Rejection by Neocarzinostatin Protein through Charges Rather than Sizes

Der-Hang Chin*[a]

Abstract: Neocarzinostatin is a very potent antitumor antibiotic from *Streptomyces carzinostaticus.* It consists of a carrier apoprotein and an active enediyne chromophore. Thiols can induce irreversible inactivation as a result of cycloaromatization of the enediyne moiety and the protein is responsible for selecting thiols with which to react. While 2-mercaptoethanol can inactivate the protein-bound chromophore, glutathione is rejected by the protein. This selective inactivation has been suggested to be the basis of the self-protective functionality of the microorganisms that produce neocarzinostatin. We have used screening tests with various thiols and investigated the pH-dependency of the reaction and have thus clearly demonstrated that neocarzinostatin apoprotein rejects thiols by means of electrostatic repulsion of negative charges. In contrast to what one would generally predict from the lock-and-key model, we have found that size or shape exclusion does not have major effects. The acidic residues of the apoprotein, in particular, Asp 33, Asp 79, and Asp 99, are located near to the site of thiolate attack. The ionized carboxylate side chains of the amino acid residues repel the negatively charged glutathione by electrostatic forces. Our model resolves the long-standing puzzle of how the neocarzinostatin apoprotein is responsible for shielding the labile chromophore from attack by the most abundant eukaryote cellular thiol before the drug reaches the target DNA.

Keywords: apoproteins · chromophores · drug research · neocarzinostatin · protection · thiols

Introduction

Neocarzinostatin (NCS) is the first identified enediyne antitumor antibiotic[1] isolated from *Streptomyces carzinostaticus.*[2] Together with other recently identified enediynes,[3–9] it currently attracts extensive interest on account of its very high potency and structural novelty. NCS consists of a carrier apoprotein and a highly labile chromophore.[10] The naturally occurring acid protein antibiotic contains a single polypeptide[11] with 113 amino acid residues (Figure 1). The small enediyne chromophore (MW 659, Figure 2) is tightly and noncovalently bound to the protein. The drug's DNA-cleaving activities are only associated with its chromophore.[10, 12] The carrier protein plays an important role in the direction, protection, and regulation of its chromophore. Without protection from the apoprotein, the NCS chromophore would be easily inactivated by heat, high pH values, light, and, in particular, thiols.[10]

In the absence of the NCS apoprotein, the attack of a thiol[13] at C12 of the chromophore generates a diradical intermediate at C2 and C6 by cycloaromatization.[14] It subsequently abstracts two hydrogen atoms, either from DNA[15] or other sources[16, 17] to form a stable tetrahydroindacene-type of product, **1** (Figure 2).[14] Another distinctive thiol – drug adduct, **2**, was discovered under protein-bound and physiological conditions.[18, 19] The cyclized structure of **2** (Figure 2) has recently been revised.[20] Because formation of **2** only occurs inside the protein environment and is not able to abstract hydrogens from DNA,[21] it has been considered to be a major waste product of the drug.[19] Interestingly, such inactivation reactions of protein-bound drugs, in which a thiol is the only

Ala-Ala-Pro-Thr-Ala-Thr-Val-Thr-Pro-Ser-Ser-Gly-Leu-Ser-Asp^{15}-Gly-Thr-Val-Val-Lys-Val-Ala-Gly-Ala-Gly-Leu-Gln-Ala-Gly-Thr-Ala-Tyr-Asp^{33}-Val-Gly-Gln-Cys-Ala-Trp-Val-Asp^{41}-Thr-Gly-Val-Leu-Ala-Cys-Asn-Pro-Ala-Asp^{51}-Phe-Ser-Ser-Val-Thr-Ala-Asp^{58}-Ala-Asn-Gly-Ser-Ala-Ser-Thr-Ser-Leu-Thr-Val-Arg-Arg-Ser-Phe-Glu^{74}-Gly-Phe-Leu-Phe-Asp^{79}-Gly-Thr-Arg-Trp-Gly-Thr-Val-Asp^{87}-Cys-Thr-Thr-Ala-Ala-Cys-Gln-Val-Gly-Leu-Ser-Asp^{99}-Ala-Ala-Gly-Asn-Gly-Pro-Glu^{106}-Gly-Val-Ala-Ile-Ser-Phe-Asn

Figure 1. The primary structure of NCS apoprotein. Acidic residues are marked with boxes for clarity.

