Key interactions in the immunoglobulin-like structure of apo-neocarzinostatin: Evidence from nuclear magnetic resonance relaxation data and molecular dynamics simulations

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Abstract

The three-dimensional structure of apo-neocarzinostatin (apo-NCS, MW: ca.11000, antitumoral chromophore carrier protein) is based on a seven-stranded antiparallel β -sandwich, very similar to the immunoglobulin folding domain. We investigated the backbone dynamics of apo-NCS by ¹³C-NMR relaxation measurements and molecular dynamics simulation. Model-free parameters determined from the experimental data are compared with a 1.5-nsec molecular simulation of apo-NCS in aqueous solution. This comparison provides an accurate description of both local and collective movements within the protein. This analysis enabled us to correlate dynamic processes with key interactions of this β -protein. Local motions that could be relevant for the intermolecular association with the ligand are also described.

Keywords: Neocarzinostatin; backbone dynamics; ¹³C-NMR relaxation; molecular dynamics; Greek key; β -sandwich

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Neocarzinostatin (NCS) is the first "enediyne" antitumor agent to be characterized. It is the first member of a chromoprotein antibiotic family that includes auromycin (AUR)

(Van Roey and Beerman 1989), actinoxanthin (AXN) (Sakata et al. 1993), C-1027 (Xu et al. 1994), and kerdacidin (Constantine et al. 1994). The clinical use of NCS is currently approved in Japan for cancers in digestive organs (stomach, pancreas, liver), urinary-bladder, brain, and for leukemia. All chromoproteins are secreted by bacteria as a complex between an enediyne compound, the chromophore, tightly but noncovalently enclosed in a protein moiety. The antitumor properties of these complexes have been shown to be due solely to the nonprotein enediyne chromophore, which causes DNA strand breaks through radical reactions (Kappen et al. 1980).

The apo-protein of NCS (apo-NCS) consists of 113 amino-acid residues and has a molecular mass of 11,000 Da.

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Abbreviations: CHARMM, chemistry at Harvard macromolecular mechanics; DEAE, di-ethyl aminoethyl weak anion exchanger; FID, free induction decay; HPLC, high performance liquid chromatography; INEPT, insensitive nuclei enhanced by polarization transfer; MD, molecular dynamics; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effects; NOESY, nuclear Overhauser enhancement spectroscopy; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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It shares extensive sequence similarity with AXN and macromycin (MCR) (>50%), resulting in similar three-dimensional structures for these apo-proteins. The three-dimensional structure of apo-NCS was determined by nuclear magnetic resonance (NMR) (Adjadj et al. 1992a; Gao 1992) and by X-ray crystallography (Teplyakov et al. 1993).

The largest domain of the protein structure consists of a seven-stranded antiparallel β -sandwich formed by an external three-strand β -sheet and an internal four-strand β -sheet arranged in a Greek key (Fig. 1). The external sheet contains strands A (residues 4–8), B (residues 18–24), and E (residues 62–68), whereas the internal sheet contains strands D (residues 53–56), C (residues 31–36), F (residues 94–98), and G (residues 108–111). The smaller of the two domains is composed of two twisted, two-stranded antiparallel β -ribbons essentially perpendicular to each other, located at the base of the sandwich. One of these ribbons is framed by the disulfide bridge Cys37–Cys47, and the other includes residues 72 to 87.

The internal β -sheet of the larger domain, the smaller domain, and the short 3_{10} helix loop between Asp48 and Phe52 define a deep U-cleft, which corresponds to the NCS-chromophore-binding site. The complex structure was determined by X-ray diffraction (Kim et al. 1993) and by NMR (Tanaka et al. 1993) (Fig. 2). The enediyne and the naphthoate moiety of the NCS-chromophore are encapsulated in this predominantly hydrophobic pocket, whereas the hydrophilic groups of the ligand face outward into the solvent.

The seven-stranded β -sandwich, which makes up the largest domain of the chromoproteins in this family, has been adopted by a variety of different superfamilies, including the immunoglobulins (Adjadj et al. 1992b; Hazes and Hol 1992). This folding pattern is the only common feature shared by these proteins, which display no sequence similarity and are not evolutionary related. Their biological functions are also very different but, in these protein families, the β -sandwich constitutes a framework supporting functional loops, the diversity of which determines interaction specificities with the substrate.

The similarity between the two protein Greek key folds is clearly shown in the topologic diagram of Figure 1. The first sheet of both β -sandwich includes A, B, and E strands and the second sheet consists of D, C, F, and G strands. The two β -sheets in apo-NCS are connected in the same way, by the crossover loops from B to C and from E to F. In addition, apo-NCS possesses the “tyrosine corner” commonly found in β -sheet proteins (Hemmingsen et al. 1994; Hamill et al. 2000a). The tyrosine corner is located in the BC loop region of apo-NCS and in the intersheet EF loop in immunoglobulin domains.

The strong structural similarity of apo-NCS with the members of the immunoglobulin fold superfamily raises questions about the nature of the stabilizing forces within the β -sandwich as these proteins share no significant sequence similarity or biological function. A more complete understanding of the fundamental features of the apo-NCS β -structure requires a description of its dynamic properties. Therefore, we studied, both experimentally and theoretically, the conformational dynamics of apo-NCS.

Heteronuclear ^{13}C or ^{15}N NMR spin relaxation spectroscopy is a powerful experimental approach for characterizing the overall conformational dynamics of proteins in solution (LeMaster and Kushlan 1996; Palmer 1997; Kay 1998). We have previously reported the backbone dynamics of apo-NCS as determined by ^{13}C -NMR relaxation at natural abundance (Mispelter et al. 1995). The possibility of producing ^{13}C -labeled apo-NCS provided us with the opportunity to carry out a more detailed study on a sample with a low concentration. We used the standard and extended model-free formalism of Lipari-Szabo to fit the data, assuming an isotropic rotational diffusion tensor (Lipari and Szabo 1982a,b; Clore et al. 1990).

As for all NMR data, relaxation parameters are averages for all the conformations present in the sample. Therefore, they give an interpretation only of the averaged characteristics of structural fluctuations. Molecular dynamics (MD), in contrast, provides a direct picture of instantaneous mo-

Fig. 1. (A) Topologic diagram of the apo-NCS structure. The larger domain contains a seven-stranded antiparallel β -sandwich formed by an external three-strand β -sheet (sheet S1: strands A,B,E) and an internal four-strand β -sheet (sheet S2: strands C,D,F,G) arranged in a Greek key. (B) Topologic diagram of an example of Ig-domain.

Fig. 2. Stereoview of the NCS complex.

tion, but the length of MD trajectories constitutes a major obstacle for the description of slow dynamic processes. Concurrent advances in theoretical force fields and computing resources have made possible realistic MD simulations of proteins in aqueous solution. The combination of NMR and MD approaches thus provides a more complete picture of the internal mobility of the protein (Chandrasekhar et al. 1992; Eriksson et al. 1993; Philippopoulos et al. 1997; Wong and Daggett 1998). In this study, we compared model-free parameters determined from the experimental data with a 1.5-nsec molecular simulation of apo-NCS in aqueous solution.

