Dynamic Structures of Granulocyte Colony-Stimulating Factor Proteins Studied by Normal Mode Analysis: Two Domain-Type Motions in Low Frequency Modes[1)]

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In this study, granulocyte colony-stimulating factor (GCSF) proteins were chosen as subjects for normal mode analysis. As helical cytokines with a four helix bundled type topology, they were classified into long chain and short chain groups by Sprang and Bazan. Normal mode calculations were also carried out with leukemia inhibitory factor (LIF), interleukin-6 (IL-6), and growth hormone (GH) as members of the long chain group and GCSF and IL-2 and IL-4 as members of the short chain group. For the GCSF families it was found that the fluctuations in the helical region are smaller than in the loop region, and it is clear that on the whole the smaller fluctuation residues belong to a large hydrophobic core region. Thus, it can be imagined how the receptor binding sites approach the receptor within the normal time-scale of pico seconds. In addition, two similar domain-type motions in low frequency modes were found with proteins in the long chain group, although we never observed any sequence similarity in the two separate two-domain regions in each protein of the long chain group. On the other hand, these two domain-type motions were not clear in proteins of the short chain group.

Key words granulocyte colony-stimulating factor (GCSF); normal mode analysis; interleukin-6 (IL-6); cytokine

Quake *et al.* have tested[2)] and demonstrated the supposition that polymer motion can be described by recording the images of partially extended 20 μm-long pieces of DNA driven by Brownian motion. This means that the motion of a biological molecule can be described by a set of normal modes. B-factors derived from X-ray crystallography are usually considered to give a picture of atomic fluctuations. However, it is difficult to identify the real internal motion of a molecule from B-factors only, because external motion, such as lattice disorder, also contributes to B-factors. With this in mind, Kidera and Go calculated[3,4)] the internal motions of human lysozyme by deducting the external motions from the B-factors and, consequently, the normal modes of a single lysozyme molecule were shown as a model of the atomic fluctuations. Many helical cytokines, such as growth hormone (GH), granulocyte colony-stimulating factor (GCSF), interleukins (IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-11), interferons (IFN-α, IFN-β, IFN-γ), leukemia inhibitory factor (LIF), and ciliary neurotrophic factor (CNTF), have been shown to adopt an "up-up-down-down" type topology. For a better understanding of cytokine functions, it is important to study not only their static but also their dynamic, structure.

In this study, GCSF proteins were chosen for normal mode analysis. GCSF was particularly useful, because there are three kinds of tertiary structures registered in the Protein Data Bank (PDB), *i.e.* structures for bovine, human, and canine GCSFs. The sequence identity among human, bovine and canine GCSFs is about 81%, and their tertiary structures are similar to each other within a *rmsd* of 2.4 Å for Cα atoms. Although the X-ray structures were all obtained at high resolution (2.3 to 1.7 Å), even in the case of bovine GCSF with the highest resolution of 1.7 Å, each has a lacking region in the cd-loop, as shown in Fig. 1. As it is common to all the GCSFs, this region may be too flexible for its structure to be defined. On the other hand, helical cytokines with a four helix bundled type topology have been classified into long chain and short chain groups.[5,6)] To elucidate the characteristics of such four helix bundled proteins, normal mode calculations were carried out on five additional proteins, with LIF, GH, and IL-6 in the long chain group together with GCSF and IL-2 and IL-4 in the short chain group. The results show that two domain-type motions in low frequency modes were observed in the long chain group. However this characteristic of the long chain group was not found in the short chain group. Thus, from a dynamic point of view our paper supports the classification of four helix bundled cytokines into two groups with the long and short chains.

Experimental

Protein Coordinates Except for IL-6 (not registered yet), the coordinates in the PDB were used for the normal mode calculations. The coordinates of bovine GCSF (1BGC), human GCSF (1RHG_A, _B, _C) and canine GCSF (1BGD, 1BGE_A, 1BGE_B) were modeled by adding each lacking region in the cd-loop using BIOCES[E].[7)] In Fig. 1, d represents the added residues which were in the lacking region. N termini (1-8) and C termini (173, 174) residues were not modeled (–). The structures were energetically optimized in two steps. In the first step, only the added residues were optimized and, next, the whole molecule was similarly treated. The modeling coordinates of IL-6 were obtained from the paper by Sumikawa and Suzuki,[8,9)] in which the structural model of IL-6, constructed based on bovine granulocyte colony-stimulating factor (bovine GCSF) and evaluated by comparison with X-ray[10)] and NMR[11,12)] experimental data, was considered to be of a high quality.

