Role of the tyrosine corner motif in the stability of neocarzinostatin

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Although the immunoglobulin-like β-sandwich fold has no specifically conserved function, some common structural features have been observed, in particular a structural motif, the tyrosine corner. Such a motif was described in neocarzinostatin (NCS), a bacterial protein the structure of which is very similar to that of the immunoglobulin domain. Compared with the other β-sheet proteins, the NCS ‘tyrosine corner’ presents non-standard structural features. To investigate the role of this motif in the NCS structure and stability, we studied the properties of a mutant where the H bond interaction had been eliminated by replacing the tyrosine with a phenylalanine. This mutation costs 4.0 kcal/mol showing that the NCS ‘tyrosine corner’ is involved in protein stability as in the other Greek key proteins. This destabilization is accompanied by remote structural effects, including modification of the binding properties, suggesting an increase in the internal flexibility of the protein. With a view to using this protein for drug targeting, these results along with those obtained previously allow us to define clearly the limitations of the modifications that can be performed on this scaffold.

Keywords: β-sheet protein/immunoglobulin fold/ neocarzinostatin/tyrosine corner

Introduction

Neocarzinostatin (NCS), isolated from Streptomyces neocarzinostaticus, belongs to a family of macromolecular chromophore-protein antibiotics that have antitumoral activity. The known members of this family are neocarzinostatin, macromycin, secreted by Streptomyces macromomyceticus, C-1027 and actinomycin, secreted by Streptomyces globisporus, madurineptin, secreted by Actinomadura madurae, and kedarcidin, secreted by an unidentified species of actinomycete. The antibiotic and antitumoral activities of these compounds are due to a low molecular weight enediyne chromophore. It has been suggested that the apoprotein stabilizes and regulates the availability of the otherwise labile chromophore (Kappen et al., 1980).

Various studies have shown that apoNCS can bind other natural chromophores (Kappen et al., 1980) or small-molecule drugs (Urbaniak et al., 2002). The ability of apoNCS to bind to other small-molecule drugs may offer opportunities for delivering other entities in vivo. However, such applications would depend not only on the ability of apoNCS to accommodate molecules other than natural chromophores within the binding cleft but also on the ability to target the drug. Before using protein engineering to remodel the loops for drug targeting, we need to know which are the main stabilizing interactions that should not be altered.

In a broader perspective, the three-dimensional structure of neocarzinostatin is based on a seven-stranded antiparallel β-sandwich, very similar to the immunoglobulin folding domain (Adjadj et al., 1992a,b; Kim et al., 1993). Although there is a large amount of data concerning the stability and folding of various immunoglobulin domain proteins (Clarke et al., 1999; Lorch et al., 1999; Fowler et al., 2002) and also about the structural determinants in the sequences of immunoglobulin variable domains (Chothia et al., 1998), few data are available for NCS or other members of this family. As NCS and the other proteins of the immunoglobulin fold family have no sequential or functional link, it is important to determine the extent to which their structural properties are similar and, conversely, the implications of differences in structural features for maintaining the stability of the fold. The similarity between the NCS and the immunoglobulin fold is clearly shown in the topological diagram in Figure 1. The first sheet of both β-sandwiches includes A, B and E strands and the second sheet consists of D, C, F and G strands.

NCS belongs to the most stable group of proteins in the Ig fold family. As we recently reported (Valerio-Lepiniec et al., 2002), the rigidity of the β-sandwich of apo-NCS is maintained by hydrophobic clusters, found at the same locations within the β-core of the protein as the hydrophobic groups implicated in maintaining the stability of the Ig domains (Izadi-pruneyre et al., 2001). In particular, residues V21 and V95 occupy a strategic position within the β-sandwich where they coordinate the association between the B and F strands (Valerio-Lepiniec et al., 2002). However, some other interactions could also be important for the stability of the protein. For example, it has been proposed that F78 and/or the natural chromophore could be implicated in the stabilization of the holo-protein (Nozaki

Fig. 1. Topological diagrams of (A) the apo-NCS structure and (B) an example of an Ig domain.