The conclusion of this work deals with the possible relationships between the dynamic behavior and the stability and functional properties of apo-NCS. These implications might be extended to the world of β -sandwich proteins.

Results and Discussion

Analysis of $C\alpha$ NMR relaxation data

Reliable quantification of peak intensities was possible for 84 of the expected 113 $C\alpha$ resonances in apo-NCS. Overlaps in ^{13}C - ^1H spectra precluded the determination of intensity values for 14 residues and those for the 15 glycine residues were not taken into account because the pulse scheme used is only valid for C-H groups. The whole experimental dataset was interpreted using the Lipari and Szabo model-free approach assuming an overall isotropic tumbling of the molecule (Lipari and Szabo 1982a,b).

To evaluate possible effects of anisotropic rotational diffusion, the principal components of the inertia tensor of apo-NCS were determined from the crystal structure PDB1NOA (Teplyakov et al. 1993). The ratios of the principal moments were 1.0:0.83:0.52 and the diffusion anisot-

ropy converged to a ratio of $D_{\text{parallel}}/D_{\text{perpendicular}}$ of 1.187. Such a low value is typical of the fitting of NMR relaxation data using the model-free approach (Schurr et al. 1994). On the basis of analysis of the $R2/R1$ ratios of 54 ^{13}C nuclei located in well-structured elements, only a slight improvement of $R2/R1$ fitting could be obtained using an axial diffusion tensor and no further improvement assuming a fully anisotropic tensor (Lee et al. 1997). A similar result was obtained using a modification of the previous method based on the assumption of an axially symmetric diffusion tensor. The effective correlation time was 4.58 nsec, very similar to the value obtained under the assumption of an isotropic diffusion tensor (Mispelter et al. 2000). The weighted mean difference in the order parameters for the two analyses was 0.012. Thus, taking into account the anisotropy of the diffusion tensor did not appear to influence the reported results.

Model-free analyses

The interpretation of relaxation data in terms of Lipari-Szabo parameters first requires estimation of the overall correlation time of the protein τ_R . Using an approach that we recently described, τ_R was optimized at 4.53 nsec for 1 mM apo-NCS at 35°C (Mispelter et al. 2000).

Appropriate models of spectral density functions were selected for the model-free analysis, according to the definition described in the Materials and Methods section. Consequently, relaxation data for 60 and 24 backbone ^{13}C nuclear spins were fit using model 4 (S^2 , τ_e , $R2_{\text{ex}}$) and model 5 (S^2_{slow} , S^2_{fast} , τ_{slow}), respectively (see Electronic Supplemental Material).

Generalized order parameter S^2

The 60 S^2 values obtained using model 4 were between 0.75 and 1 (Fig. 3). Seven of the 24 residues fit with model 5 had both S^2_{slow} and S^2_{fast} greater than 0.9, 14 residues had

Fig. 3. Generalized order parameters for apo-NCS as a function of the protein sequence. (A–C) Experimental values. (D) Simulated values. The dark boxes represent β -sheet regions of the protein.

S^2_{slow} greater than 0.9 with S^2_{fast} between 0.9 and 0.8. As previously reported (Mispelter et al. 1995), most of the main chain positions displayed limited mobility in the picosecond–nanosecond time frame, with the exception of the first three residues at the amino-terminal, and Asn113 at the carboxyl-terminal, which constitute the most mobile $C\alpha$ of the protein.

Residues undergoing restricted amplitude motions, with $S^2 > 0.9$, were mostly found within the secondary structure elements of the β -sandwich (Table 1). Mean $\langle S^2 \rangle$ values calculated for sheet S1 (strands A, B, and E) were, however, slightly lower than those for sheet S2 (strands C, D, F, and G). Mean $\langle S^2 \rangle$ values calculated for loops connecting

β -stands in the β -sandwich did not differ significantly from those of secondary structure elements. Motions of AB, BC, and DE loops were indeed constrained, although some local fluctuations were also observed. All three loops surrounding the functional cavity exhibited lower $\langle S^2 \rangle$ values. The FG loop of the β -sandwich extending from residues Asp99 to Gly107 underwent the largest amplitude of motion, consistent with the root mean square (rms) deviation determined from the NMR structure ensemble (Adjadj et al. 1992a). Finally, residues Thr89 to Ala92 framed by the disulfide bridge Cys88–Cys93, had the same (S^2_{fast} , S^2_{slow}) profile, reflecting the extra mobility of this region as a whole.

Time scales motions

As shown on Figure 4, most of the residues underwent fast rate libration motions characterized by $\tau_e \leq 50$ psec. Additional slow internal motions, τ_{slow} , associated with model 5 were localized at the amino-terminal residues Ala1, Ala2, and Pro3, for the carboxy-terminal Asn113, and in the three loops of the binding site. Residues 89 to 92, located in the small loop closed by the Cys88–Cys93 disulfide bond are also affected by slow motions. Within the β -sandwich, Val7, Ala22, and Leu67 of sheet S1 and Ser10, Thr17 of the AB loop also display additional slow components.

Although $R2_{\text{ex}}$ values do not permit quantitative interpretation of the microsecond–millisecond internal motions, previous studies have shown that they provide valuable qualitative information (Redfield et al. 1992; Cheng et al. 1993; Farrow et al. 1994; Yamasaki et al. 1995; Carr et al. 1997). Thirty-one $^{13}\text{C}\alpha$ exhibited apparent chemical exchange in excess of 0.5 Hz. These residues were strongly clustered in three spatial regions of the protein, within the

Table 1. Mean experimental and simulated order parameters for secondary structural elements, loops, and binding site loops

	Sequence	Number of residues with exp. data	$\langle S^2 \rangle_{\text{NMR}}$	$\langle S^2 \rangle_{\text{MD}}$
Strand A	4–8	4	0.96	0.90
Strand B	18–24	5	0.91	0.90
Strand C	31–36	5	0.97	0.93
Strand D	53–56	2	0.92	0.92
Strand E	62–68	6	0.89	0.90
Strand F	94–98	3	0.90	0.88
Strand G	108–111	4	0.92	0.90
A–B loop	9–17	6	0.91	0.88
B–C loop	25–30	4	0.89	0.88
D–E loop	57–61	3	0.93	0.89
Binding site				
	37–47	9	0.89	0.88
	72–87	12	0.90	0.86
F–G loop	99–107	5	0.84	0.81

Fig. 4. Effective correlation times for internal motions and exchange contributions to R_2 (Hz) for apo-NCS as function of the protein sequence. The dark boxes represent β -sheet regions of the protein.

binding site loops, at the base and top of the β -sandwich (carboxy-terminal, AB loop, BC loop, and the beginning of strand E). This spatial clustering is consistent with the probable occurrence of collective dynamic processes on the microsecond–millisecond time frame. The largest R_{2ex} contributions were displayed by the $C\alpha$ of the aromatic residues (Trp39, Phe76, Phe78 and Trp83 and Phe112) and most of the other $C\alpha$ nuclei exhibiting apparent conformational exchanges are located in the spatial vicinity of these residues.