Normal Mode Analysis The thermal fluctuations were calculated using the molecular mechanics method.[13,14)] The molecular structure was energetically optimized with a slightly modified force field of AMBER[15)] by using our program of molecular mechanics and normal mode analysis.[16)] A threshold of 0.17 kJ/mol Å was used as the maximal

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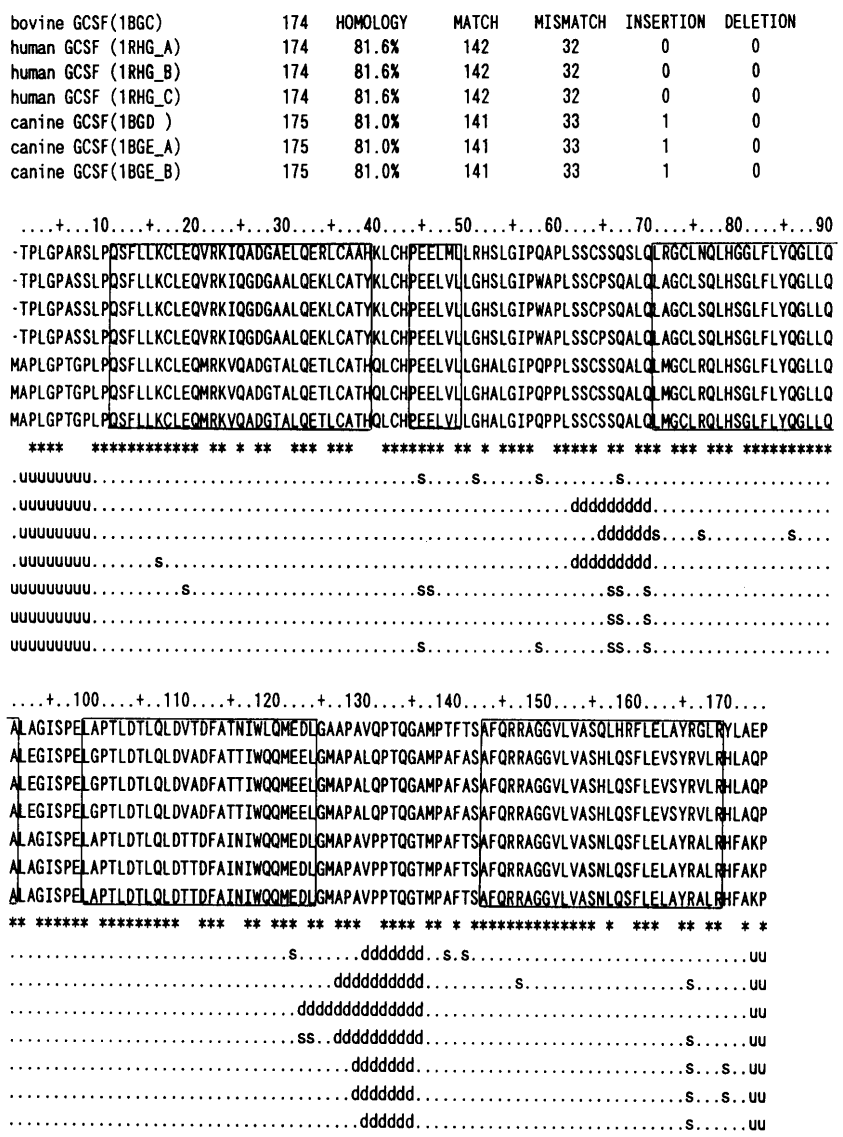

Fig. 1. The Region Lacking Coordinates on Bovine, Human and Canine GCSFs in PDB

d, lacking all the atoms of the amino acid residue; s, lacking the side chain; *, the same residue in all the GCSFs. u, N termini and C termini which were not constructed.

component of the atomic gradients. This modification may prevent the drastic collapse of the molecules from their initial conformation, since the constraints on the atoms are gradually reduced during the energy optimization. In our calculation, we assumed that the molecule was *in vacuo*. The electrostatic potential and the van der Waals' potential were cut off at 9.0 Å and switched smoothly and continuously to a value of zero at 10.0 Å. Therefore, all interactions beyond 10.0Å were neglected, and the non-bond atom-pair lists were updated at every step of the energy minimization. Although the energy minimization was performed with Cartesian coordinates of 3N freedoms, where N is the number of atoms, for normal mode analysis all the freedoms were described by the torsion angles except for the peptide C–N bond. We modified the parameters of the S–S bond since the bending and torsion parameters are assumed to be zero only during normal mode analysis. The fluctuation of the atoms was calculated by assuming a temperature of 300°K using normal modes and vibration frequencies. Normal mode calculations with bovine GCSF were carried out for the six structures energetically optimized under different conditions. The average of each *rmsd* and its standard deviation between the six structures were 0.4 and 0.07 Å, respectively. The motional correlation coefficients of each Cα atom pair were calculated by the Wako method,[17] and the fluctuation directions from Molcat.[18] The classification of domain-type motions into groups was carried out using the algorithm calculation proposed by Hayward *et al.*[19]

Results and Discussion

Cα Deviation The secondary structures of GCSF are all α-helical, and GCSF possesses four helices bundled in an "up-up-down-down" type of topology. The structure of bovine GCSF is illustrated in Fig. 2.[20] There are four helices and two long loops, forming a large hydrophobic core, and a short helix in the ab-loop called helix E. For each residue, a discrimination is made by coloring hydrophobic or hydrophilic residues with magenta or blue, and this is shown in Fig. 3. It can be seen that the hydrophobic core is compact formed with the helices bundled together.

