A New Model for Ligand Release

ROLE OF SIDE CHAIN IN GATING THE ENEDIYNE ANTIBIOTIC*

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Antitumor antibiotic chromoproteins such as neocarzinostatin involve a labile toxin that is tightly bound by a protective protein with very high affinity but must also be freed to exert its function. Contrary to the prevalent concept of ligand release, we established that toxin release from neocarzinostatin requires no major backbone conformational changes. We report, herein, that subtile changes in the side chains of specific amino acid residues are adequate to gate the release of chromophore. A recombinant wild type aponeocarzinostatin and its variants mutated around the opening of the chromophore binding cleft are employed to identify specific side chains likely to affect chromophore release. Preliminary, biophysical characterization of mutant apoproteins by circular dichroism and thermal denaturation indicate that the fundamental biophysical characterization of mutant apoproteins by circular

The extent of DNA cleavage in vitro corresponds well to the rate of chromophore release. The results provide the first clear-cut indication of how toxin release can be controlled by a specific side chain of a carrier protein.

The search for stable and nontoxic carrier-based drug delivery systems is a new trend in the recently emerging field of ligand-targeted therapeutics (1). The protein-mediated ligand-selective and high affinity carriers offer a promising strategy in drug targeting and controlled delivery of small therapeutic chemical agents (2). In light of the rapid advancements, understanding the naturally ingenious ways by which the bioactive ligands are released and controlled has been the subject of a few examples; see Refs. 6–8 for reviews and data base).

We anticipate that the possible candidates responsible for gating the chromophore release should satisfy two criteria. First, they must reside on the surface rather than the interior of the protein. Second, they are likely to be located in the opening of the binding cleft rather than being distributed in other parts of the surface. Physiological release of a noncovalently bound ligand from a protein complex is often triggered by a specific contact with some trans-acting agents in a cell. Such contact, if present, is more likely to occur on the solvent-exposed surface than the interior. Next, when backbone conformation remains unchanged, association or dissociation of a ligand must be through the opening of the binding cleft. Fig. 1 illustrates an aqueous model of holoNCS (20) adapted from the crystal structure (pdb.1nco.ent) (see supplementary Fig. S1 on line for the stereo view of holoNCS complex). The NCS–C is clamped in between two β-hairpin loops, designated as Loop 1 (between strands 7 and 8) and Loop 2 (between strands 9 and 10). NMR studies on
of the labile chromophore, and the integrity of the NCS-C after reconstitution was examined by HPLC analysis.

*pCAL-n-EK-apoNCS Construct*—The genomic DNA template was isolated from vegetative cultures of *S. carzinostaticus* (ATCC 15944). The sequence of the *ncsA* gene (GenBank^TM^, National Institutes of Health genetic sequence data base [gi420324]) (27), which encodes the 147-amino acid pre-apoNCS with a signal peptide at the N terminus, was used for primer design. The native gene of the 113-amino acid apoNCS was amplified by PCR with the following primers: forward, 5'-GACGACGACAAAGCCGGCCGCACGCTACGGT-3'; and reverse, 5'-GGAACAGACCCGTCGGCGAGGATCCTCCGATCA-3'. The 12-nucleotide upstream and 13-nucleotide downstream flanking sequences were included for ligation-independent cloning in T7/lac promoter-based *Escherichia coli* expression vector, pCAL-n-EK (Stratagene, La Jolla, CA). The vector (50 ng), after it is digested with *Eam*III and gel-purified, was incubated at 72 °C for 10 min in a solution containing 1 mM dTTP and *Pfu* DNA polymerase (ligation-independent cloning kit; Stratagene, La Jolla, CA) to produce 12- and 13-nucleotide ligation-independent cloning single-stranded overhears. Similarly, 175 ng of the gel-purified PCR amplicons was treated with *Pfu* DNA polymerase in the presence of 1 mM dATP. The vector and insert were annealed at room temperature for 1 h of incubation, and the product was transformed into *E. coli* DH5*α*. The apoNCS gene was fused downstream to the sequence encoding the N-terminal calmodulin affinity peptide (CBP) tag that was combined with the EK cleavage site (28, 29). The inclusion of the ES site allowed complete removal of the CBP tag and generated apoNCS with the native N terminus (i.e. Ala^1^ in apoNCS). The DNA sequencing of PCR amplicons and pCAL-n-EK-apoNCS construct was performed twice on automated ABI prism sequencer model 310 (Applied Biosystems, CA) to verify the correctness of the sequence.