For residues displaying negative effective correlation times, apparently negative exchange contributions R_{2ex} , or eventually $S^2 > 1$, the assumption underlying the model-free treatment ($\tau_e < \tau_c$) is violated; however, their data were not better fit by model 5. This “blindness” of NMR studies is, however, meaningful because it can be interpreted as an indirect probe for the involvement of internal motions oc-

curring on a time scale of $\sim 10^{-8}$ to $\sim 10^{-3}$ sec, in the relaxation processes of the residues (Constantine et al. 1993).

Analysis of the simulated $C\alpha$ NMR relaxation data

Before drawing conclusions about the nature of a particular dynamic process, we checked that the simulation models were representative of the structural properties of apo-NCS in solution. This examination was achieved by comparing the spatial proximities between two residues of apo-NCS, as derived from nuclear Overhauser enhancement spectroscopy (NOESY) spectra, with the $C\alpha$ correlated motions deduced from analysis of the simulated data (McCammon 1984). Figure 5 shows the superimposition of the experimental data mapping all magnetization transfers [nuclear Overhauser effects (NOE)-connectivities] with the map of cross-correlations indicating the fast motions that give rise to these transfers. The large similarity between the two maps definitively established the validity of our MD model.

Atoms undergo correlated motions if their displacements have similar amplitude and direction. In other words, the correlations are indicative of collective contributions that may affect local motions over larger time intervals (McCammon 1984). For apo-NCS, consistent with what is expected for all proteins, residues in the loop regions exhibited the greatest cross-correlations, but residues Ala24, Tyr32, Val55, Thr56, Ser62, Ala63, and Leu97, although located in β -strands, also appeared to be involved in such a larger collective process (Fig. 6). As most of these residues are lying in the surrounding of the Tyr32 ring, correlation between these collective displacements with the ring motions cannot be ruled out.

Simulated order parameters S^2_{MD}

After a rapid drop to ~ 0.95 , corresponding to small amplitude motions on a sub-picosecond time scale, the correlation functions for internal motions, $C_i(t)$, differed significantly but not systematically in shape from residue to residue and could be roughly divided into three categories shown in Figure 7 (only the 84 residues with known experimental S^2 values were considered in the following study): (1) 42 residues, illustrated by Val19, displayed a converged and flat correlation function over the correlated time (most had order parameters above 0.9); (2) 31 residues, illustrated by Asn103, displayed converged correlation functions with slow decay, oscillations, and order parameters below 0.9; and (3) for the third category, illustrated by Phe78, no plateau value was reached by the correlation function within 500 psec.

Comparison between experimental and simulated order parameters

A variety of comparisons between simulation and experimental studies of protein dynamics have been made (Eriks-

Fig. 5. Superimposition of cross-correlations between $C\alpha$ in the apo-NCS structure deduced from the analysis of the 1.5-nsec dynamic simulation (contour line) and experimental NOE connections (dots) that characterize the cross relaxation of specific pairs of nuclei. The correlation of residues i and j is computed as the normalized quantity: $C_{ij} = \langle R_i R_j \rangle / \langle R_i^2 \rangle^{1/2} \langle R_j^2 \rangle^{1/2}$ where R_i is the instantaneous displacement of the centroid of residue i from the mean position of the centroid. The contour was drawn for $C_{ij} = 0.8$ (McCammon 1984).

son et al. 1993; Philippopoulos et al. 1997; Chatfield et al. 1998; Wong and Daggett 1998). In general, the pictures derived from the two types of study are consistent. As both simulation and the analysis of experimental studies involve approximations, differences in certain detailed results are inevitable. Approximations in the simulations include those in the underlying potential functions, those arising from the use of classic rather than quantum dynamics, and those associated with the finite lengths of the simulations. Approximations in the experimental NMR work arise largely from the Lipari–Szabo formalism used to interpret the data. Nevertheless, bearing in mind the limits to validity of the two approaches, a combination of experimental and theoretical information provides a more complete picture of the motions occurring over the various different time scales.

Of the 84 simulated S^2 values, 73 were from 0.8 and 0.95, confirming the high rigidity of the apo-NCS structure. As shown by comparing the two mean value profiles in Table 1, the agreement between mean experimental and simulated order parameter values was overall very good. The small

amplitude motions within loops, together with the largest mobility of the FG loop were also well reproduced by the simulation.

Fig. 6. Number of cross-correlations per residue deduced from analysis of the simulated data as a function of protein sequence.

Fig. 7. Typical examples of correlation functions for each of the three categories listed in the text. The horizontal line indicates the extracted order parameter.

Optimal agreement between the two approaches ($S^2_{\text{exp}} \sim S^2_{\text{MD}}$) was observed for 35 residues fit with model 4. This convergence suggests that atomic displacements are fast and occur around an average conformation. However, these motions may also coincide with fast but infrequent distortions of the protein distributed over many residues with small relative displacements of neighboring atoms in certain large-scale motions. Therefore, this may account for the $R2_{\text{ex}}$ contribution detected in the Lipari–Szabo analysis for some residues, albeit associated with low amplitude fast motions. It should be noted that, on the time scale used for NMR, a number of microstates are sampled, whereas for the shorter time scale used for simulation, only one microstate is sampled (Chatfield et al. 1998). The striking example is Trp83, which showed a very high exchange contribution of almost 9 Hz superimposed to its constrained motions. The degree of instability created by the β -bulge seen in the vicinity of this residue is a possible explanation for these particular slow motions. Among the physical processes that may also generate such exchange terms, proximity to reorienting aromatic side chains may produce varying chemical shifts, even at rigid backbone sites (Constantine et al. 1993). For example, the $R2$ exchange contribution displayed by Arg71 may be related to the ring motion of Phe112, close proximity of which is indicated by the unusually high-field shifted resonance of its $H\alpha$ (2.18 ppm; Adajadj et al. 1992a).

Convergence between MD simulations and NMR experiments was also observed for residues fit with model 5 that is, residues undergoing motions on two time scales (picoseconds and nanoseconds). Agreement was indeed observed between fast S^2_{fast} and S^2_{MD} values for 17 of the 24 residues fit with this model. This is consistent with the fact that the MD trajectory adequately described fast-restricted motions on a picosecond time scale, whereas it could not accurately

sample slow motions on the nanosecond time scale (Philippopoulos et al. 1997).

Conversely, large differences between the order parameters obtained from NMR and MD were observed for some residues, mostly located in the amino terminal part of the molecule and in the binding site loops. For some of them, MD has shown higher mobility than NMR. As well described by Chandrasekhar et al. (1992), MD may reveal rare and large conformational transitions, not detectable by NMR, which deals with a population of molecules as a whole. Dihedral angle fluctuations were indeed observed for Phe78, Asp79, and Ser98 and agreement between the MD and the NMR S^2 values were found for shorter simulation time periods during which no transition occurred. On the other hand, the MD has produced reduced mobility compared to NMR. The significant exchange contributions to $R2$ displayed by most residues of this class, suggested a possible implication of microsecond–millisecond processes into the relaxation of these residues, which could not be characterized because of the too short duration of the MD trajectory.