Figure 4-1a shows the fluctuations of Cα atoms obtained from the normal modes of bovine GCSF. The upper bars in Fig. 4 show the helical regions. To inspect the four main helices more precisely, Fig. 5 is a blowup of the helical region in Fig. 4-1a. It can be seen that the fluctuation in the helical region is smaller than in the loop region, and the fluctuation of the short helix E on the ab-loop is great than those of the main helices A, B, C and D. Moreover,

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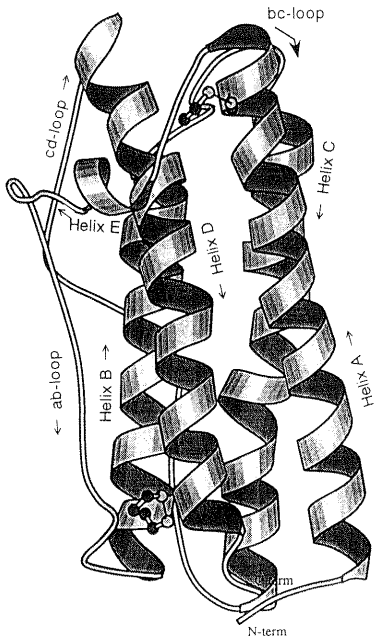

Fig. 2. A Schematic Diagram of the GCSF Structure

The figure was produced using the MOLSCRIPT[20] program. A, B, C, and D are the four main helices, and E is the short helix on the ab-loop. A pair of balls and sticks represents an S–S bond.

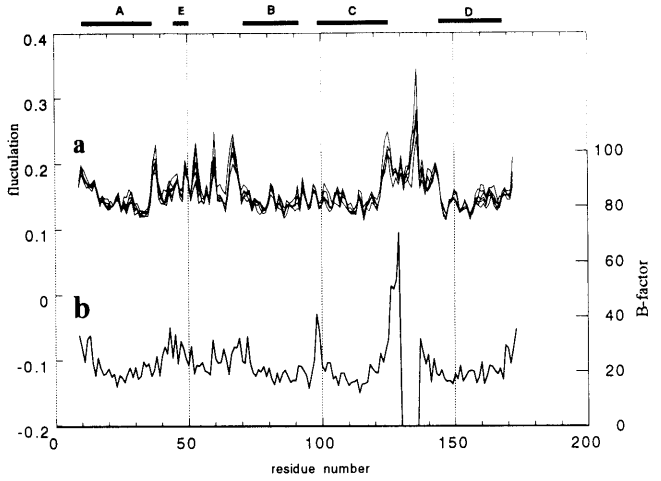

Fig. 4-1a. Fluctuations of Cα Atoms Obtained from the Normal Modes of Bovine GCSF for Six Structures Obtained under Various Energetically Optimized Conditions (Left Side Axis) and 4-1b. B-Factors of Bovine GCSF(1BGC) (Right Side Axis)

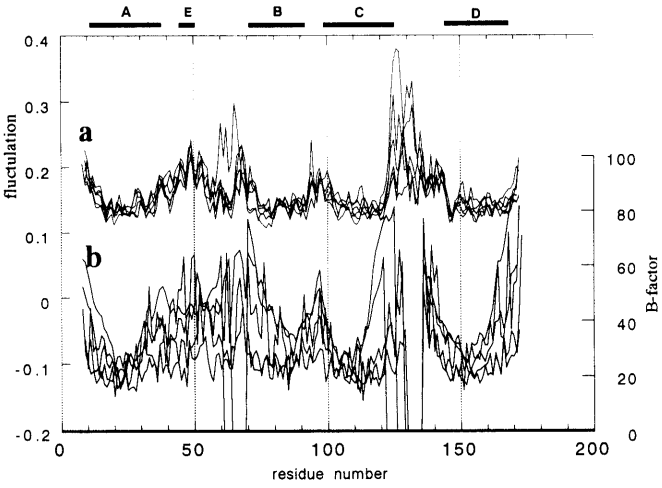

Fig. 4-2a. Fluctuations of Cα Atoms Obtained from the Normal Modes of Human and Canine GCSFs for Each of the Three PDB Structures (Left Side Axis) and 4-2b. B-Factors of Human and Canine GCSF for Each of the Three PDB structures (Right Side Axis)

A lacking region in the cd-loop was modelled for each of the human, bovine, and canine GCSFs. B-factors are large in the region with no coordinates.