733
et al., 2002). More specifically related to the Ig fold, apoNCS has a Tyr corner-like motif, a structure contributing to the stability and fold of Greek key structure (Hemmingsen et al., 1994) and that has not been seen elsewhere (Hemmingsen et al., 1994; Hamill et al., 2000). This motif occurs at the end of a β-arch in most Greek key β-sheet proteins. Various tyrosine corner classes have been proposed, the most common corresponding to a tyrosine that forms a hydrogen bond with residue Y-4 (defined as Δ4 tyrosine corner). Whereas this structural motif has been described in various proteins, its exact role has only been studied in a few proteins (Hemmingsen et al., 1994; Hamill et al., 2000). The OH moiety of the tyrosine corner is not conserved as a folding nucleus but is related to the structural restraint of the loop connecting the β-sheet. In NCS, Tyr32 located in the BC loop region makes a hydrogen bond from its side chain OH to the backbone NH of residue Glu27, in the Y-5 position (Figure 2). The NCS ‘tyrosine corner’ has different properties to other β-sheet proteins. Clarke et al. showed that the tyrosine corner is located at the base of the F-strand and that the hydrogen bond stabilizes the E–F loop (Clarke et al., 1999). In apo-NCS, the motif is in the BC loop region. To investigate the role of this motif in NCS stability, we studied the stability properties of a mutant with no H bond interaction. The H bond interaction with Glu27 was eliminated by replacing Tyr32 with phenylalanine. Thermal and chemical denaturation together with NMR analysis of the mutant and wild-type (WT) clearly show that this tyrosine corner is also highly implicated in protein stability.

Materials and methods

Mutagenesis and protein purification

The cloning of the neocarzinostatin gene has been described previously (Heyd et al., 2000). Mutagenesis was performed using the Stratagene Quickchange kit. Point mutation was identified using restriction digestion (silent removal site) and DNA sequencing of complete NCS DNA. The 6× His-tagged protein was over-produced in *Escherichia coli* BLR21 (Novagen) and then purified on an Ni-NTA matrix (Qiagen). All experiments were performed in 20 mM phosphate buffer, pH 5.4.

Physicochemical properties

Circular dichroism (CD) spectra were recorded from 185 to 250 nm on a JASCO dichrograph equipped with a thermostatically controlled cell holder and connected to a computer for data acquisition. Data were acquired from 13 μM sample solutions in quartz cells of 1 mm pathlength.

All NMR spectra were acquired on a Varian Unity 500 spectrometer under the conditions described elsewhere (Adjadj et al., 1990, 1991).

Ethidium bromide (EtBr) binding to WT and mutant apo-NCS was studied by fluorimetry with an Amino SLM 8000 fluorimeter by monitoring the intrinsic fluorescence of a EtBr solution (5 μM final concentration, λex = 479 nm, λem = 620 nm, bandwidth 2 nm) at various apo-NCS concentrations. Saturation curve data were analyzed by using the following equation:

\[
\Delta F = \Delta F_{\text{max}} \frac{P_0 - B_0 \Delta F}{K_d + P_0 - B_0 \Delta F_{\text{max}}}
\]

where \(\Delta F_{\text{max}} = F_{\text{max}} - F_0\), \(\Delta F = F - F_0\), and \(F_0\) and \(F_0\) are the fluorescence intensity measured in the presence and absence of the protein, respectively. \(P_0\) is the total protein concentration, \(B_0\) is the total ethidium bromide concentration and \(K_d\) is the dissociation constant. Experimental data were fitted according to Equation 1 by using a simplex procedure based on the Nelder and Mead algorithm (Heyd et al., 2000).

Stability of apo-NCS WT and mutant

Thermal stability was studied by differential scanning calorimetry (DSC) on a Microcal instrument with a 1 mg/ml apo-NCS solution dialyzed overnight against standard phosphate buffer. Buffer solution from the dialysis bath was used as a reference. All solutions were degassed just before loading into the calorimeter. Scanning was performed at 1 K/min.

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Fig. 2. (A) Apo-NCS structure showing the hydrogen bond between the Tyr32 and Glu27. The residues displaying significant differences in their proton chemical shifts between the mutant and the wild-type protein are colored. (B) Holo-NCS structure showing Tyr32, the natural ligand and identifying the residues 70, 71 and 88.
Reversibility of the denaturation process was checked by re-scanning a denatured sample after cooling.