Dynamic analysis of the β -sheet regions

Sheet S1

Within strand A, experimental and calculated S^2 mean values differ slightly because the simulated order parameters for Thr4 and Thr6 are significantly lower than those derived from the NMR relaxation data. The particularly low τ_e values exhibited by these two residues, together with the apparent negative $R2_{\text{ex}}$ for Thr6 suggest that Lipari–Szabo formalism fails to correctly interpret their dynamic behavior. The greater mobility of the beginning of strand A, as suggested by the largest motions sampled by MD, may indeed be influenced by the great flexibility of the amino-terminal segment. Conversely, Val7 and Thr8 at the end of strand A both display highly restrained motions on at least two time scales.

Within strand B, the residue profiles of the two order parameters are strongly similar. The large side chain peptide segment ranging from Val18 to Lys20, at the beginning of the strand displayed constrained motions, whereas residues Ala22 to Ala24 showed higher mobility. Except for Ala24, the whole residues of this strand displayed at least two time scale motions. The unexpected chemical exchange term observed for Val19 may arise from a modulation in the chemical environment resulting from the mobility of the AB loop, by hydrophobic interactions with Leu13.

Within strand E, residues 62 to 64 exhibited a large mobility on the picosecond–nanosecond time scale and significant exchange terms. Their motions are highly correlated with the end of strand B as shown by the MD calculations and, overall, by the Ala24/Ser62 and Ala22/Ser64 $H\alpha$ – $H\alpha$

NOE connectivities (Adjadj et al. 1990). NMR relaxation data for Ser66 were not quantified but its dynamic properties could be similar to those of Lys20, because of their high correlation, as suggested by $H\alpha$ – $H\alpha$ NOE and MD. Leu67 and Ser68 displayed very constrained motions on at least two time scales. For Thr65, the NMR predicted higher mobility than the MD, suggesting implications of slow motions. However, this residue, whose relaxation data could not fit with model 5, did not display any exchange contribution to R2. Thus, Thr65, located in the middle of β -strand E and surrounded by two types of motion, may be affected by specific local mobility, which could not be interpreted by either approach.

Sheet S2

Strand C displayed the highest mean experimental and simulated S^2 values. Ala31 and Val34 displayed rapid motions, whereas Tyr32, Asp33, and Gln36 are subjected to additional slow motions, not interpretable by the Lipari–Szabo formalism.

Residues Val55 and Thr56 at the end of strand D showed constrained motions on the picosecond–nanosecond time scale. The dynamic properties of the beginning of strand D is less clear because of the absence of NMR relaxation data for Ser53 and Ser54. However, Ser54 probably displayed a low level of motions, correlated with the restrained mobility of Asp33, as suggested by the $H\alpha$ – $H\alpha$ NOE observed between the two residues.

Within strand F, Gln94 showed restrained picosecond–nanosecond time scale motions and a significant exchange contribution to R2, indicating a microsecond–millisecond time scale. The dynamic properties of Leu97 remained unclear. Its rather large flexibility detected by NMR was not seen by MD. This discrepancy suggested that Leu97 was subjected to slow motions, however, not qualitatively revealed by the NMR data. As mentioned above, the motions of Ser98 could be disturbed by dihedral angle fluctuations. Noteworthy, Leu97 and Ser98 are located at the end of strand F and at the beginning of the most mobile FG loop. Their motions may, therefore, be perturbed by the large fluctuations of the FG loop; however, both residues have hydrophobic contacts with Tyr32 and influence of complex motions of this neighbor cannot be excluded.

Strand G displayed reduced motions on the picosecond–nanosecond time scale, as shown by the mean experimental and simulated S^2 values. Most also show significant exchange contribution to R2. The beginning of strand G shares similar dynamic properties that the beginning of strand F, consistent with the cross-correlation between the $C\alpha$ motions of Ala109 and Gln94, revealed by NMR and MD. Fluctuations at the other extremity are rather concerted with the loop connecting strands A and B. Therefore, the $R2_{ex}$ contributions may result from a spatial microstate sampling by the AB loop; however, other types of processes may

contribute to the slow (microsecond–millisecond) movements globally animating this strand. The end of strand G, corresponding to the carboxy-terminal part of the protein, may be involved in equilibrium between slightly distinct conformers, and proximity to the aromatic side chain of Phe112 may result in various chemical shifts at the surrounding site.

Dynamic analysis of the loop regions

The motions of loop AB are constrained, as shown by the mean experimental and calculated S^2 . Ser10 and Thr17, located at each end of the loop, both display motions on two time scales that required fit with model 5. Ser11, Ser14, and Asp15 displayed also significantly high exchange contributions to R2. The closeness of the reorienting side chain of Phe112 and backbone motions of the end of strand G, which also displayed $R2_{ex}$, may account for these exchange contributions. However, loop AB may be also affected by multistate exchange processes.

As the BC and DE loops interact strongly together, their motions are highly concerted. Experimental and simulated S^2 values were in agreement for most of the residues for which NMR relaxation data were available, showing fast restrained motions of these two loop regions as a whole. Leu26 and Gln27 of loop BC displayed significant exchange contributions to R2. Thr30 and Asp58 displayed negative $R2_{ex}$, suggesting at least nanosecond fluctuations and Ala57 showed two time scale motions, well described by model 5.

Dynamic analysis of the binding site

The binding pocket is formed by the internal β -sheet 2, including the large FG loop, and by two twisted, antiparallel β -sheets essentially perpendicular to each other, which protrude at the base of the β -sandwich. One of these sheets is closed by the Cys37–Cys47 disulfide bridge, and the other extends from residues Ser72 to Asp87. The cleft also contains a short bubble loop formed by residues Asn48 to Phe52. All of these structural elements have been described in detail in previous papers (Adjadj et al. 1992a,b; Gao 1992).

The β -ribbon joined by the disulfide bridge Cys37–Cys47 displayed experimental and simulated mean values >0.87 , indicative of their rather restrained motions on the picosecond–nanosecond time scale. However, from the NMR data, the mobility of Cys37 and Trp39 seemed to have a larger amplitude, suggesting contributions of slow motions. Cys37 displayed a significantly reduced order parameter of 0.773, a large τ_e value of around 2 nsec, and a very large exchange contribution to R2, also detected for Trp39. The NMR relaxation data for Val40, Thr42, Val44, and Thr46 fit with model 5, consistent with the motion of these residues on two-time scales. For each residue, the slow con-

tribution was constrained, whereas the fast component had a larger amplitude, consistent with the lower simulated S^2 values.

Although the aromatic ring of Phe52 is relatively free to rotate, the backbone motions of Asp51 and Phe52 are highly constrained, in accordance with the well-defined structural properties described for this protein region (Adjadj et al. 1992a; Gao 1992).

The second β -ribbon, ranging from Ser72 to Asp87, also displayed a rather reduced mobility on the picosecond–nanosecond time scale as demonstrated by NMR and MD. However, the two S^2 profiles differed significantly for some residues. For Phe78 and Asp79, the lower S^2_{MD} value enabled us to draw attention to dihedral fluctuations, whereas the lower experimental S^2 values found for Phe76 and Arg82 led us to suppose that slow motions could contribute to the relaxation of their $C\alpha$. Significant contributions to R_2 were also depicted for these residues, in particular Trp83, which exhibited the largest R_{2ex} value (8.9 Hz). The NMR relaxation data for Glu74, Thr81, Thr85, and Asp87 were fit with model 5, suggesting two time scale motions.