*Site-specific Mutant Constructs of apoNCS*—A PCR-based quick change mutagenesis method was applied to obtain site-specific mutant constructs of apoNCS. The codon GCC and CUG were used for the replacement to Ala and Leu, respectively, according to the codon usage of *E. coli* (30). Followed by PCR, DpnI was used to deplete the unmutagenized DNA. The mutant construct was transformed into *E. coli* DH5*α*, and the constructed plasmids were sequenced at least twice until the correct sequence was repeatedly confirmed.

*Expression and Purification of Recombinant apoNCS Proteins*—The pCAL-n-EK expression construct of native or mutated apoNCSs was transformed into *E. coli* BL21 Codon Plus (Stratagene, La Jolla, CA). The expression of CBP-apoNCS fusion protein was induced by 0.2 mM isopropyl β-D-thiogalactopyranoside at 30 °C for 5 h. After ultrasonication of the isopropyl β-D-thiogalactopyranoside-induced *E. coli* cells, the CBP-apoNCS fusion protein was found in soluble fraction of the cell extract. The CBP-apoNCS fusion protein was purified using calmodulin affinity resin (Stratagene, La Jolla, CA) (Stratagene, La Jolla, CA) (28, 29). The protein was subsequently desalted and concentrated through Amicon or Centricron cellulose membrane (MWCO: 3000) (Millipore, Bedford, MA). The quantity of the recovered CBP-apoNCS fusion protein was evaluated by UV spectrophotometry. The extinction coefficient of the fusion protein at 278 nm, ε_{278} = 21,400 M^{-1} cm^{-1}, was estimated from the reported value for apoNCS, ε_{278} = 14,400 M^{-1} cm^{-1} (31, 32), and the calculated value for the CBP tag protein, ε_{278} = 7000 M^{-1} cm^{-1} (ProtParam) (33). For each mg of the CBP-apoNCS fusion protein, 1 unit of EK (Invitrogen, CA, or GenScript, Piscataway, NJ) was added, and the solution was incubated at room temperature for several days. When the enzymatic cleaving of the CBP tag was more than 90% complete (checked by SDS-PAGE), the mixture was separated by DEAE-Sepha-
rose (Fast Flow) resin (Amersham Biosciences AB, Uppsala, Sweden) with a linear gradient of 0—400 mM NaCl. The final protein product was collected after dialysis against water or filtration through Amicon or Centricon cellulose membrane (MWCO: 3000). The purified protein was analyzed by SDS-PAGE and HPLC. The averaged purity was greater than 95%. The authenticity of each protein was verified by mass spectrometry and disulfide linkage test, as described below. The final yield of recombinant apoNCS and mutants was at a range of 0.25—4 mg/liter of LB culture. Aggregation was frequently observed during purification of wild type (WT) recombinant apoNCS and mutants. This caused low yield from some mutants. For instance, the average yield of D79A was only 0.25 mg/liter of LB culture. Among all mutants purified for the study, F78L showed the highest yield (4 mg/liter of LB culture).

Expression and Purification of 15N-Labeled Proteins—Uniform 15N labeling was achieved by growing the host bacteria, E. coli BL21 Codon Plus, with vitamin B1 in M9 minimal medium containing 0.25 g/liter of 15NH4Cl (Cambridge Isotope Laboratories, Inc., MA) as the sole nitrogen source. The purification of the labeled apoNCS and mutants was carried out following the same procedure as described above. The yield was about half of that of the corresponding unlabeled protein. Changing ammonium chloride from 0.50 to 0.125 g/liter of medium did not significantly reduce or improve the protein yield.

Mass Spectrometry—All of the mass spectrometries were done on a Finnigan LCQ mass spectrometry detector (Thermo Electron, San Jose, CA) equipped with atmospheric pressure ionization source using electrospray ionization (+)-charge mode. The protein samples were diluted in 30% acetonitrile containing 0.1% trifluoroacetic acid for analysis.