[a] Prof. D.-H. Chin
Department of Chemistry
National Changhua University of Education
Changhua 50058, Taiwan (Republic of China)
Fax: (+886) 47-211178 or 211190
E-mail: chdhchin@cc.ncue.edu.tw

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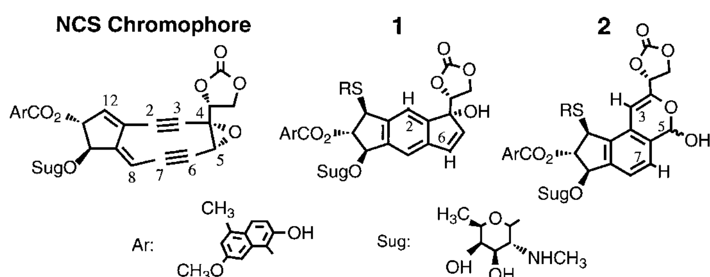

Figure 2. The NCS chromophore and its cycloaromatization products.

cofactor, was found to be dependent on the attacking thiol. While the addition of 2-mercaptoethanol (BME) causes inactivation of the drug, glutathione (GSH) is not able to induce such a reaction with holo-NCS.[21] In the absence of NCS apoprotein, the inactivation of the chromophore by cycloaromatization occurs rapidly with both thiols.

Many prokaryotes utilize small thiols in their metabolism. It is probably vitally important for the organism *Streptomyces carzinostaticus* to produce NCS apoprotein along with the biologically active chromophore. Coincidentally, the NCS apoprotein does permit small thiols, such as BME, to penetrate into the protein cavity and inactivate the chromophore. Such a fascinating character allows the microorganism that produces neocarzinostatin as a potent antibiotic to protect itself. In contrast to prokaryotes, eukaryotes typically employ GSH. NCS apoprotein is also ingeniously designed to shield the chromophore from attack by the most abundant cellular thiol GSH. Thus, the antibiotic produced enables the preservation of its activity before it reaches the cellular target DNA. Little is known about this long-standing and puzzling mechanism of the naturally occurring chromoprotein. The chemical basis of how two entirely different actions, that is either to permit proper thiols for inactivation or to reject certain thiols for preservation, can be concurrently served by the very same NCS apoprotein.

Abstract in Chinese: 新抑癌素是近期發現的強力抗腫瘤抗生素。它包含一個酸性蛋白，以及蛋白內具有活性的烯雙炔生色團。硫醇類能引發烯雙炔的環化作用，導致生色團失去活性，而蛋白具有把守關口，選擇硫醇種類的能力。２－乙醇硫醇能進入蛋白，引發生色團的環化，使這種強烈細胞毒素失去殺傷力，麩胺基硫－真核細胞中含量最多的硫醇類，卻被排斥在外，沒有反應。對製造新抑癌素的原核微生物而言，這種功能會產生奇妙的自我保護作用。同時，在攻擊核酸殺死細胞的過程中，蛋白又能保護生色團，不會先被細胞中的麩胺基硫破壞而失去活性。蛋白如何發揮這種神妙功能，一直是個謎，我們的研究，解開了長期懸而未解的疑惑。實驗結果顯示，電荷互斥是蛋白選擇的最重要機理，形狀及大小，反而不是蛋白選擇的重要因子，與一般推斷的鑰匙與鎖的模型不同。蛋白的酸性胺基酸殘基，尤其在三十三，七十九，及九十九位置的天門冬胺酸，座落在結合穴口附近，可能是保護生色團的主力，其上的負電荷，使生色團免於受到帶負電的麩胺基硫的侵襲。

Results and Discussion

Why and how the thiol-screening test was undertaken: Considering that the thiol is the only cofactor in the thiol-induced drug reactions, we decided to test the various thiols for their ability to deactivate the NCS chromophore. Thiol GSH (Figure 3) is noticeably larger than BME. Therefore, it is

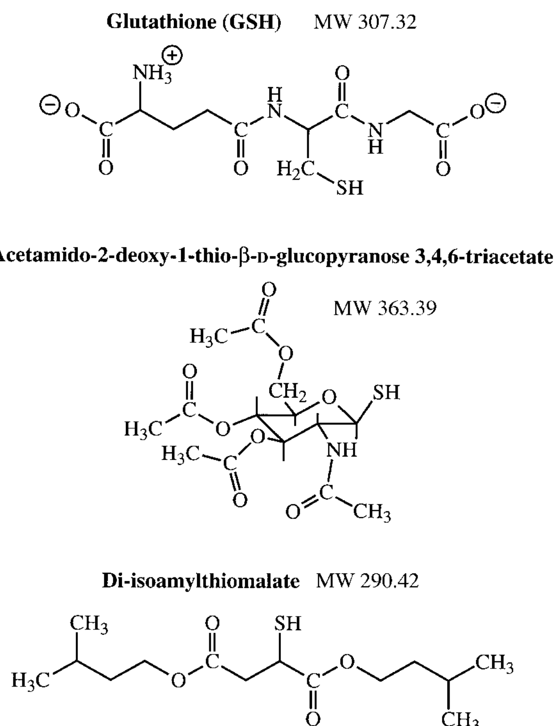

Figure 3. GSH and thiols of comparable size or shape.