As revealed by NMR and MD, the FG loop displayed the largest mobility on the picosecond–nanosecond time scale. However, the dynamic properties of this peptide segment could not be well resolved, mainly because of the lack of NMR data for three glycines and for Ala100.

The loop 72–87 is fixed on the end of strand E by the tripeptide Val69–Arg70–Arg71 and on the beginning of strand F by a small loop closed by the Cys88–Cys93 disulfide bridge. These specific regions are stabilized to the rest of the β -core by hydrophobic interactions involving the aromatic ring of Phe112 (Adjadj et al. 1992a). The experimental and simulated parameters for Val69–Arg71 are typical of highly constrained segments on the picosecond–nanosecond time scale. However, the exceeding experimental S^2 values, together with the negative R_{2ex} values observed for Val69 and Arg70, suggested that the relaxation processes of these residues were influenced by additional internal motions occurring on a time scale of $\sim 10^{-8}$ to $\sim 10^{-3}$ sec (Constantine et al. 1993). The dynamic processes of the residues enclosed by the Cys88–Cys93 disulfide bridge involved two time scale motions, accurately fit by model 5. The slow components were constrained ($S^2_{slow} \approx 0.94$), whereas fast contributions were associated with large amplitudes ($S^2_{fast} \approx 0.86$), in very good agreement with the simulated order parameters.

Conclusions

Analysis of the intramolecular motions of apo-NCS provided us with an opportunity to deduce fundamental properties that could be relevant to both the structural stability and the biological activity of this β -protein.

The first conclusive remark that can be derived from the results presented above concerns the β -sandwich domain of apo-NCS. On the basis of both the experimental and simulated order parameter values, two distinct types of internal mobility were clearly evidenced. Residues displaying reduced S^2 values were observed exclusively at the top of the sheet S1. The stretch of small side chain residues (Ala22–Ala24), located at the end of strand B, are subjected to large amplitude nanosecond motions correlated with the mobility of the beginning of strand A (Thr4–Thr6) and extensive two time scale movements at the start of strand E (Ser62–Thr65). Overall, our results, in particular the significant R_{2ex} terms observed for the first three residues of the well-defined strand E, strongly suggest that this part of the sheet is subject to collective breathing motions (Cheng et al. 1993). With the exception of this structural part, the β -sandwich of apo-NCS is rather rigid. This compactness is maintained by three spatial clusters of structurally related residues displaying restrained motions.

One group fills the center of the interface between the two β -sheets S1 and S2 and is formed by Val7, Thr8 of strand A, Val18 to Lys20 of strand B, Leu67, Thr68 of strand E, Val34 and Gln36 of strand C, Gln94 of strand F, Val 108 and Ala109 of strand G. These residues are tightly packed together through hydrogen bonds or hydrophobic interactions and constitute a compact band, which helps to maintain the dynamics of this protein region highly correlated. The lack of NMR relaxation data for Val21 and Val95 precluded quantification of their dynamic behaviors; however, they likely undergo restrained motions, as their side chains are fully buried into this cluster.

A second group contains the whole AB loop, the tripeptide Val69 to Arg71, and the carboxy-terminal part of the protein ranging from Ile110 to Phe112. The *cis*-configuration of the Pro9 allows the AB loop to be closely packed against both the base of the β -sandwich and the carboxy-terminal region. This spatial proximity leads to hydrophobic contacts with the aromatic ring of Phe112, to hydrogen bonds connecting Ser10 and Ser111, and gives the possibility to a salt bridge between Asp15 and Arg70.

A third group encloses the BC and EF loops, at the top of the β -sandwich. The structural properties of this part of the protein directly result from those of the “Tyrosine-corner” motif, ubiquitously and exclusively found in Greek key β -sandwich proteins (Hemmingsen et al. 1994; Hamill et al. 2000a). In apo-NCS, the aromatic side chain of Tyr32 is wrapped by a pattern of interactions involving Leu26, Gln27, Thr30, Ala31, Ala57, Ala63, Leu97, and Ser98. This cage of residues helps the two loops to pack tightly together against sheets S1 and S2. Noteworthy, the additional slow motions they display could be due to necessary displacements provoked by the damped ring rotation of the buried tyrosine residue (McCammon and Harvey 1989).

The second conclusive remark concerns the dynamics of the binding site loops. Of the three loops, the FG loop exhibits the largest amplitude motions. This greater flexibility may be explained by the preponderance of short residues, such as glycines and alanines, in particular, at the center of the loop. Flexibility may be also enhanced because of the motion freedom of some side chain residues in the absence of ligand (Gln106). However, within the NCS complex, hydrophilic side chain residues of the FG loop are in close contact with the hydrophilic part of the chromophore (Tanaka et al. 1996). Thus, the enhanced dynamic properties of the FG loop would permit considerable accommodation between the cleft and the substrate. Conversely, the rigidity of the backbone of both highly hydrophobic loops 37–47 and 72–87 could be relevant for packing the aromatic residues of the protein against the hydrophobic moiety of the NCS-chromophore.

The last conclusive remark deals with the dynamics properties of the residues lying in the structural interface between the β -sandwich and the functional loops. Their strategic positions lead to suppose that they could play a key role in controlling the dynamic behavior of the protein-binding regions upon complexation with the chromophore. As described above, Tyr32 takes an important part in maintaining the rigidity of the top of the β -sandwich; however, as it also makes contact with Asp99, Asn103, Pro105 of the loop FG, we can speculate that this aromatic residue may have a critical contribution in governing the mobility of this loop. In the same way, Gln36, located in the highly constrained strand C, is one of the most conserved residues among the sequences of all the known NCS-related members of the protein family. Together with its implication in maintaining strand C intimately packed to the base of the protein structure through tight contacts with Val69, Arg70, Val95, and Phe112, Gln36 strongly interacts with the aromatic side chain of Phe73 of the loop 72–87, thereby inducing a high dynamic correlation between the end of strand C and this loop. It is interesting to note that, whereas the beginning of strand C displays concerted motions with the top and the center of the protein, Gln36 is correlated with the other side of the structure. The sandwiched Gly35 could be thus necessary to provide conformational plasticity to the strand. Noteworthy, Gly96, which faces Gly35 in the structure, is also wrapped by the constrained Gln94–Val95 and the more mobile dipeptide segment Leu97–Ser98.

The other common feature of all known NCS-related members of the protein family is the little peptide loop framed by the disulfide bridge Cys88–Cys93. Although the residues of this region display the same (S^2_{fast} , S^2_{slow}) profile, fluctuations of Cys88 and Thr89 are associated with those of Arg71 and Phe112, whereas the mobility of Thr90, Ala91, and Ala92 is instead correlated with that of Ala38, Trp39, Val40, and Val86 located in the binding cavity. These results strongly suggest that this region performs

hinge motions, which could be relevant to the biological function of the protein. In particular, this loop may permit up-and-down motions of the two relatively rigid perpendicular functional loops, 37–47 and 72–87.