NMR Spectroscopy—One-dimensional 1H NMR spectra of unlabeled WT apoNCS and mutants were recorded at room temperature on a Varian 600-MHz NMR spectrometer (Palo Alto, CA). The two-dimensional 15N-1H heteronuclear single quantum coherence (HSQC) NMR spectra were recorded on a Bruker DMX 600-MHz spectrometer (Rheinstetten, Germany) at 25 °C. The NMR samples were prepared by dissolving lyophilized protein in 20 mM sodium phosphate, pH 7.0, in 10% D2O, 90% H2O. The cross-peaks for WT apoNCS were identified based on the reported assignments (25). The cross-peak of Tyr32, which is in a tyrosine corner-like motif and is considered to contribute stability, was about half of that of the corresponding unlabeled protein. Changing ammonium chloride from 0.50 to 0.125 g/liter of medium did not significantly reduce or improve the protein yield.

CD Spectroscopy—All of the CD measurements were carried out on a Jasco J-715 spectropolarimeter (Tokyo, Japan) equipped with a circulating water bath (Neslab, model RTE-140) (Portsmouth, NH). For apoNCS and its mutants, a 200-μl protein solution in 20 mM sodium phosphate, pH 7.0, with a concentration level of 10—15 μM for far UV CD and 50 μM for near UV CD, was used for measurement. The spectrum was recorded at 25 °C using a temperature controlled water-jacketed cell with 0.1-cm-path length at a scan speed of 20 nm/min. For reconstituted WT and mutated holoNCSs, a 10 μM sample in 20 mM ammonium acetate, pH 4.0, was used for measurement. Because of the instability of holoNCSs, the scan speed was increased to 100 nm/min to reduce time. All of the spectra were accumulated minimum five times and are corrected for the respective buffer blanks. The data are expressed as the mean residue ellipticity for both apoNCS and holoNCS (19).

Fluorescence Spectroscopy—The fluorescent changes from a holoNCS sample in kinetic release study were monitored by a SLM Aminco Bowman Series II luminescence spectrometer (SLM Aminco Bowman, Urbana, IL). The sample temperature was controlled at 25 °C by a thermostatic cell holder equipped with a refrigerated thermostatic bath circulator. The sample of volume 150 μl in a 3-mm square cell was used. A single point acquisition of emission data at 440 nm was collected once per 30 s by excitation at 340 nm. To avoid the degradation of holoNCSs by excessive exposure to the light source, the light shutter was controlled by modification of time scan mode using an in-house written macro language commands so that it can be closed in between data collections. In a 100-min monitoring, exposure of NCS to light was minimized to 2.33 min. A rapid kinetics setup was used for fast releasing mutant F78L, where the shutter control was suspended for the initial 3 min to collect data once per 5 s. After that, the shutter control was enabled as described.

Disulfide Linkage Test—A facile disulfide evaluation procedure was developed for checking the integrality of the produced recombinant apoNCS and its mutants. To 100 μl of solution of 10 μM apoNCS protein in 0.1 M sodium phosphate, pH 8.0, guanidine hydrochloride was added to a final concentration of 4 M. After mixing, 80-fold molar excess of iodoacetamide over apoNCS (20-fold excess/Cys residue) was added at 37 °C for 2 h. Under this treatment, alkylation can occur on any un-oxidized Cys residues in apoNCS. The protein sample was then desalted and analyzed by mass spectrometry.

Thermal-induced Denaturation—The thermal-induced denaturation of 50 μM of WT apoNCS and mutants, in 10 mM sodium phosphate buffer, pH 7.0, was monitored by CD spectroscopy at 224 nm from 25—91 °C with 3 °C increments. The temperature was controlled by a microprocessor and a temperature sensor. The equilibrium time was 15 min, and the ellipticity was recorded for 30 s at each temperature setting.

Kinetic Study on the Release of NCS-C—The rate of NCS-C release from natural, reconstituted WT or mutated holoNCSs was determined using the method previously published (19). The reconstituted holoNCSs (10 μM) were prepared in 100 mM Tris-HCl, pH 7.0, and the release of NCS-C was monitored by fluorescence spectroscopy at 25 °C after the addition of 5 mM GSH. The final reading of the emission was obtained after the sample was treated with 80% isopropanol (to complete the release of NCS-C). The release kinetics was also independently examined by HPLC analyses. The samples (0.5 nmol), prepared as above, were drawn at different time points and were analyzed by reverse phase HPLC. The quantity of the intact NCS-C and GSH-induced adduct, which represent population of the protein-bound and released species, respectively, were estimated using the method described (35).