reasonable to assume that the size of the thiol would be the main selective criterion of the NCS protein. In addition to the induced-fit theory as well as the enzyme-transition state complementarity, many proteins follow a lock-and-key model. This implies that only thiol molecules with the correct size and shape are chosen as a reactive cofactor by the NCS protein. Therefore, we systematically varied the structure and size of the thiol in a screening reaction with holo-NCS in order to reveal the mystic selective power of the NCS protein. The thiol-screening test, although tedious, can reveal the potential of each individual thiol to induce inactivation of holo-NCS. Analysis of the results should pinpoint the factors that control the selectivity of the apoprotein.

The cyclized drug products **1** and **2** from most thiols can be detected easily by their strong fluorescent emission. By using the spectroscopic and HPLC methods we have previously established,[22] the extent of the thiol-induced drug inactivation can be estimated by the quantitative analyses of the products **1** and **2** and of the unreacted NCS chromophore. A few thiols, in particular those with an ester group, were able to inactivate the NCS chromophore efficiently under protein-bound, aqueous conditions; however, the HPLC analysis failed to detect the fluorescent drug products. In these cases we estimated the extent of the NCS reaction solely by measuring the disappearance of the NCS chromophore.

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Although we have examined almost all the commercially available thiols, many nonpolar thiols were found to induce only poor reactions of holo-NCS. The reason for this may not lie in the protective effect of the NCS apoprotein, but rather the fact that most of the chromophore remained unmodified could also be the result of the low solubility of nonpolar thiols under aqueous conditions. In order to avoid miscounting such non-reactive systems, the results from nonpolar thiols were excluded from the analyses of the present screening test.

Effect of the size and shape of a thiol: The results of the thiol-screening test (Table 1) surprisingly show that size or shape of the applied thiol does not appear to be a major control factor for the selectivity of the NCS apoprotein. Some of the large thiols with molecular weights up to 360, such as 2-acetamido-2-deoxy-1-thio-β-D-glucopyranose 3,4,6-triacetate (Figure 3), were found to be able to penetrate into the NCS protein cluster to induce cycloaromatization of the drug under regular reaction conditions.[23] Some of the small thiols, even those with only a two-carbon chain, were found to have difficulties to induce the irreversible inactivation reaction. For instance, mercaptoacetic acid is not able to inactivate the protein-bound chromophore, and the drug virtually remains unmodified after a long period of incubation for up to 7 days at 0 °C

Table 1. Thiol-induced inactivation of the chromophore in holo-NCS.[a]