Because apo-NCS and immunoglobulin-like domains display structural similarities, they may also share similar stability properties. Hamill et al. (2000b) recently compared immunoglobulin-like β -sandwich proteins and reports that: “The BCEF motif is an efficiently packed unit of structure, which is capped on both ends by hydrophobic residues in the BC and EF loops, and it is probably the stability that results from formation of this structure that drives the folding of Ig-like proteins.” From the analysis reported here, we showed that the corresponding residues in apo-NCS constitute the more rigid parts of the protein structure. Further studies are needed to clarify the relationships between the rigidity and the stability of the immunoglobulin-like structure of apo-NCS.

Materials and methods

Production and purification of apo-NCS secreted by Escherichia coli

^{13}C -labeled proteins were produced using Celton-C liquid medium (Martek) under the same culture conditions as for the unlabeled protein (Heyd et al. 2000). Ammonium sulfate (45%) was added to the culture-supernatant to eliminate some of the contaminants by precipitation. The precipitate was collected by centrifugation for 30 min at 12,000 g and the supernatant was subjected to precipitation with 95% ammonium sulfate. The precipitate was collected by centrifugation and the pellet was dissolved in a minimal volume of 25 mM sodium phthalate at pH 5.5. The resulting solution was thoroughly dialyzed against 25 mM sodium phthalate at pH 5.5. The dialysate was loaded on to a G25 (Pharmacia) column (33 by 2.2 cm). The protein was eluted with 25 mM sodium phthalate at pH 5.5. Fractions containing protein were pooled, diluted twice, and the pH was adjusted to 6.5 with KOH. The resulting solution was loaded onto a DEAE TSK 650S column (1.6 by 22 cm) equilibrated with 12.5 mM sodium phthalate at pH 6.5. The protein was eluted with a NaCl gradient of 0 to 0.3 M (2×200 mL) at a flow rate of 100 mL/H. Fractions containing NCS were concentrated on YM3-Diaflo (Amicon) and loaded onto a G-50 (Pharmacia) column (95 by 2.5 cm) equilibrated with 10 mM NH_4HCO_3 buffer at pH 8. Fractions containing purified NCS were pooled, concentrated, and lyophilized. All purification steps were performed at +4°C. The presence of NCS at each step of purification was detected by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and analytical high performance liquid chromatography (HPLC) (Beckman).

NMR relaxation measurements and data analysis

All NMR spectra were acquired on a Varian Unity500 spectrometer, equipped with a pulsed field gradient triple resonance probe. Pure lyophilized samples of labeled protein were dissolved in 50 mM sodium phosphate buffer at pH 5.5 (100% $^2\text{H}_2\text{O}$). The labeled sample concentration was ~1 mM. The NMR sample tube was

flushed with pure nitrogen gas and sealed, to minimize the amount of dissolved oxygen.

NMR sequences and data processing

R1, R2 and steady-state NOE measurements were carried out at one magnetic field, using the available sequences with delays settled as described by Yamazaki et al. (1994). Specifically, the constant period was set at 13.3 msec ($1/J_{\text{C}\alpha\text{C}\beta}$). Magnetization transfer from ^1H to ^{13}C was achieved during an INEPT delay of 2 by 1.7 msec ($1/2\text{CH}$). The recovery delay before the pulse sequence was set at 2 sec for R1 and R2 experiments and at 4 sec for steady-state NOE measurements. For NOE measurements, individual free induction decays (FIDs) were interleaved, alternatively with and without proton saturation. Proton saturation was applied during the 4-sec relaxation delay and was achieved by a train of 120° ^1H pulses at a reduced RF field strength of 9.7 kHz separated by a 2.5-msec free period. Values of T_1 were determined on the basis of spectra recorded with 22 delays of 5.03 msec to 1363.8 msec, whereas T_2 values were based on 18 experiments recorded with delays of 4 msec to 120.4 msec.

The three relaxation parameters were analyzed in terms of internal motions, using the simple or extended model-free Lipari-Szabo approach. If the correlation function for the fluctuations of the dipolar magnetic interaction within the C-H bond can be described by a single exponential (Lipari and Szabo 1982a,b), the spectral density is expressed as a function of ω by:

$$J(\omega) = \frac{2}{5} \left[S^2 \frac{\tau_R}{1 + \omega^2 \tau_R^2} + (1 - S^2) \frac{\tau_e}{1 + \omega^2 \tau_e^2} \right] \quad (1)$$

The order parameter S^2 specifies the degree of spatial restriction of the bond vector, with the values from 0 for isotropic internal motions to 1 for completely restricted motion. The effective correlation time τ_e is related both to τ_R and to the correlation time for the internal motion τ_i by:

$$\tau_e^{-1} = \tau_i^{-1} + \tau_R^{-1}$$

If the residues display internal motions in a time window close to 1 nsec, the correlation function of the internal motions cannot be described by a single exponential. In this case, the correlation function becomes biexponential and $J(\omega)$ can be expressed as (Clore et al. 1990):

$$J(\omega) = \frac{2}{5} \left[S^2 \frac{\tau_R}{1 + \omega^2 \tau_R^2} + (1 - S_f^2) \frac{\tau_f}{1 + \omega^2 \tau_f^2} + (S_f^2 - S^2) \frac{\tau_s}{1 + \omega^2 \tau_s^2} \right]$$

where

$$S^2 = S_s^2 S_f^2 \quad (2)$$

S_s^2 and S_f^2 are the generalized order parameters for slow (τ_{slow} , nanosecond time scale) and fast (τ_{fast} , picosecond time scale) internal motions. For the backbone $\text{C}\alpha$, τ_f can usually be assumed to be short enough for equation [2] to be reduced to:

$$J(\omega) = \frac{2}{5} \left[S_f^2 S_s^2 \frac{\tau_R}{1 + \omega^2 \tau_R^2} + S_f^2 (1 - S_s^2) \frac{\tau_s}{1 + \omega^2 \tau_s^2} \right] \quad (3)$$

With three relaxation parameters, therefore, it is possible to obtain S_s^2 , S_f^2 and τ_s for each site. To determine the dynamic parameters

for apo-NCS, we first assumed that the correlation function for the internal motion could be described by a single exponential (Lipari-Szabo model 4) (Lipari and Szabo 1982a,b; Mandel et al. 1995; Philippopoulos et al. 1997). The corresponding model-free parameters S^2 and τ_e , which exactly fit the LS equation ($\chi^2 = 0$), were obtained from the NOE (η) and R1 data alone. R2 was then calculated and compared with the experimental value for each residue. The difference between the two R2 values led to the exchange contribution, $R2_{\text{ex}}$. In the case of negative R2 values, the extended model-free parameters (S_{slow}^2 , S_{fast}^2 , τ_{slow}) were then considered (extended Lipari-Szabo model 5).