Kinetic Release Data Processing Information—The rate of release is expressed as

$$\frac{d[\text{holoNCS}]}{dt} = -k_{\text{obs}}[\text{holoNCS}] = -k_1[\text{holoNCS}]$$

(Eq. 1)

where $k_{obs}$ and $n$ are the observed rate constant and reaction order for the rate of disappearance of holoNCSs, respectively, and $k_1$ is the first order rate constant after approximation. However, the release rate decreased gradually with time when ratio of apoNCS to NCS-C increased with progressing lose of the intact NCS-C. For consistency, all of the rate constants were calculated from the initial 10% release that fit into a first order linear plot (the averaged $r = 0.97$ analyzed by the Kaleidagraph program (Synergy Software, Reading, PA)).

holoNCS-induced DNA Damage—The DNA cleavage ability of each chromoprotein complex was assessed by the proportion of conversion from form I DNA (supercoiled) to form II (relaxed; caused by single strand breaks) and form III (linear; caused by double-stranded breaks). The supercoiled pBR322 DNA was isolated from E. coli using Mini-Prep plasmid isolation kit (Qiagen Inc.). A 16-μl DNA drug reaction mixture was prepared by mixing 5 μM (final concentration) of freshly reconstituted holoNCS, 5 mM GSH, 100 mM Tris-HCl, pH 7.0, and 40 ng/μl of DNA.
profiles shown were obtained from recombinant WT (---), D79A (--), S98A (--), and D99A (--). The thermal denaturation, monitored by ellipticity changes at 224 nm, indicates compatible stability of WT and mutated apoNCSs. The profiles shown were obtained from: recombinant WT (---), F76A (--), L77A (--), F78A (--), F78L (--), D79A (--), S98A (--), and D99A (--).}

HPLC Analyses—When release kinetics was followed by HPLC, the amount of the protein bound (intact form) and the released NCS-C (inactivated by GSH into an adduct) were analyzed by a Waters Millennium HPLC equipped with a 600E solvent delivery system, a 996 photodiode array detector, and a Waters 474 or a Jasco FP-1520 fluorescence detector using the described method (19, 35). The purity of WT and mutated apoNCSs (0.5–1 nmol) were examined using a gradient that starts with a sharp increase from 0 to 30% of CH3CN (containing 0.1% trifluoroacetic acid) in 2 min, followed by a shallow linear increase to 80% in 16028 min.

RESULTS

Cloning and Characterization of Recombinant apoNCS Proteins—After a long attempt, we successfully isolated the native gene of the 113-amino acid apoNCS from S. carzinostaticus. The use of pCAL-n-EK allowed expression of a fusion protein from which apoNCS could be retrieved by a proteolytic removal of its tag by EK. The recombinant apoNCS thus obtained possessed the native termini without any extraneous residues. After DEAE-Sepharose ion exchange chromatography, the purified recombinant apoNCS and mutants showed a single band corresponding to the band of the natural apoNCS in SDS-PAGE. A single peak was observed by HPLC analysis, indicating the homogeneity of the purified proteins. The mono isotopic mass number of the recombinant WT apoNCS is shown as 11085.2. The mono isotopic mass numbers of all mutants and 15N-labeled proteins are also in good agreement with their theoretical molecular weights.

Disulfide Assay—To avoid any possible artifacts, it was crucial to examine whether all disulfide linkages were properly formed in the produced proteins. This assay was specifically developed for apoNCS, and the sensitivity and validity have been evaluated and confirmed. As evidenced by mass spectrometry measurement, no alkylated species was observed for all purified proteins obtained in the current investigation. The results demonstrate the absence of free —SH in cysteine residues and indicate that the WT and all mutated apoNCSs contain two disulfides as the natural one does.

CD Spectra of WT and Mutated apoNCS—The structural characterization of WT and mutant apoNCSs was carried out by CD. The far UV CD spectrum of the recombinant WT apoNCS (Fig. 2A) revealed a predominant β-sheet structure. The profile is consistent with that reported for the native apoNCS (19, 23, 36, 37). The far UV CD spectra of F78A, F78L, D79A, and S98A mutants also closely resembled that of WT apoNCS. Only a slight deviation was observed in the spectra of F76A and D99A mutants. Overall, the characteristic features of the far UV CD of WT apoNCS were retained in all of the mutants, suggesting WT-like secondary structures existing in all the mutants.