Name	Formula[b]	Result
positively charged thiols		
2-aminoethanethiol	$^+H_3NCH_2CH_2SH$	R_1[c]
(2-aminoethylisothiouronium) bromide	$H_2NC^+(=NH_2)NHCH_2CH_2SH$	R_1
L-cysteine methyl ester	$HSCH_2CH(NH_3)^+COOCH_3$	R_1
L-cysteine ethyl ester	$HSCH_2CH(NH_3)^+COOC_2H_5$	R_1
2-(butylamino)ethanethiol	$CH_3(CH_2)_3NH_2^+CH_2CH_2SH$	R_1
2-diethylaminoethanethiol	$(C_2H_5)_2NH^+CH_2CH_2SH$	R_1
2-dimethylaminoethanethiol	$(CH_3)_2NH^+CH_2CH_2SH$	R_1
neutral thiols		
2-mercaptoethanol (BME)	$HSCH_2CH_2OH$	R_1
dithiothreitol	$HSCH_2CH(OH)CH(OH)CH_2SH$	R_1
3-mercapto-1,2-propanediol	$HSCH_2CH(OH)CH_2OH$	R_1
methyl thioglycolate	$HSCH_2COOCH_3$	R_1
ethyl 2-mercaptoacetate	$HSCH_2COOC_2H_5$	R_1
1-mercapto-2-propanol	$CH_3CH(OH)CH_2SH$	R_1
methyl 3-mercaptopropionate	$HSCH_2CH_2COOCH_3$	R_1
3-mercapto-2-butanol	$CH_3CH(SH)CH(OH)CH_3$	R_1
2-acetamido-2-deoxy-1-thio-β-D-glucopyranose 3,4,6-triacetate	$HSCHCH(NHR)CH(OR)CH(OR)CHCH_2OR$ O $R = -COCH_3$	R_1
1,4-dithioerythritol	$HSCH_2CH(OH)CH(OH)CH_2SH$	R_1
trimethylolpropane tris-(3-mercaptopropionate)	$(HSCH_2CH_2CO_2CH_2)_3CC_2H_5$	R_2[d]
glycoldimercaptoacetate	$(HSCH_2COOCH_2)_2$	R_2
pentaerythritol tetrakis-(2-mercaptoacetate)	$(HSCH_2CO_2CH_2)_4C$	R_2
pentaerythritol tetrakis-(3-mercaptopropionate)	$(HSCH_2CH_2CO_2CH_2)_4C$	PR[e]
2-ethyl-2-(hydroxymethyl)-1,3-propanediol tris(3-mercaptopropionate)	$(HSCH_2CH_2CO_2CH_2)_3CC_2H_5$	PR
ethyl 3-mercapto-propionate	$HSCH_2CH_2COOC_2H_5$	R_2
butyl 3-mercaptopropionate	$HSCH_2CH_2COO(CH_2)_3CH_3$	R_2
di-isoamylthiomalate	$(CH_3)_2CHCH_2CH_2OOCCH_2CH\text{-}(SH)COOCH_2CH_2CH(CH_3)_2$	R_2
n-propyl 3-mercaptopropionate	$HS(CH_2)_2COO(CH_2)_2CH_3$	R_2
ethyl 2-mercaptopropionate	$CH_3CH(SH)COOC_2H_5$	PR
zwitterionic thiols		
Cys-Gly	$^+H_3NCH(CH_2SH)CONHCH_2COO^-$	PR
L-cysteine	$HSCH_2CH(NH_3)^+COO^-$	PR
negatively charged thiols		
glutathione (GSH)	$^-OOCCH(NH_3)^+CH_2CH_2CONH\text{-}CH(CH_2SH)CONHCH_2COO^-$	N[f]
3-mercaptopropionic acid	$HSCH_2CH_2COO^-$	N
2,3-dimercapto-1-propane sulfonic acid, sodium salt	$HSCH_2CH(SH)CH_2SO_3^-$	N
mercaptoacetic acid, or sodium salt	$HSCH_2COO^-$	N
N-(2-mercaptopropionyl) glycine	$CH_3CH(SH)CONHCH_2COO^-$	N
mercaptosuccinic acid	$^-OOCCH_2CH(SH)COO^-$	N
3-mercapto-1-propanesulfonic acid, sodium salt	$HS(CH_2)_3SO_3^-$	N
2-mercaptopropionic acid	$CH_3CH(SH)COO^-$	N
N-acetyl-L-cysteine	$HSCH_2CH(NHCOCH_3)COO^-$	N

[a] General reaction conditions were as follows: A solution of 1 – 2.5 nmol of holo-NCS (final concentration, 10 – 50 μM was mixed with 5 – 10 mM of thiol. The mixture was maintained at pH 7 – 8 by adding 50 – 100 mM (final concentration) of Tris-HAc and/or Tris-HCl buffer and was incubated either at 37 °C for 30 – 60 min or 0 °C for 24 – 48 h. [b] The thiol formula in aqueous form under neutral condition is shown in this table. R_1 stands for reacted. More than 90 % of the NCS chromophore disappeared after incubation and the cycloaromatized products were detected by HPLC analyses. R_2 also stands for reacted. More than 90 % of the NCS chromophore disappeared after incubation but the cycloaromatized products, either **1** or **2**, were not detected by HPLC in significant quantities. The drug products were probably either degraded or undetectable in our analytical system. [e] PR stands for partially reacted. More than 50 % of the NCS chromophore had reacted after incubation. [f] N stands for no reaction. Most NCS chromophore was preserved. Less than 10 % had been inactivated after incubation.

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under regular neutral or slightly alkaline reactive conditions. Appreciable amounts of the inactivated thiol–drug adduct **2** are only observed under quite extreme conditions, such as incubation under very acidic pH at 25 °C for 12 h.

To substantiate the hypothesis that size or shape does not play a major role, di-isoamylthiomalate (Figure 3), which has a similar size and shape as GSH, was chosen for a close comparison. Figure 4 illustrates the disappearance of the

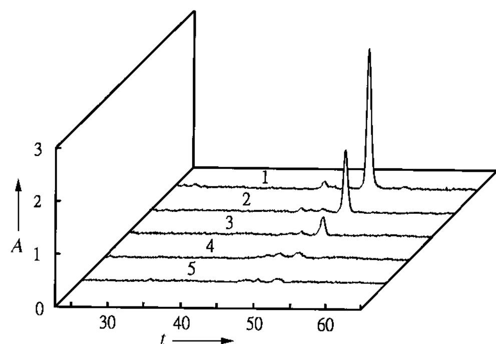

Figure 4. HPLC profiles displaying the thiol-induced inactivation of the chromophore in holo-NCS. The chromatography was carried out on a photodiode array UV detector at 340 nm. Samples containing 1 nmol of holo-NCS (50 μM) and 100 mM of Tris-HCl/Tris-HAc buffer at pH 7.4 were incubated with: (line 1) 10 mM of GSH at 37 °C for 90 min; (line 2) 10 mM of di-isoamylthiomalate at 37 °C for 30 min; (line 3) 10 mM of di-isoamylthiomalate at 37 °C for 60 min; (line 4) 10 mM of di-isoamylthiomalate at 37 °C for 90 min; (line 5) 10 mM of GSH and 10 mM of di-isoamylthiomalate at 37 °C for 90 min. A = UV absorbance ($\times 10^{-3}$). t = elution time in min.