To determine the most appropriate procedure for modeling the relaxation parameters of each residue, a Monte-Carlo simulation was performed, using 500 generated “experimental” values, providing an error estimate for the microdynamic parameters. If convergence was not reached for 500 trials, model 4 was considered. Indeed, using the extended model, the values of the microdynamic parameters had to exactly fit the experimental values ($\chi^2 = 0$). In addition, if conformational averaging or chemical exchange effects contributed to the transverse relaxation, this criterion was not satisfied and we kept model 4, to estimate the R2 exchange contribution.

MD simulations

MD simulations were performed using the program CHARMM (Brooks et al. 1983) version 24. The initial set of atomic coordinates was obtained from the 1.5 Å crystal structure of NCS (Teplyakov et al. 1993), referred to 1NOA in the protein data bank (Bernstein et al. 1977). Polar hydrogen atoms were built in using the HBUILD algorithm (Brünger and Karplus 1988). The protein was embedded in an equilibrated rectangular water box (6142 molecules) $57 \times 61 \times 58$ Å in size. Bond lengths were kept rigid using the SHAKE-method (Ryckaert et al. 1977) and the integration time step was 1.0 fs. Periodic boundary conditions were applied to eliminate edge effects using the IMAGE facility of CHARMM. A nonbonded cutoff of 11.5 Å was used and the nonbonded list was updated automatically. After the system reached the final temperature of 308K by periodical rescaling of velocities, it was equilibrated for 100 psec. This equilibration was followed by a 1.5-nsec production run without velocity rescaling. The calculation was performed on a SiliconGraphics Indigo2 R10000 during 4300 h of CPU time. Assuming that internal motion is independent of overall tumbling, the reorientation of the CH vector, which contributes to the ^{13}C relaxation can be described by the angular autocorrelation function

$$C(t) = C_r(t)C_i(t)$$

where $C_r(t)$ and $C_i(t)$ are correlation functions for overall rotational tumbling and internal motion, respectively. Before analysis of the trajectory for motion parameters, the overall translation and rotation of apo-NCS was eliminated so that correlation functions depended on internal motions only. The generalized order parameters S^2 were evaluated by estimating the plateau value of the calculated autocorrelation function $C_i(t)$ at one-third of the whole trajectory (Wong and Daggett 1998).

Electronic supplemental material

Supplemental material includes a table with the backbone dynamic parameters (S^2 , τ_e , $R2_{\text{ex}}$, S_{slow}^2 , S_{fast}^2 , τ_{slow}) for apo-NCS calculated from ^{13}C -NMR relaxation data acquired at 35°C.