from Fig. 2 it can be determined that, since the two long loops of the ab- and cd-loops are against helices B and D, these loops suppress the fluctuations of helices B and D. Hence, the fluctuations of helices A and C are a little greater than those of helices B and D as shown in Fig. 5. The lower residues in Fig. 2, namely the N termini of helices A and B, and the C termini of helices C and D, seem to fluctuate more. Figure 4-2a shows the fluctuations of Cα atoms obtained from the normal mode calculations of human and canine GCSFs. The results are very similar to that of bovine GCSF. Regarding each Cα atom on the protein surface with a large solvent accessibility value, the fluctuations are smaller in the helices than in the loops. It is clear that, on the whole, the smaller fluctuating residues in the four helices belong to the hydrophobic core region. The B-factors of bovine GCSF (1BGC), shown in Fig. 4-1b, are smaller in the helical region than in the loop region, which is the same as the fluctuation results obtained by normal mode analysis. The B-factors in the main helices were about 20, which is appropriate for a rigid structure. As regards the bc-loop, which is seven residues shorter than the ab- and cd-loops, the B-factor is slightly greater, although the C α fluctuations of the bc-loop are similar to those of the B and D helices in Fig. 4-1a. The B-factors of human and canine GCSFs (1RHG, 1BGD and 1BGE) are shown in Fig. 4-2b, with the same points shown as in Fig. 4-1b.

S–S Bridges As shown in Fig. 2, there are four Cys residues (36C, 42C, 64C, 74C) in bovine GCSF. Both the former and latter two residues are a pair of S–S bridges. However, while the former is not necessary for biological activity, the latter is.[21] Cα fluctuations were found to be larger in 42C and 64C which belong to the ab-loop and smaller in 36C and 74C which belong to the A and B helices, respectively. There seems to be no motional restriction from 36C and 74C to 42C and 64C, respectively, as the calculation of normal modes for bovine GCSF with no S–S bridges gave similar results. Thus, it appears that the S–S bridges have no effect on fluctuations within the pico-second time-scale in low frequency modes.

Receptor Binding Sites Using alanine scanning, Young *et al.* reported on the receptor binding sites of human GCSF which include 16K, 19E, 23K, 109D and 112D, and they showed that 19E is the most important.[22] These residues are shown in Fig. 6, and are located on the surface which is exposed to the solvent and central regions of helices A and C. It can be seen that these residues show smaller fluctuations. The motional correlation coefficients of each Cα atom pair are shown in Fig. 7-1, and in Fig. 7-2 which is a blowup of Fig. 7-1 involving the area crossing 15—25 and 105—115. The correlations between any two residues of 16K, 19E, and 23K in helix A and 109D and 112D in helix C are positive, which means that the

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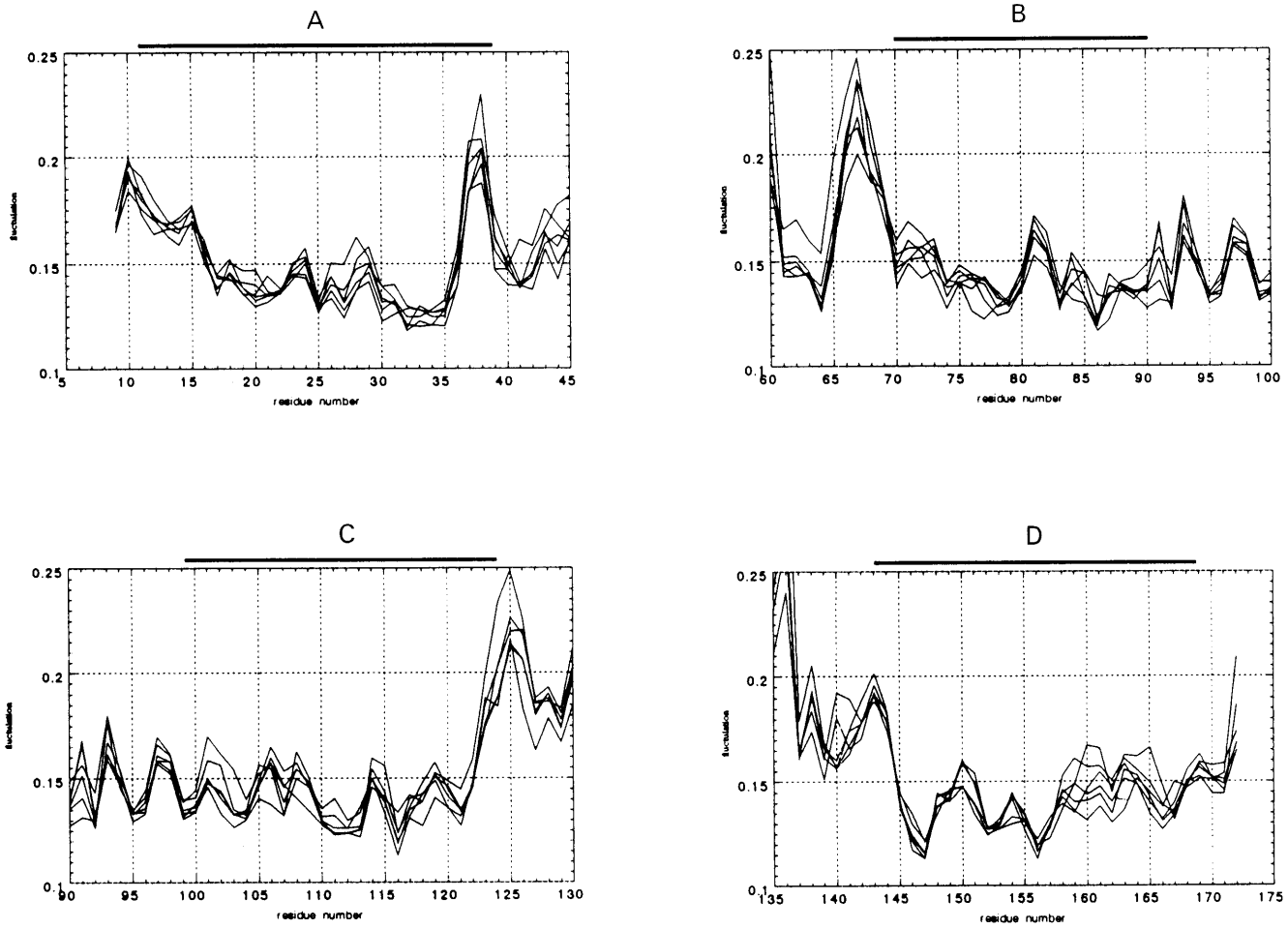