chromophore in holo-NCS induced by both thiols. While the chromophore remained almost intact under incubation with GSH alone (Figure 4, line 1), with di-isoamylthiomalate the same amount of the drug was decomposed almost completely (Figure 4, line 4). In contrast to holo-NCS, which does not react with GSH, the extracted chromophore reacts rather rapidly with GSH to form product **1**. Thus, the examination of the formation of **1** from GSH is a good criterion for judging the extent of release of the chromophore from NCS protein cleft.[23] Figure 4, line 5, shows the analysis of the reaction of holo-NCS with mixed thiols of GSH and di-isoamylthiomalate. None of **1** from GSH (elutes at 26 min) was detected, and most chromophore was decomposed by di-isoamylthiomalate as usual. This strongly indicates that the inactivation of the drug occurs inside the protein cluster, and that the NCS apoprotein allows di-isoamylthiomalate to penetrate into the binding site even though it has a similar size and molecular shape to GSH.

Effect of the chemical functional groups attached to the thiol: GSH differs from other thiols not only in terms of its large size but also due to the presence of multiple chemical functional groups. It consists of peptide bonds, amino and carboxylic groups, etc., which result in net negative charge under physiological aqueous conditions. However, when we attempted to arrange the screening data according to the functional groups on the employed thiols, we failed to get a fully consistent result within the same category of functional group. For instance, Cys-Gly, which is a dipeptide thiol that contains the same functional groups as GSH, is partially reactive with holo-NCS. In contrast to L-cysteine, which is also partially reactive, N-acetyl-L-cysteine is unreactive, while L-cysteine ethyl or methyl esters are reactive. These results suggest that the presence of a specific chemical functional group may not be the key factor in controlling the selectivity of the NCS protein.

Effect of the type of charge of a thiol: GSH differs from the nonelectrolyte BME not only in terms of its large size and functional groups but also in terms of its net negative charge. After arranging our screening results according to the inherent charge types of the employed thiol, we found a surprisingly high consistency within the same category of charge (Table 1). Thiols were organized into four categories (Table 1) based on their charges under aqueous and neutral conditions, that is, (A) positively charged, (B) neutral, (C) zwitterionic, and (D) negatively charged. The results clearly demonstrate that charge plays the most important role for the selectivity of the NCS apoprotein. All the positively charged or neutral thiols can readily attack the protein-bound chromophore even though their size, shape and functional groups present are diverse. In contrast, the negatively charged thiols are efficiently shielded by the NCS protein despite their varying sizes and acidic functional groups. Apparently, the NCS apoprotein protects its labile chromophore through its acidic nature which results in electrostatic repulsion of the negatively charged species.

The pH-dependence of the thiol-induced inactivation of holo-NCS: To further confirm that charge plays a key role in shielding the chromophore, we measured the extent of the thiol-induced drug inactivation at different pH levels. Proper thiols, either nonelectrolytes or electrolytes with different charge types, were chosen for a pH-dependent study. Each batch of the reaction solution for a selected thiol was incubated under a series of pH values. The formation of the drug products and the consumption of the chromophore were then analyzed to measure the extent of the drug reaction as a percentage. Figure 5 shows the pH-dependence curves for

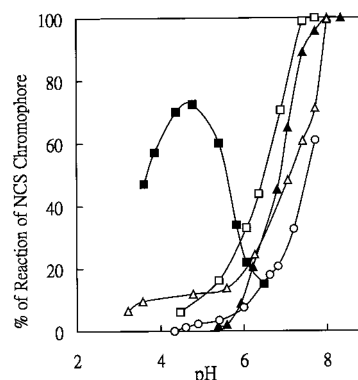

Figure 5. The pH-dependence study of the thiol-induced reactions of holo-NCS. Reaction conditions were: (□) 2 mM of 2-aminoethanethiol (a positively charged thiol), 10 μM of holo-NCS, and 100 mM of Tris-HCl/Tris-HAc buffer at 0 °C for 60 min; (△) 5 mM of methyl thioglycolate (a neutral thiol ester), 50 μM of holo-NCS, and 100 mM of Tris-HCl/Tris-HAc buffer at 10 °C for 60 min; (○) 10 mM of L-cysteine (a zwitterionic thiol), 435 μM of holo-NCS, and 100 mM of Tris-HCl/Tris-HAc buffer at 37 °C for 60 min; (▲) 10 mM of 3-mercapto-1,2-propanediol (a neutral thiol), 10 μM of holo-NCS, and 100 mM of Tris-HCl/Tris-HAc buffer at 0 °C for 120 min; (■) 20 mM of mercaptoacetic acid (a negatively charged thiol), 10 μM of holo-NCS, and 200 mM of Tris-HCl/Tris-HAc buffer at 25 °C for 12 h.