Similarly, the near UV CD spectrum (Fig. 2A, inset) of recombinant WT apoNCS exhibited the same broad negative signal around 271 nm as observed for native apoNCS. All of the mutants exhibited a similar broad negative band with similar intensity in the mean residue ellipticity. The results reveal that the aromatic side chains of Tyr32, Trp39, and Trp83 experience the same steady and compact tertiary environment as seen in the native apoNCS.

Thermal Denaturation Experiments—To compare the conformational stability with that of natural apoNCS, thermal denaturation of recombinant WT and all of the mutated apoNCSs was studied by CD spectroscopy. The Tm of the recombinant WT apoNCS is shown as 64.0 °C, which is consistent with the Tm (63.5 °C) of the natural apoNCS measured by CD (37) but is slightly lower than the Tm (68 °C) measured by differential scanning calorimetry (38). The denaturation curves (Fig. 2C) show that both side limbs (25–52 and 73–91 °C) and the rapid unfolding phase (52–73 °C) are nearly the same for all mutants. The measured Tm values are 63.5, 65.4, 65.8, 65.7, 60.4, 64.5, and 63.3 °C for F76A, L77A, F78A, F78L, D79A, S98A, and D99A mutants, respectively.

\[3\] C.-J. Tseng and D.-H. Chin, unpublished data.
The observed $T_m$ values for L77A and D79A are slightly smaller (6 and 4 °C) than that of WT apoNCS. This is not surprising, considering the well known fact that charged residues at surface can stabilize proteins (39). Likewise, replacing the large hydrophobic residues with small ones can also reduce stability (39, 40).

**CD Spectra of Reconstituted WT and Mutated holoNCSs**—The CD spectra of apoNCS, unbound NCS-C and holoNCS are distinctively different (32, 36, 41). To inspect the binding properties between mutated apoNCS and NCS-C, the CD spectra of freshly reconstituted complexes were recorded at pH 4, where NCS-C is more stable than at pH 7. The spectrum of recombinant WT holoNCS showed a characteristic and broad positive signal around 255 nm (Fig. 2B), which is consistent with that of its natural counterpart, but is distinct from that of apoNCS. The prominent intensity of this signal was sensitive to the binding molar ratio of NCS-C to apoNCS (14). Because NCS-C is labile only when released, this fact implies that the release rate should also rely on the stability of NCS. The role of Phe78 side chain in gating the release of NCS-C. The observed $T_m$ values for L77A and D79A are slightly smaller (6 and 4 °C) than that of WT apoNCS. This is not surprising, considering the well known fact that charged residues at surface can stabilize proteins (39). Likewise, replacing the large hydrophobic residues with small ones can also reduce stability (39, 40).

**Kinetic Release Monitored by Fluorescence**—GSH failed to interact with the protein-bound NCS-C (20) but could quickly inactivate freed NCS-C by GSH. The profiles shown were obtained from holoNCSs made of WT (●), F76A (○), L77A (□), F78A (●), F78L (●), D79A (●), S98A (▲), and D99A (▲). All of the other mutants exhibit similar profiles as WT (data not shown). The rate constant ($k$) of the chromophore release from the natural, recombinant WT, and mutated holoNCSs was calculated from the initial 10% release that fit into a first order linear plot.

**TABLE 1**

| Sources of holoNCS chromoproteins | $k$ for the initial 10% chromophore release |
|-----------------------------------|--------------------------------------|
| Natural holoNCS from *S. carzinostaticus* | 0.20 ± 0.01 |
| Reconstituted holoNCS chromoproteins |                                       |
| From recombinant WT | 0.19 ± 0.01 |
| From F76A mutant | 0.16 ± 0.02 |
| From L77A mutant | 0.18 ± 0.02 |
| From F78A mutant | 1.32 ± 0.18 |
| From F78L mutant | 16.5 ± 2.9 |
| From D79A mutant | 0.18 ± 0.04 |
| From S98A mutant | 0.15 ± 0.03 |
| From D99A mutant | 0.21 ± 0.04 |