Fig. 5. Fluctuations of Cα Atoms in Regions of the Four Helices from Bovine GCSF

This is a blowup of the helical region from Fig 4-1a.

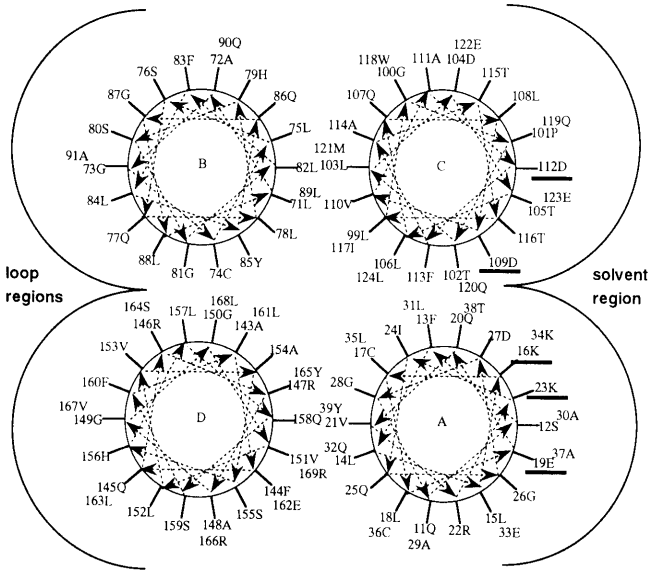

Fig. 6. Wheel Plots for the Four Main Helices from Human GCSF

Solvent region near helices A and C and loop regions of ab- and cd- loops near helices B and D are shown in the figure.

fluctuational correlations are the same. Similar results were obtained for bovine and canine GCSFs, as shown in Figs. 7-3 and 7-4. Thus, it can be imagined that the receptor binding sites approach the receptor within the normal mode time-scale in low frequency modes.

Fluctuations Separated into Two Domains The lower frequency modes which involve the whole molecule were extracted from the calculation results of mouse epidermal growth factor.[23] In general, for a better understanding of the dynamical aspects of the bimolecule it is important to extract low frequency modes using normal mode calculations. It has been supposed that the GCSF structure consists of just one domain because the large hydrophobic core is formed compactly with the four helices bundled together. However, the lower frequency vibrational modes were shown to include two domain-type motions, and three rotational motions for the main part as shown in Figs. 8-1, 8-2, and 8-3. Using the algorithm calculation proposed by Hayward *et al.* as described in the Experimental, the residues forming the two domain-type motions were distinguished for human GCSF, as shown in Fig. 9. The residue numbers separated into the two domain regions with the rotational motions are described in Table 1. From the two domain motions in Fig. 8 it appears that the four bundled helices are bending and twisting with the vibration center located in the middle of each helix. Normal mode calculations with bovine GCSF were carried out on six structures, and each of the three structures for human and canine GCSFs, yielding the result that GCSFs have two such separate domain-type motions. Figure 10 shows