neutral, zwitterionic, positively charged, and negatively charged thiols. All reactions except for that with a negatively charged thiol exhibit very similar pH profiles. In the other reaction the rate dropped rapidly as the pH level decreased. The *N*-methyl-D-fucosamine group in the NCS chromophore has been suggested to participate in the thiol activation of the drug as an internal base.[24] The reported crystal structure of NCS shows that the protonated amino group, which is pH-dependent, orients above C12 at a distance equivalent to the van der Waals diameter of a sulfur atom.[25] Since the cycloaromatization reaction is initiated by a thiolate attack at C12 of the chromophore, a decrease of the reaction rate at lower pH is generally expected since fewer thiolate nucleophiles are available under acidic conditions. However, the negatively charged thiol behaves unlike the others by showing an initially increased reaction rate as the pH level decreases. The reaction rate drops again with the continuous decrease of the pH after it reaches a maximum. With mercaptoacetic acid the extent of NCS reaction reaches a maximum at pH 4.7 (Figure 5). The reaction of 3-mercaptopropionic acid also shows a similar curve with a maximum at a pH value of almost 4.0 (data not shown). Apparently, lower pH levels favor protonation at the ionized carboxylate side chains of amino acid residues as well as at the acidic functional group of the thiol. This leads to partial neutralization of the charge and could substantially reduce the negative electrostatic repulsion between the protein and acidic thiols. It allows the neutralized thiol molecules to have a chance to penetrate the protein barrier and thus be able to induce inactivation of the chromophore. The low availability of the nucleophilic thiolate at low pH probably accounts for the decreased rate of the NCS reaction at the pH level below the maximum point. Control studies show that most of the NCS chromophore is preserved under the described acidic reactive conditions without the addition of mercaptoacetic acid or 3-mercaptopropionic acid. Addition of GSH under the same conditions did not lead to significant formation of the thiol – drug adduct **1,** and most of the chromophore remained unmodified. This rules out the possibility of releasing the NCS chromophore from the protein cavity. The pH-dependence data fully support our electrostatic charge repulsion mechanism of the NCS apoprotein.

Our findings could also account for the early observations made nearly twenty years ago, which showed that acylation of amino groups (Ala 1 and Lys 20) of the NCS protein did not affect the activity of the drug, but modification of the ten carboxyl groups into carboxamides resulted in the loss of activity.[26, 27] Apparently, the ten carboxylic amino acid residues, each of which exhibits one negative charge, play the key role in protecting the NCS chromophore.

Model studies: The crystal structures of both holo-NCS and apoprotein have recently been determined at 0.18 and 0.15 nm resolution, respectively.[25, 28] The three-dimensional holo-NCS structure was downloaded from the Brookhaven data bank (file pdb.1nco.ent), which had been determined from crystals that were grown in a 2-methyl-2,4-pentanediol solution (70 %) under acidic pH. With a help of a molecular modeling simulation program, the acidified solid-state form was used as the basis to simulate the form expected under aqueous conditions and neutral pH. The structure was modified by adding charges to all four basic and ten acidic residues as well as to N and C termini. The resulting molecule was then simulated within an environment of three layers of water molecules. The potential energy calculated by employing a simple minimize strategy was -3015.32 kcal mol^{-1} with a maximum derivative well below 0.001. The resulting model has the following properties: electrostatic -5089.86; van der Waals repulsive 5776.64 kcal mol^{-1}; van der Waals dispersive -4983.29 kcal mol^{-1}; hydrogen bond 0.00; nonbond (total) -4296.51 kcal mol^{-1}; angle 743.76; bond 400.87; torsion 131.79; out of plane 4.76.