The heat capacity of the solvent alone was subtracted from that of the protein sample. These corrected data were analyzed using a cubic spline as a baseline in the transition. Thermodynamic parameters $\Delta H_{\text{cal}}$ and $\Delta H_{\text{ch}}$ were determined by fitting the following equation to the data:

$$\Delta C_p(T) = \frac{K_\text{d}(T) \Delta H_{\text{cal}} \Delta H_{\text{ch}}}{[1 + K_\text{d}(T)]^2 RT^2}$$

where $K_\text{d}$ is the equilibrium constant for a two-state process and $\Delta H_{\text{cal}}$ is the measured enthalpy, corresponding to

$$\Delta H_{\text{cal}} = \int_{T_1}^{T_2} C_p(T) dT$$

and $\Delta H_{\text{ch}}$ is the enthalpy calculated on the basis of a two-state process. If the measured transition corresponds to a two-state process, the values of the two enthalpies $\Delta H_{\text{cal}}$ and $\Delta H_{\text{ch}}$ are equal. Values of the ratio $\Delta H_{\text{cal}}/\Delta H_{\text{ch}}$ other than 1 imply the presence of intermediates or multimolecular processes (Freire, 1995).

For spectroscopic measurements, the fraction unfolded $f_u$ was calculated from the signal $\Theta$ using the standard equation

$$f_u = \frac{\Theta_N - \Theta}{\Theta_N - \Theta_D}$$

where $\Theta_N$ and $\Theta_D$ represent, respectively, the signal value of the native and denatured species at each temperature, taking into account the baselines preceding and following the transition region. $\Delta H_{\text{ch}}$ and $T_1/2$ were calculated from the transition curves using the standard equation

$$\frac{\partial \ln K}{\partial \frac{1}{T}} = \frac{-\Delta H_{\text{ch}}}{R}$$

where $K$ is defined as

$$K = \frac{f_u}{1 - f_u}$$

and $\Delta H_{\text{ch}}$ is assumed to be temperature independent and $R$ is the gas constant. $T_m$ was estimated for $\ln K = 0$.

The unfolding induced by guanidinium chloride (GdmCl) of 5 $\mu$M protein solutions in standard buffer was monitored by fluorescence spectroscopy. Fluorescence was measured with an Aminco SLM 8000 fluorimeter, after 12 h of incubation at 4°C in solutions of various GdmCl concentrations. Ultra-pure GdmCl was obtained from Pierce; the denaturant concentrations were checked by refractometry, using the relationship provided by Nozaki (Nozaki, 1970). The transition curves were constructed by plotting the position of maximum fluorescence emission ($\lambda_{\text{exc}} = 295$ nm, bandwidth 2 nm) against denaturant concentration.

The model of the linear dependence of $\Delta G$, upon denaturant concentration, $x$, according to Pace (Pace, 1986) was used for thermodynamic analysis:

$$\Delta G_x = \Delta G_0 - mx$$

Assuming that the linear dependence of the free energy change on denaturant concentration observed in the transition region can be extrapolated to zero denaturant concentration, $\Delta G_0$ represents the standard variation of free energy in the absence of denaturant and $m$ is a constant proportional to the increase in the accessible surface area of the protein to the solvent on denaturation. An equation derived from Equation 7, taking into account the baselines and the transition region, was used to analyze the data:

$$y_x = y_n + s_n x + \left\{ \frac{[\Delta G_0 - mx]}{RT} \right\} \left\{ \frac{1}{1 + e^{[\Delta G_0 - mx]/RT}} \right\} + A + (s_d - s_n)x$$

where $y_x$ is the experimental signal in the presence of $x$ M GdmCl, $y_n$ is the signal of the native form, $s_n$ and $s_d$ are the solvent effects on the native and denatured protein signal, respectively, and $A$ is the amplitude of the transition. Experimental data were fitted according to Equation 8 by using a simplex procedure based on the Nelder and Mead algorithm.

**Results**

**Structural properties of the Y32F mutant**

The overall structure of the mutant was evaluated by comparing the CD spectra of the mutant and the wild-type protein (Figure 3). The spectra are fully superimposable and have a signal characteristic of an all-$\beta$-protein with a maximum at 195 nm and a minimum at 210 nm. The mutant Y32F spectrum also displays a positive contribution at ~223 nm, which is specific to the apo-NCS. This maximum has been proposed to result from typical secondary structure (Heyd et al., 2000).

The three-dimensional structure of Y32F was further analysed by $^1$H NMR spectroscopy. The NOESY spectra of the mutant and wild-type indicate that the overall structure of the protein is preserved, which is consistent with the CD data. Chemical shift differences between the $^1$H resonances of the variant and the wild-type proteins were identical in almost all cases (Figure 2). Deviations are observed for the protons...
located in the immediate sequential (backbone protons of the strand C and loop BC) and spatial vicinity (backbone and side-chain protons of the FG loop) of the mutation site. In addition, the residues located at the base of the β-sheet (70–71, 88, the end of strand F and strand G) in the mutant displayed significant deviations in their proton chemical shifts compared with the WT proteins.