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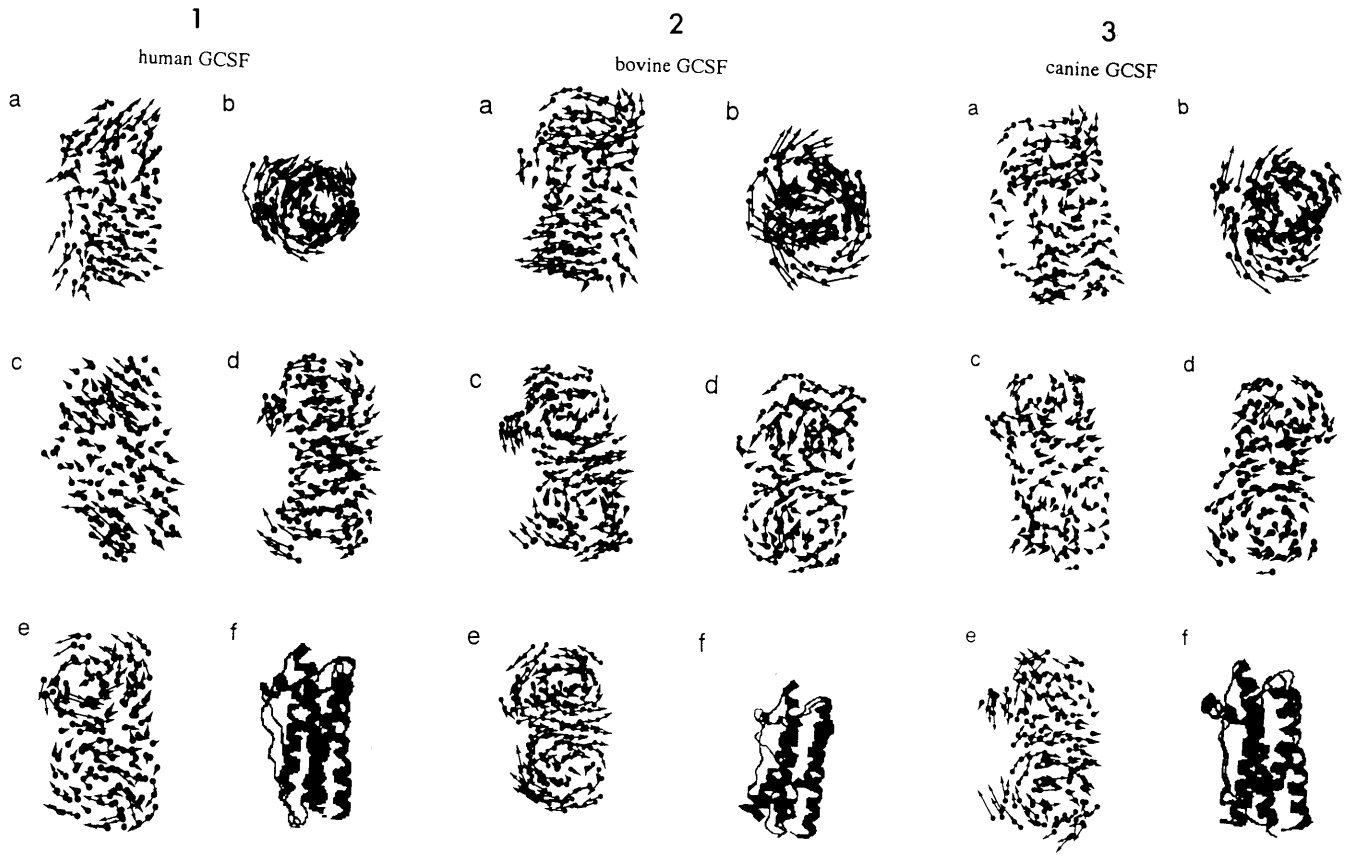

Fig. 8. Two Domain-Type Motions in Low Frequency Modes for Human, Bovine, and Canine GCSFs

The viewpoint in a, c, and e is the same as in f which is a schematic diagram of GCSF; b is a top view of a; the viewpoint in d is rotated by 90 degrees around the y-axis anti-clockwise from c. The wavenumber frequencies (cm^{-1}) were as follows; 1a, b: 14.1 cm^{-1}; c, d: 16.9 cm^{-1}; e: 14.5 cm^{-1}; f: schematic diagram of human GCSF; 2a, b: 15.2 cm^{-1}; c, d: 18.0 cm^{-1}; e: 19.0 cm^{-1}; f: schematic diagram of bovine GCSF; 3a, b: 13.9 cm^{-1}; c, d: 14.1 cm^{-1}; e: 17.7 cm^{-1}; f: schematic diagram of canine GCSF. Normal mode vectors for Cα atoms in which the amplitude was multiplied by 400 are shown with arrows.

that human GCSF has 32 residues for the large hydrophobic core region surrounded by four main helices, namely 18 residues in the upper domain and 14 in the lower domain. The residues which have a strong hydrophobic interaction with other residues in the hydrophobic core are in the central region of the helices and belong to both the upper and lower domains.

Comparison with Other Cytokines Similar two domain-type motions were found with GH and LIF which belong to the long chain group as shown in Figs. 11a, and 11b, but this was not clear with IL-2 and IL-4 in the short chain group (Figs. 11c, d). From these results, it may be a rule that the two domain-type motions are characteristic of the long chain group with the four helices bundle. The normal mode calculation of the modeled IL-6 structure in the long chain group, which was the target of our previous modeling study and normal mode analysis, although it has no experimental PDB coordinates yet, also gave such two domain-type motions as shown in Fig. 12. This observation supports the above rule in which the two domain-type motions are included in the long chain group. Although cytokines such as GCSF, LIF, GH, and IL-6 in the long chain group have low sequence similarity to each other, the two domain-type motions in low frequency modes were found to be common to all. Moreover, the regions in which two domain-type motions are observed are also conserved between them. Thus, the classification into long and short chain groups that Sprang and Bazan proposed for