The stability of NCS is known to be influenced by its concentration and molar ratio of NCS-C to apoNCS (14). Because NCS-C is labile only when released, this fact implies that the release rate should also rely on these factors. Because the released NCS-C is continuously inactivated by excess GSH in solution, accumulation of free apoNCS molecules causes molar ratio of apoNCS to NCS-C to increase and thus gradually slows down the release rate. For the purpose of comparison, all of the rate constants herein were obtained from 10 μM freshly reconstituted chromoproteins and were calculated from the initial 10% NCS-C release that fit into a first order linear plot (Table 1). Except for Phe78 mutants, all of the other mutants showed release rates comparable with that of WT holoNCS ($k = 0.19$ h$^{-1}$), with variations in rate no larger than 20%. Some mutants, such as S98A, even showed a slightly suppressed release rate than WT. However, an amazingly large increase of nearly 8- and 9-fold in release rate was constantly obtained in mutants F78A and F78L, respectively.

**Time Course Analyses of Remaining Bound NCS-C by HPLC**—The reverse phase chromatography resulted in good separation between the intact NCS-C (those remained as protein-bound prior to analysis) and the GSH-induced adduct (those released and inactivated by excess...
GSH. Thus, the separation and quantification of the intact protein-bound NCS-C by HPLC-based analysis became a straightforward approach for detecting the progressive release of the chromophore (Fig. 3B). The results from the recombinant WT holoNCS are in good agreement with that from natural holoNCS secreted by S. carzinostaticus (data not shown). When compared with WT holoNCS, the Phe⁷⁸ mutants show significant decrease of intact NCS-C. As a whole, the HPLC analyses showed a trend in releasing behavior similar to that obtained from kinetic studies monitored by continuous changes in fluorescence emission. In addition, the HPLC experiments also served in analyzing the cyclization products of NCS-C in the presence of activating thiols (35). The HPLC analysis of the kinetic reactions in the presence of GSH confirmed the absence of the protein-assisted cyclization products of NCS-C in all the reconstituted mutant holoNCS complexes, including the fast releasing Phe⁷⁸ mutants. All of the spectroscopic and chromatographic characteristics of the observed GSH-induced cyclized product correspond with the reaction of the unbound NCS-C (20, 35). The above results ensure proper cyclization of NCS-C by GSH following its release and further confirm the validity of the kinetic release measurements.

DNA Cleavage Experiments—In NCS-mediated DNA cleavage reaction, the chromophore release is the first step in the action of NCS (14). The extent of NCS-induced DNA cleavage thus reflects the rate of NCS-C release, provided such release is the rate-determining step. We performed NCS-DNA reaction employing freshly reconstituted WT and mutated holoNCSs. Fig. 3C shows the agarose gel electrophoretic profile of plasmid pBR322 cleavage after exposure to 5 μM of each reconstituted holoNCS at 16 °C. The results show that F78A and F78L holoNCS mutants significantly enhance the DNA cleavage activities. In product from reaction with F78A, the quantity of full-length DNA was substantially reduced and is seen as a smear below the full-length DNA. With F78L, no full-length DNA is visible, and the size of the cleaved DNA is below 500 bp, as indicated by the arrow in Fig. 3C. The DNA cleavage activity of some mutants such as S98A was slightly suppressed, in comparison with that of WT. The results correspond well with the observed chromophore release rate as followed by both fluorescence and HPLC. It should be noted that DNA cleavage reactions at low concentrations (20 and 40 nM) of NCS, either at 25 °C or 0 °C, consistently showed no difference in the cleavage efficiency among WT and all mutated holoNCSs. When the concentration of holoNCS is low enough, where the release is fast and is no longer the rate-determining step, the extent of DNA cleavage would not depend on the release rate.