**Integrity of the binding site**
The integrity of the cleft was checked by measuring EtBr binding. This compound binds apo-NCS stoichiometrically in the natural chromophore cleft (Mohanty et al., 1994) and this is therefore a convenient tool for monitoring the functional properties of apo-NCS and its variants. The $K_d$ value obtained at pH 5.4 for the Y32F mutant (29 μM) is different from that of the wild-type protein (16 μM) determined under the same conditions. The slight but significant increase in $K_d$ indicates that this mutation induces changes in the conformation and/or dynamic properties of the binding site of the EtBr.

**Stability of the mutant**

**Thermal stability of the mutant Y32F followed by DSC.** The thermal stabilities of mutant Y32F and wild-type NCS were compared using DSC (Figure 4). The transition was fitted with a non-two-state model. At pH 5.4, the midpoint temperature of the transition for the Y32F mutant is $T_m = 60.3 \pm 0.4$ versus 69.3 ± 0.4°C for the wild-type. The calorimetric enthalpy of the mutant ($\Delta H_{cal} = 94.5 \pm 1.1$ kcal/mol) was lower than that of the wild-type (111.7 ± 1.1 kcal/mol). The $\Delta H_{cal}/\Delta H_{cal}$ ratio for both proteins was close to one (1.05 for the Y32F and 0.96 for the WT). These results suggest that the Y32F mutation has significant effects on the overall stability of the protein.

**Thermal denaturation of the mutant Y32F followed by fluorescence.** The thermal transition was also monitored by following the variation of the fluorescence intensity and the variation of the wavelength, $\lambda_{max}$, which is the maximum fluorescence emission wavelength for NCS and mutant Y32F. Both transitions obtained from the two proteins were similar. The fluorescence spectra of the WT and the mutant Y32F, obtained by excitation at 295 nm, were recorded in the temperature range 25–80°C (Figure 5). For the WT, transition occurred between 60 and 75°C, with one midpoint transition, $T_m = 71.7 \pm 1.3°C$. For the Y32F mutant, a double transition was observed with two midpoints: $T_{m1} = 60.4 \pm 1.5°C$, which corresponds to that obtained in DSC, and $T_{m2} = 71.9 \pm 2.2°C$, probably related to local structure denaturation. The results suggest that in the mutant protein, there is an intermediate during unfolding.

**Chemical denaturation of the mutant Y32F followed by fluorescence.** Denaturation of the WT and mutant Y32F induced by GdmCl was also studied. Figure 6 shows the normalized variation of the maximum emission fluorescence wavelength of NCS and Y32F as a function of the denaturant concentration. The transition for NCS occurred between 2 and 4 M GdmCl with a midpoint transition, $C_m = 3.02 \pm 0.05$ M. The calculated Gibbs free energy of unfolding, $\Delta G_\text{unf}$, is 9.65 ± 0.06 kcal/mol. For the mutant Y32F, there is a large downward shift of the transition midpoint, $C_m = 2.09 \pm 0.05$ M. Conjointly, there was also a loss of 4.0 kcal/mol in free energy of unfolding. For the two proteins the m value is between 3 and 4 kcal/mol/M, the value for the mutant Y32F being slightly lower.

**Discussion**

**Effect of the mutation on the structure of the protein**
The substitution Y→F does not change the overall structural features of the protein. At 25°C the fluorescence properties of
the mutant are comparable to those of the WT, suggesting that the mutation does not significantly modify the accessibility of W39, the main contributor to fluorescence. The CD spectrum of the mutant protein is identical with that of the WT protein, indicating that the mutation does not change the overall secondary structure content of the protein. Hence the observed changes in protein stability are mainly related to changes in local interactions.

Effects of the mutation on protein stability
Removing the tyrosine hydroxyl moiety reduces the stability of NCS as assessed by microcalorimetry and denaturation monitored by CD and fluorescence. DSC measurements indicate that the $T_m$ of the Y32F mutant is ~9°C lower than that of the wild-type protein; this shift in $T_m$ is accompanied by a decrease of 16 kcal/mol in $\Delta H_{m}$. This decrease in stability corresponds also to a change in the denaturation process. Thermal denaturation monitored by fluorescence clearly shows a double transition, the first one with a $T_m$ corresponding to that determined by microcalorimetry and the second with a higher $T_m$, corresponding to the denaturation of a more stable local residual structure.