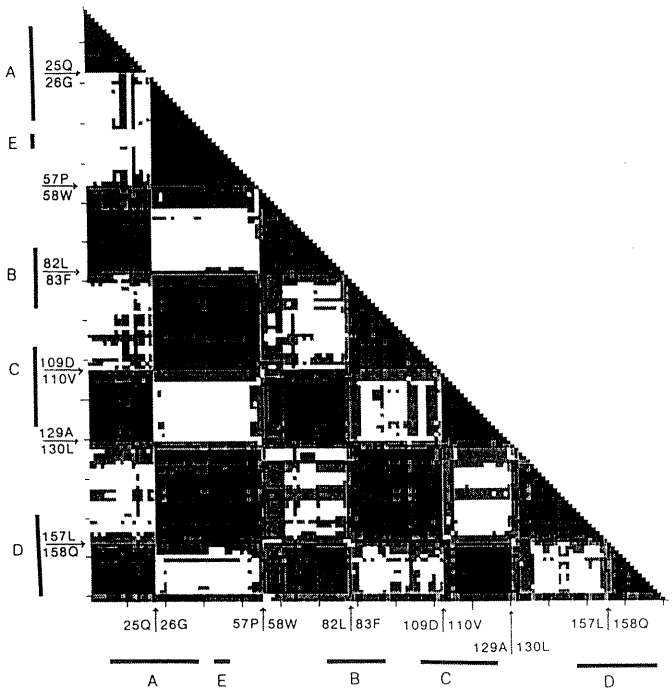

Fig. 9. Two Domain-Type Motions

Two domain-type motions are described in white and black using the algorithm calculation by Hayward *et al.*[19] The amino acid residue numbers forming the boundary regions between the two domain-type motions are shown in the figure. Secondary structures of α helices are represented as thick bar lines.

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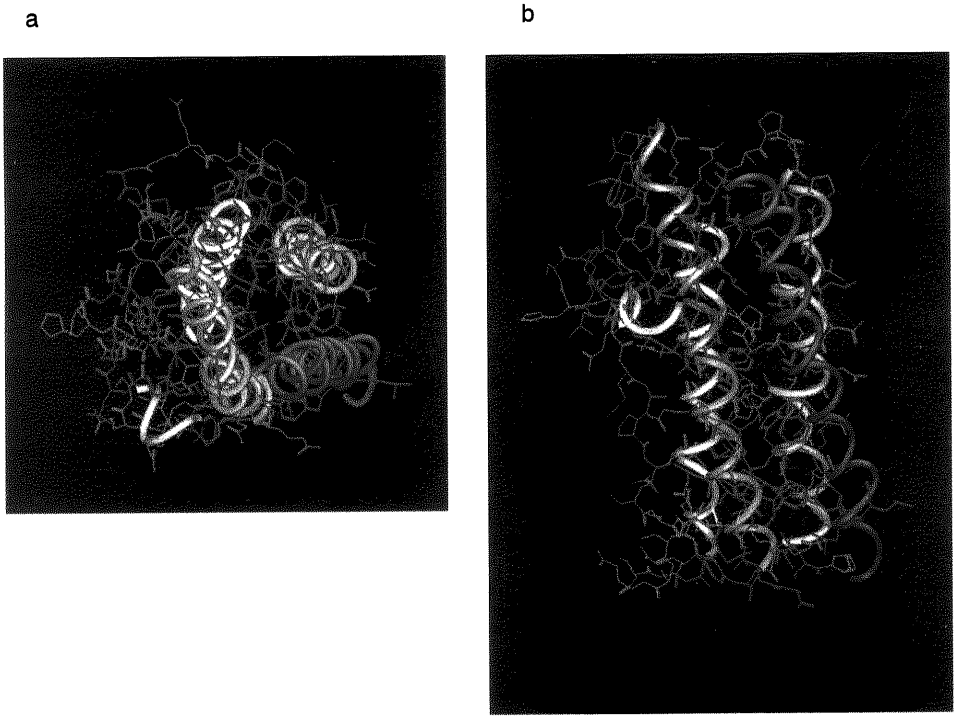

Fig. 3. Hydrophobic and Hydrophilic Representation of Bovine GCSF

Hydrophobic residues are shown in magenta, and hydrophilic residues in cyan. a is the top view of b, and hydrophobic residues are associated with the inside. b is the same viewpoint as in Fig. 2.