Two-dimensional ¹⁵N-¹H HSQC NMR Experiments—Two-dimensional ¹⁵N-¹H HSQC NMR spectroscopy enables one to closely inspect the changes in binding pocket structure, in particular in those mutants that show enhanced chromophore release. We therefore carried out NMR experiments for WT and mutant apoNCSs under conditions similar to that of release experiments. The ¹⁵N and ¹H chemical shifts of apoNCS at pH 7 showed good agreement with those at pH 5 (25), as predicted by the fact that apoNCS is stable in a wide pH range of 4–10 (37). Close comparisons among the ¹⁵N-¹H HSQC spectra obtained from WT, F78A, and F78L apoNCS are shown in Fig. 4. The results reveal that most cross-peaks remain dispersed and unperturbed by mutations along the entire region. The chemical shift of residues at both terminals, which are, in general, more dynamic than other portions, also appears to be unchanged upon mutation. The Thr⁴ residue at the N terminus and Asn³¹³ at the C terminus shown in Fig. 4 are such examples. A number of residues, including Gly³⁵, Trp⁹⁹, Leu⁴⁵, Pro⁴⁹, Phe⁵², Phe⁷⁶, Leu⁷⁷, Gly⁹⁹, Gin⁹⁴, Val⁹⁵, Gly⁹⁶, Ser⁹⁸, Ala¹⁰¹, and Gly¹⁰² can form van der Waals’ contact or H-bond with NCS-C (18, 42). Among those, the residues that have close contacts with naphthoate group of NCS-C, namely, Phe⁵², Ser⁹⁸, Gly⁹⁶, Gin⁹⁴, and Trp⁹⁹, are particularly

![FIGURE 4. Evaluation of the binding structural integrity at the residue level on the fast releasing mutants of NCS. Two-dimensional ¹⁵N-¹H HSQC NMR spectra of WT and Phe⁷⁸ apoNCS mutants at 25 °C and pH 7 are compared with identical axis scales. The cross-peaks were assigned based on the reported chemical shifts (25). The cross-lines in the spectrum of WT, F78A, and F78L apoNCS show that residues involved in NCS-C binding are barely perturbed by mutations.](image-url)
important for binding (43). Fig. 4 also shows that the resonances from these binding residues remain mostly unchanged upon mutation at Phe78. There are a few perturbations observed in the residues neighboring the point mutation in both mutants. A small shift at the cross-peak of Gly80 in the F78A mutant is one example. Such changes are common phenomena (40) and are often not significant in changing the side chain packing in a mutant (44). The results of HSQC NMR experiments revealed that the overall binding backbone conformation of apoNCS is unchanged, despite mutation at Phe78. This excludes the possibility that the fast releasing caused by the mutation at the side chain of Phe78 results from the backbone conformational changes in the binding cleft.

**DISCUSSION**

The antineoplastic activity of NCS largely depends upon the release and availability of NCS-C (14, 45). The similarity between the structure of apoNCS and holoNCS (18) implies that a nearly "lock and key" model is applicable for the NCS-C association or dissociation occurring in the binding cleft. This feature allows NCS to be placed in a unique category, where major structural transition is not a prerequisite for ligand release (19).

When a protein-bound ligand is freed without a major preceding change in protein conformation, the ligand must escape through an existing "opening." The residues around the opening at the protein surface therefore play a major role in gating the release. In this study, we focus on mutation in NCS around the opening of the binding pocket, constituted mainly by two loops (Gly75-Phe76-Leu77-Phe78-Asp79) and Gly80 in Loop 1 and Ser98-Asp99-Ala100-Ala101-Gly102 in Loop 2 (Fig. 1). The small residues, such as Gly80, Ala100, Ala101, and Gly102, were excluded from the alanine-scanning mutagenesis employed in this study. Because all of the mutants except Phe78 show no significant effect, the inclusion of leucine replacement of Phe78 brings one more factor for our current investigation.

Although employing an *E. coli*-derived recombinant protein as a substitute for the natural NCS produced by *S. carzinostaticus*, we avoided bringing in any extraneous residues at the termini to dismiss any possible obscurity in protein conformation. Disulfide assay demonstrated that disulfide linkage is properly formed in all of the produced proteins. Structural comparison of WT and mutated apoNCSs indicated that they are similar in secondary and tertiary structures, as evidenced by the results of CD spectroscopy (Fig. 2A). Moreover, the closely matching 

**FIGURE 5.** A new model of drug release illustrated by the enediyne containing antibiotic chromoprotein NCS. The schematic surface view (pdb:1Yco.ent) of holoNCS (left) and apoNCS (right) shows the strategic bridging position of the benzene ring of Phe78 (shown in blue) in gating the release of NCS-C (shown in yellow). The flipping motion between the "open" and "closed" conformation of Phe78 could be triggered by a release stimulant.
Enediyne Chromophore Release from Neocarzinostatin

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