Figure 6 illustrates the holo-NCS model with all acidic residues labeled. The distances between one of the carboxylic oxygen atoms and the C12 atom of the chromophore are 7.01,

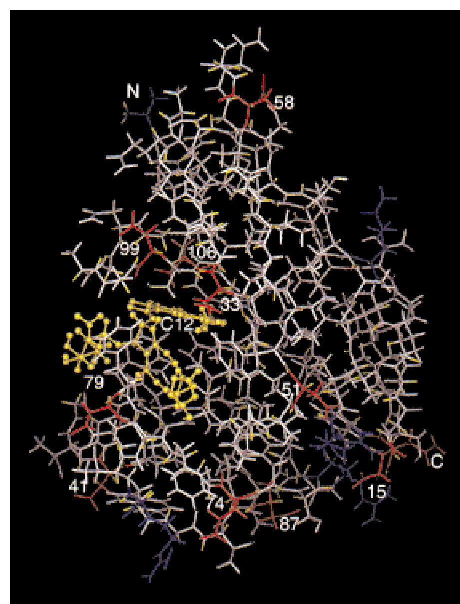

Figure 6. View of holo-NCS model in an aqueous environment under neutral pH. All charged residues are colored. The chromophore is represented by ball-and-stick model with C12 labeled. All acid residues and both N and C termini are labeled.

10.04, and 8.16 Å for Asp 33, Asp 79, and Asp 99, respectively. The three amino acid residues can be viewed as sitting at the door of the binding site, acting as three major sentries guarding the chromophore from an attack at the C12 position by a negatively charged species. Four of the acidic amino acid residues, Asp 41, Asp 51, Glu 74, and Glu 106, were located near the binding site at distances ranging from 14 to 18 Å from C12. These residues are probably good chaperons helping to keep the labile chromophore from becoming inactive. The remaining three acid residues, Asp 15, Asp 58, and Asp 87, were relatively far from C12 at distances beyond 20 Å. Their main purpose is probably to maintain the folded tertiary structure of the protein rather than to protect the chromophore. We have initiated a more advanced calculation involving point charges and overall electrostatic potential of the protein. Such treatment should produce a more detailed picture taking into account the accumulative effect of many other small contributions.

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Conclusion

Our results explain well the long-standing and puzzling observation of why nature makes NCS as a chromoprotein and why the apoprotein is acidic. The production of the NCS apoprotein in addition to the active enediyne warhead is important for the organism *Streptomyces carzinostaticus*. The labile chromophore, which is a deadly weapon, needs to be controlled by the microorganism through the thiol-selective ability of the apoprotein, that is, either to inactivate or to preserve it. Our thiol-screening and pH-dependent data reveal that electrostatic charge repulsion is the key factor played by the NCS apoprotein. In this way it can decide whether to shield or to destroy the chromophore. Neither the size or shape exclusion nor chemical functionality has a major effect on the protecting mechanism of the chromoprotein. The model study suggests that most acidic amino acid residues are located near the binding cavity. In particular Asp33, Asp79, and Asp99 cover the door of the attacking site. The apoprotein strongly repels negative species through its acidic nature and thus protects the chromophore from the attack by the negatively charged GSH in a cellular system until the drug reaches its target DNA. The data provide very valuable information that may help a clinician to design an effective way to reduce the unwanted inactivation of the drug.

Experimental Section

NCS and reagents: NCS powder was a gift from Kayaku Co., Ltd. (Japan). The fluffy powder was dissolved in water and stored in aliquots at $-80\,^{\circ}C$ in the dark. Its concentration (0.87 mM) was determined by absorption at 340 nm (ε, 10800 $M^{-1}cm^{-1}$). Care was taken to avoid exposure of fluorescent light during all handling related to the NCS chromophore. Other chemical reagents, from Aldrich, Sigma, Lancaster, and Fluka, were purchased at optimum purity and used as received.

Thiol-screening test for NCS reactions: A typical reaction solution contained 20–100 µL aqueous mixture of 5–10 mM of thiol and 10–50 µM of holo-NCS. The solution was adjusted to neutral or slightly basic by the addition of 50–100 mM of tris(hydroxymethyl)aminomethane acetate (Tris-HAc) and/or Tris-HCl buffer at pH 7–8. After incubation, either at 37 °C for 30–60 min or at 0 °C for 24–48 h, the thiol-induced drug reaction was analyzed by the described HPLC method. The reproducibility of the result for each thiol was based on 3 to 10 repeat tests.

The pH-dependence study: Each thiol–drug reaction was conducted in at a series of different pH values. The incubation temperature and the time periods were kept the same in one set of reactions. To avoid using different types of buffer over a wide range of pH values, a fair amount of acetic acid was added with equimolar concentration of Tris acetate, Tris-HCl, and Tris base to prepare mixed Tris-HAc/Tris-HCl buffers with pH values of 3–8. Each buffer capacity was tested and approved by using 0.1 M hydrochloric acid. Since the Tris buffer system is usually temperature-sensitive, the real pH value at the incubation temperature was measured before collecting data. The percentage values given from the reactions were calculated from the molar ratio of the cyclized drug products and the unreacted NCS chromophore as described in the section on the HPLC analysis. For each thiol, a minimum of three sets of pH-dependence experiments were performed. The deviations were less than ±10%.