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References

- Adjadj, É., Mispelter, J., Quiniou, É., Dimicoli, J.L., Favaudon, V., and Lhoste J.M. 1990. Proton NMR studies of apo-neocarzinostatin from *Streptomyces carzinostaticus*. Sequence-specific assignment and secondary structure. *Eur. J. Biochem.* **190**: 263–271.
- Adjadj, É., Quiniou, É., Mispelter, J., Favaudon, V., and Lhoste, J.M. 1992a. Three dimensional solution structure of apo-neocarzinostatin from *Streptomyces carzinostaticus* determined by NMR spectroscopy. *Eur. J. Biochem.* **203**: 505–511.
- . 1992b. The seven-stranded β -sandwich structure of apo-neocarzinostatin as compared to the immunoglobulin domain. *Biochimie* **74**: 853–858.
- Bernstein, F.C., Koetzle, T.F., Williams, G.J., Meyer, E.E. Jr., Brice, M.D., Rodgers, J.R., Kennard, O., Shimanouchi, T., and Tasumi, M. 1977. The Protein Data Bank: A computer-based archival file for macromolecular structures. *J. Mol. Biol.* **112**: 535–542.
- Brooks, B.R., Bruccoleri, R.E., Olafson, B.D., States, D.J., Swaminathan, S., and Karplus, M. 1983. CHARMM: A program for macromolecular energy minimization and dynamics calculations. *J. Comput. Chem.* **4**: 187–217.
- Brünger, A.T. and Karplus, M. 1988. Polar hydrogen positions in proteins: Empirical energy placement and neutron diffraction comparison. *Proteins* **4**: 148–156.
- Carr, P.A., Erickson, H.P., and Palmer, A.G. 3rd. 1997. Backbone dynamics of homologous fibronectin type III cell adhesion domains from fibronectin and tenascin. *Structure* **5**: 949–959.
- Chandrasekhar, I., Clore, G.M., Szabo, A., Gronenborn, A.M., and Brooks, B.R. 1992. A 500 ps MD simulation study of interleukin-1 beta in water. Correlation with nuclear magnetic resonance spectroscopy and crystallography. *J. Mol. Biol.* **226**: 239–250.
- Chatfield, D.C., Szabo, A., Bernard, R., and Brooks, B.R. 1998. Molecular dynamics of staphylococcal nuclease: Comparison of simulation with ^{15}N and ^{13}C NMR relaxation data. *J. Am. Chem. Soc.* **120**: 5301–5311.
- Cheng, J.W., Lepre, C.A., Chambers, S.P., Fulghum, J.R., Thomson, J.A., and Moore, J.M. 1993. ^{15}N NMR relaxation studies of the FK506 binding protein: Backbone dynamics of the uncomplexed receptor. *Biochemistry* **32**: 9000–9010.
- Clore, G.M., Szabo, A., Bax, A., Kay, L.E., Driscoll, P.C., and Gronenborn, A.M. 1990. Deviations from the simple two-parameter model-free approach to the interpretation of nitrogen-15 nuclear magnetic relaxation of proteins. *J. Am. Chem. Soc.* **112**: 4989–4991.
- Constantine, K.L., Friedrichs, M.S., Goldfarb, V., Jeffrey, P.D., Sheriff, S., and Mueller, L. 1993. Characterization of the backbone dynamics of an anti-digoxin antibody V_L domain by inverse detected ^1H - ^{15}N NMR: Comparisons with X-ray data for Fab. *Proteins* **15**: 290–311.
- Constantine, K.L., Colson, K.L., Wittekind, M., Friedrichs, M.S., Zein, N., Tuttle, J., Langley, D.R., Leet, J.E., Schroeder, D.R., and Lam, K.S. 1994. Sequential ^1H , ^{13}C , and ^{15}N NMR assignments and solution conformation of apokardacin. *Biochemistry* **33**: 11438–11452.
- Eriksson, M.A., Berglund, H., Härd, T., and Nilsson, L.A. 1993. Comparison of ^{15}N NMR relaxation measurements with a molecular dynamics simulation: Backbone dynamics of the glucocorticoid receptor DNA-binding domain. *Proteins* **17**: 375–390.
- Farrow, N.A., Muhandiram, R., Singer, A.U., Pascal, S.M., Kay, C.M., Gish, G., Shoelson, S.E., Pawson, T., Forman-Kay, J.D., and Kay, L.E. 1994. Backbone dynamics of a free and phosphopeptide-complexed Src homology 2 domain studied by ^{15}N NMR relaxation. *Biochemistry* **33**: 5984–6003.
- Gao, X. 1992. Three-dimensional solution structure of apo-neocarzinostatin. *J. Mol. Biol.* **225**: 125–135.
- Hamill, S.J., Cota, E., Chothia, C., and Clarke, J. 2000a. Conservation of folding and stability within a protein family: The tyrosine corner as an evolutionary cul-de-sac. *J. Mol. Biol.* **295**: 641–649.
- Hamill, S.J., Steward, A., and Clarke, J. 2000b. The folding of an immunoglobulin-like Greek key protein is defined by a common-core nucleus and regions constrained by topology. *J. Mol. Biol.* **297**: 165–178.
- Hazes, B. and Hol, W.G. 1992. Comparison of the hemocyanin β -sandwich with other Greek key β -sandwichs: Possible importance of the " β -zipper" in protein structure and folding. *Proteins* **12**: 278–298.
- Hemmingsen, J.M., Gernert, K.M., Richardson, J.S., and Richardson, D.C. 1994. The tyrosine corner: A feature of most Greek key β -sandwich proteins. *Protein Sci.* **3**: 1927–1937.
- Heyd, B., Lerat, G., Adjadj, É., Minard, P., and Desmadril, M. 2000. Reinvestigation of the proteolytic activity of neocarzinostatin. *J. Bact.* **82**: 1812–1818.
- Kappen, L.S., Napier, M.A., and Goldberg, I.H. 1980. Roles of chromophore and apo-protein in neocarzinostatin action. *Proc. Natl. Acad. Sci.* **77**: 1970–1974.
- Kay, L.E. 1998. Protein dynamics from NMR. *Nature Struct. Biol.* (Suppl.) **5**: 513–517.
- Kim, K.H., Kwon, B.M., Myers, A.G., and Rees, D.C. 1993. Crystal structure of neocarzinostatin, an antitumor protein–chromophore complex. *Science* **262**: 1042–1046.
- Lee, L.K., Rance, M., Chazin, W.J., and Palmer, A.G. 3rd. 1997. Rotational diffusion anisotropy of proteins from simultaneous analysis of ^{15}N and ^{13}C alpha nuclear spin relaxation. *J. Biomol. NMR* **9**: 287–298.
- LeMaster, D.M. and Kushlan, D.M. 1996. Dynamical mapping of *E. coli* thioredoxin via ^{13}C NMR relaxation analysis. *J. Am. Chem. Soc.* **118**: 9255–9264.
- Lipari, G. and Szabo, A. 1982a. Model-free approach to the interpretation of nuclear magnetic resonance relaxation in macromolecules. I. Theory and range of validity. *J. Am. Chem. Soc.* **104**: 4546–4559.
- . 1982b. Model-free approach to the interpretation of nuclear magnetic resonance relaxation in macromolecules. 2. Analysis of experimental results. *J. Am. Chem. Soc.* **104**: 4559–4570.
- Mandel, A.M., Akke, M., Palmer, A.G. III. 1995. Backbone dynamics of *Escherichia coli* ribonuclease HI: Correlation with structure and function in an active enzyme. *J. Mol. Biol.* **246**: 144–163.
- McCammon, J.A. 1984. Protein dynamics. *Rep. Prog. Physics* **47**: 1–46.
- McCammon, J.A. and Harvey, S.C. 1989. *Dynamics of proteins and nucleic acids*. Cambridge University Press, Cambridge, UK.
- Mispelter, J., Izadi-Pruneyre, N., Quiniou, É., and Adjadj, É. 2000. Simple and accurate determination of global tau(R) in proteins using (13)C or (15)N relaxation data. *J. Magn. Reson.* **143**: 229–232.
- Mispelter, J., Lefevre, C., Adjadj, É., Quiniou, É., and Favaudon, V. 1995. Internal motions of apo-neocarzinostatin as studied by ^{13}C NMR methine relaxation at natural abundance. *J. Biomol. NMR* **5**: 233–244.
- Palmer, A.G. 3rd. 1997. Probing molecular motion by NMR. *Curr. Opin. Struct. Biol.* **7**: 732–737.
- Philippopoulos, M., Mandel, A.M., Palmer, A.G., and Lim, C. 1997. Accuracy and precision of NMR relaxation experiments and MD simulations for characterizing protein dynamics. *Proteins* **28**: 481–493.
- Redfield, C., Boyd, J., Smith, L.J., Smith, R.A., and Dobson, C.M. 1992. Loop mobility in a four-helix-bundle protein: ^{15}N NMR relaxation measurements on human interleukin-4. *Biochemistry* **31**: 10431–10437.
- Ryckaert, J.P., Ciocotti, G., and Berendsen, H. 1977. Numerical-integration of cartesian equations of motion of a system with constraints-molecular dynamics of N-alkanes. *J. Comput. Phys.* **23**: 327–341.
- Sakata, N., Mase, T., Ikeno, S., Hori, M., and Otani, T. 1993. The amino acid sequence of actinoxanthin apoprotein deduced from the base sequence of the gene. *J. Antibiotics* **46**: 1475–1477.
- Schurr, J.M., Babcock, H.P., and Fujimoto, B.S. 1994. A test of the model-free formulas. Effects of anisotropic rotational diffusion and dimerization. *J. Magn. Reson. B* **105**: 211–224.
- Tanaka, T., Hiram, M., Fujita, K., Imajo, S., and Ishiguro, M. 1993. Solution structure of the antitumor antibiotic neocarzinostatin, a chromophore-protein complex. *J. Chem. Soc.; Chem. Comm.* 1205–1207.
- Tanaka, T., Hiram, M., Imajo, S., and Ishiguro, M. 1996. Supramolecular neocarzinostatin complex. In *Comprehensive supramolecular chemistry*, vol. 4, pp. 287–309. Elsevier Science Ltd., Oxford, UK.

- Teplyakov, A., Obmolova, G., Wilson, K., and Kuromizu, K. 1993. Crystal structure of apo-neocarzinostatin at 0.15-nm resolution. *Eur. J. Biochem.* **213**: 737–741.
- Van Roey, P. and Beerman, T.A. 1989. Crystal structure analysis of auromycin apoprotein (macromycin) shows importance of protein side chains to chromophore binding selectivity. *Proc. Natl. Acad. Sci.* **86**: 6587–6591.
- Wong, K.B. and Daggett, V. 1998. Barstar has a highly dynamic hydrophobic core: Evidence from molecular dynamics simulations and nuclear magnetic resonance relaxation data. *Biochemistry* **37**: 11182–11192.
- Xu, Y.J., Zhen, Y.S., and Goldberg, I.H. 1994. C1027 chromophore, a potent new enediyne antitumor antibiotic, induces sequence-specific double-strand DNA cleavage. *Biochemistry* **33**: 5947–5954.
- Yamasaki, K., Saito, M., Oobatake, M., and Kanaya, S. 1995. Characterization of the internal motions of *Escherichia coli* ribonuclease HI by a combination of ^{15}N -NMR relaxation analysis and molecular dynamics simulation: Examination of dynamic models. *Biochemistry* **34**: 6587–6601.
- Yamazaki, T., Muhandiram, R., and Kay, L.E. 1994. NMR experiments for the measurement of carbon relaxation properties in highly enriched, uniformly ^{13}C , ^{15}N -labeled proteins: Application to $^{13}\text{C}\alpha$ carbons. *J. Am. Chem. Soc.* **116**: 8266–8278.