The substitution of tyrosine by phenylalanine leads to the occurrence of intermediate states which were not detected in the wild-type. This has been observed for apo-pseudoazurin, another protein with a Δ5 tyrosine corner, in which intermediate states were observed after substituting tyrosine with tryptophan (Jones et al., 2000). It must be emphasized that NCS does not undergo a true two-state heat denaturation process and presents an unexpected complex unfolding transition with temperature (Perez et al., 2001). Hence the presence of two transitions could simply be related to a change in the relative stability of one or more intermediates that have been detected by SAXS experiments (Perez et al., 2001).

CD and NMR measurements show that both secondary structure and overall folding are identical in mutant Y32F and wild-type protein. However, some chemical shift variations were observed for residues in contact with the ‘tyrosine corner’. Remote effects are observed at the base of the β-sandwich and in particular for residues 70–71 and 88. In addition, the integrity of the ligand-binding site is affected by the substitution of tyrosine. The value of the dissociation constant obtained for mutant Y32F is about twice as high as that obtained for the wild-type. The structure of the mutant is similar to, if not identical with, that of the wild-type and therefore the slightly reduced affinity for EtBr of mutant Y32F is probably due to a weakening of attractive interactions between the protein and ligand, which could be due to an increase in the internal flexibility of the protein. Indeed, it has been shown that Y32 takes an important part in maintaining the rigidity of the top of the β-sandwich (Izadi-pruneyre et al., 2001). Removing the OH-bond could release this internal constraint. This hypothesis is consistent with change in the $m$ values for the mutant Y32F, characteristic of an increase in the mean accessible surface area of the native protein.

Comparison with other tyrosine corners
The substitution Y32F has various effects on the stability of the Ig fold. Hamil et al. have worked on various proteins of the fibronectin type III superfamily (Hamil et al., 2000). They have shown that the mutation Y→F costs between 1.5 and 3 kcal/mol. In some cases, the destabilization induced by this replacement is so large that the protein cannot fold, as is the case for pseudoazurin (Hazes and Hol, 1992). Although for NCS the destabilization is fairly large (4 kcal/mol), this mutant is able to acquire a native fold. This raises the question as to why the tyrosine corner motif is found ubiquitously and exclusively in Greek key proteins and why the hydrogen bond is necessary for their stability. It has been shown that the tyrosine corner is not a folding nucleus and that this structure has mainly a stabilizing role (Hamil et al., 2000). Indeed, the diverse effects of the Y→F substitution in closely related local structures suggest that, as observed for other interactions such as N- and C-cap, the environment can modulate the stabilizing role of the hydrogen bond. The pseudoazurin tyrosine corner consolidates a β-zipper structure between strands VI and VII and is thus essential to the protein stability (Hazes and Hol, 1992; Jones et al., 2000). In NCS, although the Tyr corner is important for the stability of the protein, it is not essential. This could be related to the localization of the Tyr corner in this protein. In NCS, the Tyr corner is not localized on the E–F loop, where it is observed for the majority of Ig fold proteins, but on the B–C loop. In this protein, the stabilization of the E–F loop is obtained via a disulfide bridge (Cys88–Cys93) that is essential for NCS stability, its elimination being ‘lethal’ for the protein. As described in a study of the dynamic properties of apo-NCS (Izadi-pruneyre et al., 2001), the tyrosine corner allows a structural cohesion with the other side of a protein. Substitution with phenylalanine induces greater flexibility of the loop and this is tolerated in the protein structure.

Although our data do not rule out the possibility that the tyrosine corner could also play a role in the folding process, we suggest that the differences between the role of the tyrosine corners in related proteins may originate from their specific functions. For immunoglobulins, the B–C loop (CDR1) is stabilized by a disulfide bridge, providing the rigidity required for correct recognition of the antigen. In contrast, for NCS, the tyrosine corner has a stabilizing role in the same loop, but also plays a role in the correlated internal movements needed for correct ligand binding. With a view to using this protein for drug targeting, these results, combined with those obtained previously (Valero-Lepiniec et al., 2002), allow us to define clearly the limitations of the modifications that can be performed on this scaffold.

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References


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