HPLC analyses: The thiol-induced drug reactions were assayed by Waters Millennium systems with a model 600E multisolvent delivery system equipped with a model 996 photodiode array (PDA) detector and a model 474 fluorescence detector as previously described.[22] The PDA detection recorded 0.25 spectrum per second at a resolution of 1.2 nm. The fluorescent excitation was set at 340 nm and the emission was detected at 440 nm with a bandwidth of 40 nm. The mobile phase composed of 5 mM of ammonium acetate (pH 4) in H_2O/CH_3OH and the typical gradient started with a sharp increase (0–48% methanol) over 3 min followed by a less pronounced linear increase (80% methanol) over 62 min. Separations were performed by a Waters μ-Bondapak C18 column (10 µm; 125 Å; 0.39 × 30 cm) with a 0.4 cm guard column at a flow rate of 1 mL min^{-1}.

The extent of inactivation of the drug was determined by measuring quantities of the cyclized products **1** and **2** and the remaining NCS chromophore. Each reaction was repeated until at least three consistent results were obtained. The previously established chromatographic assignments and spectroscopic fingerprints[22] were employed for peak identification. The area of either UV absorbance or fluorescence emission was integrated to estimate the molar quantities of each species.

The major UV absorptivity of the β-band at 226 nm displayed very little hypsochromic or bathochromic shift and almost no hyperchromic or hypochromic shift among the NCS chromophore and its various derivatives.[22] Thus, the ratio of the integrated absorbance of each elution peak on a chromatographic profile extracted from the PDA at 226 nm, A_{226}, can be directly converted to their molar ratios. The extent of the thiol-induced drug reaction was expressed as the sum of A_{226} from the cyclized drug products as a percentage of the sum of A_{226} from the products and the remaining NCS chromophore. However, when the drug reaction is poor due to the shielding effect of the NCS protein, accurate measurement of A_{226} from small UV bands of the drug products is often limited by low sensitivity. We have previously reported[22] the relative fluorescent quantum efficiency Φ_F measured at the excited wavelength of 340 nm. The Φ_F values for both types of thiol-induced cyclized drug product are more than 60 times stronger than that for the NCS chromophore A, which is the major component of native NCS. We therefore estimated the yield of the drug product based on the fluorescent emission for poor drug reactions. In order for the measured fluorescent emission to be converted into molar ratios, the fluorescence detector was connected to a PDA UV detector. The ratio of the integrated UV absorbance at 226 nm to that of the correspondent fluorescent emission in a same chromatographic run from reactions that display good yields was taken as a conversion factor, c, to convert the fluorescent ratios into molar ratios. For example, ethyl 2-mercaptoacetate induced thiol–drug adducts have average c values of 0.1044 and 0.1055 for **1** and **2**, respectively. The extent of the drug reaction was calculated as given in expression (1), where F_1 and F_2 are the integration values of the fluorescent bands from products **1** and **2**, respectively.

$$\%\ \text{NCS reaction} = (F_1 \times 0.1044 + F_2 \times 0.1055)/(F_1 \times 0.1044 + F_2 \times 0.1055 + A_{226}\ \text{from the remaining NCS chromophore}) \qquad (1)$$

As shown in Figure 4, some thiols can induce good drug reactions but failed to produce detectable amount of the drug products by HPLC analyses. In such cases we measured the extent of the NCS reactions based on the disappearance of the NCS chromophore from the HPLC analyses. An inert fragment of the NCS chromophore, 2-hydroxy-7-methoxy-5-methyl-1-naphthoic acid, has been found to be present as an impurity (in trace amounts) in the original NCS powder supplied. The amount of this impurity remains about constant for reactions that were incubated under similar conditions. Since it elutes after about 20 min by using our HPLC method and can be easily distinguished from the NCS chromophore and all the cyclized products (**1** or **2**), it was considered as a conventional internal standard when applicable. The error of this method is in general within ±10%.

Simple minimization energy of aqueous holo-NCS model: The crystal structure of holo-NCS[26] was downloaded from the Brookhaven pdb.1nco.ent file. It was converted by using the MSI Biosym program and was modified in the following way: i) The co-crystallized solvent molecule (2-methyl-2,4-pentanediol) in the crystal structure was deleted; ii) The atom type of the NCS chromophore was corrected; iii) The N terminus and the four basic residues, Lys^{20}, Arg^{70}, Arg^{71} and Arg^{82} were modified into a protonated and positively charged form; iv) The C terminus and the ten acidic residues, Asp15, Asp33, Asp41, Asp51, Asp58, Glu74, Asp79, Asp87, Asp99, and Glu106, were modified into a deprotonated and negatively charged form; v) Three layers of water molecules were added; vi) The energy was minimized by using the force field CVFF and the simple minimize strategy method of CG (Polak–Ribiere) with a derivative set at 0.0001 and iterations set at 10000.

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