophore binds to deoxyribonucleic acid by intercalation. *Biochemistry.* 20:4007–4014.

Povirk, L. F., and I. H. Goldberg. 1980. Binding of the nonprotein chromophore of neocarzinostatin to deoxyribonucleic acid. *Biochemistry.* 19:4773–4780.

Privalov, P. L. 1990. Cold denaturation of proteins. *Crit. Rev. Biochem. Mol. Biol.* 25:281–305.

Privalov, P. L., V. Griko Yu, S. Venyaminov, and V. P. Kutyshenko. 1986. Cold denaturation of myoglobin. *J. Mol. Biol.* 190:487–498.

Privalov, P. L., and G. I. Makhatadze. 1993. Contribution of hydration to protein folding thermodynamics. II. The entropy and Gibbs energy of hydration. *J. Mol. Biol.* 232:660–679.

Remerowski, M. L., S. J. Glaser, L. C. Sieker, T. S. Samy, and G. P. Drobny. 1990. Sequential $^1$H NMR assignments and secondary structure of aponeocarzinostatin in solution. *Biochemistry.* 29:8401–8409.

Robinson, C. R., Y. Liu, R. O'Brien, S. G. Sligar, and J. M. Sturtevant. 1998. A differential scanning calorimetric study of the thermal unfolding of apo- and holo-cytochrome $b_{562}$. *Protein Sci.* 7:961–965.

Russell, B. S., and K. L. Bren. 2002. Denaturant dependence of equilibrium unfolding intermediates and denatured state structure of Horse ferricytochrome *c*. *J. Biol. Inorg. Chem.* 7:909–916.

Samuel, D., T. K. S. Kumar, T. Srimathi, H. Hsieh, and C. Yu. 2000. Identification and characterization of an equilibrium intermediate in the unfolding pathway of an all $\beta$-barrel protein. *J. Biol. Chem.* 275:34968–34975.

Santoro, M. M., and D. W. Bolen. 1988. Unfolding free energy changes determined by the linear extrapolation method. I. Unfolding of phenylmethanesulfonyl $\alpha$-chymotrypsin using different denaturants. *Biochemistry.* 27:8063–8068.

Santoro, M. M., and D. W. Bolen. 1992. A test of the linear extrapolation of unfolding free energy changes over an extended denaturant concentration range. *Biochemistry.* 31:4901–4907.

Schellman, J. A. 1978. Solvent denaturation. *Biopolymers.* 17:1305–1322.

Schellman, J. A. 1987. The thermodynamic stability of proteins. *Annu. Rev. Biophys. Biophys. Chem.* 16:115–137.

Shen, B., W. Liu, and K. Nonaka. 2003. Enediyne natural products: biosynthesis and prospect towards engineering novel antitumor agents. *Curr. Med. Chem.* 10:2317–2325.

Shortle, D. R. 1996. Structural analysis of non-native states of proteins by NMR methods. *Curr. Opin. Struct. Biol.* 6:24–30.

Sieker, L. C., L. H. Jensen, and T. S. Ananthasamy. 1976. Neocarzinostatin antitumor protein: a preliminary x-ray diffraction study. *Biochem. Biophys. Res. Commun.* 68:358–362.

Sivaraman, T., T. K. S. Kumar, G. Jayaraman, C. C. Han, and C. Yu. 1997. Characterization of a partially structured state in an all-$\beta$-sheet protein. *Biochem. J.* 321:457–464.

Sreerama, N., and R. W. Woody. 2004. Computation and analysis of protein circular dichroism spectra. *Methods Enzymol.* 383:318–351.

Sudhahar, G. C. P., K. Balamurugan, and D.-H. Chin. 2000. Release of the neocarzinostatin chromophore from the holoprotein does not require major conformational change of the tertiary and secondary structures induced by trifluoroethanol. *J. Biol. Chem.* 275:39900–39906.

Swint, L., and A. D. Robertson. 1993. Thermodynamics of unfolding for turkey ovomucoid third domain: thermal and chemical denaturation. *Protein Sci.* 2:2037–2049.

Tanaka, T., M. Hirama, K. Fujita, S. Imajo, and M. Ishiguro. 1993. Solution structure of the antitumor antibiotic neocarzinostatin; a chromophore protein complex. *J. Chem. Soc. Chem. Commun.*1205–1207.

Tanford, C. 1970. Protein denaturation. C. Theoretical models for the mechanism of denaturation. *Adv. Protein Chem.* 24:1–95.

Teplyakov, A., G. Obmolova, K. Wilson, and K. Kuromizu. 1993. Crystal-structure of apo-neocarzinostatin at 0.15-nm resolution. *Eur. J. Biochem.* 213:737–741.

Tsai, C. J., J. V. Maizel, Jr., and R. Nussinov. 2002. The hydrophobic effect: a new insight from cold denaturation and a two-state water structure. *Crit. Rev. Biochem. Mol. Biol.* 37:55–69.

Woody, R. W. 2004. Circular dichroism of protein-folding intermediates. *Methods Enzymol.* 380:242–285.

Xi, Z., and I. H. Goldberg. 1999. DNA-damaging enediyne compounds. *In* Comprehensive Natural Products Chemistry. D.S. Barton and K. Nakanishi, editors. Elsevier, New York. 553–592.