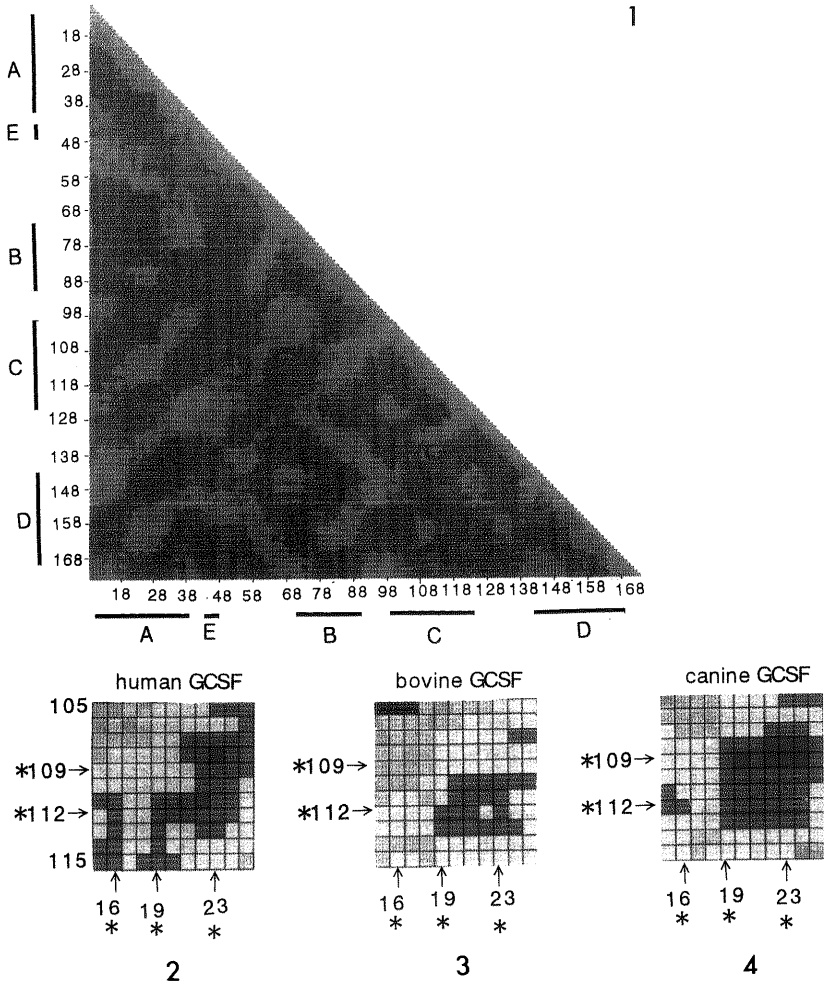

Fig. 7. Motional Correlation Coefficients of Each Cα Atom Pair

1: The whole area of human GCSF. The regions of helices A, B, C, and D are shown as thick bar lines; 2: The area crossing 15—25 and 105—115 in human GCSF; 3: The area crossing 15—25 and 105—115 in bovine GCSF; 4: The area crossing 15—25 and 105—115 in canine GCSF. Red: positive value (deep red : highest positive value). Blue: negative value.

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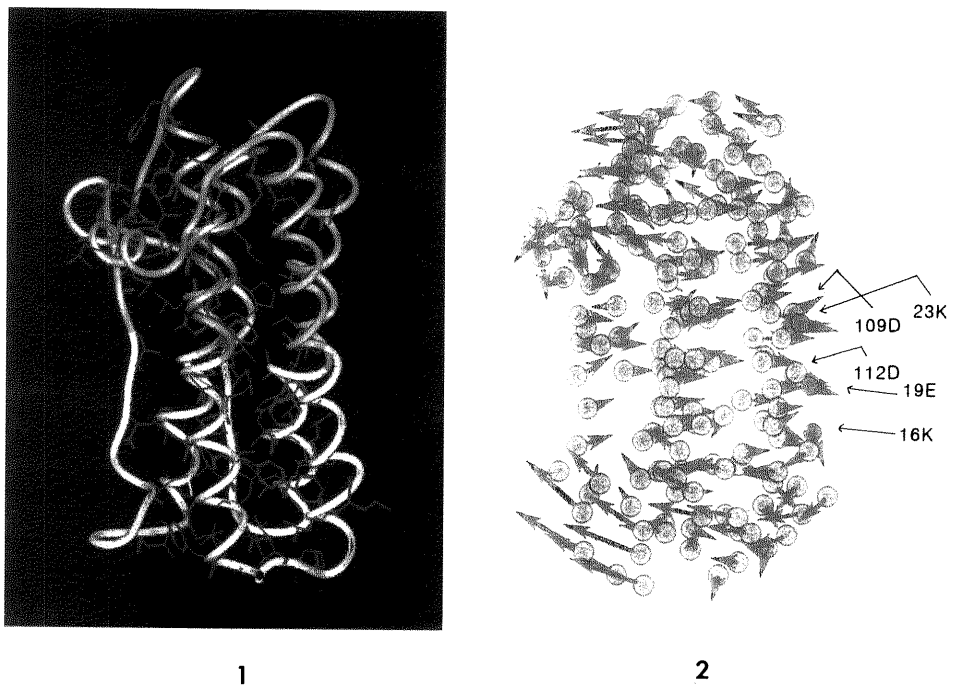

Fig. 10-1. Hydrophobic Residues in the Two Domains-Type Motions of Human GCSF and 10-2. The Amino Acid Residues, 16K, 19E, 23K, 109D and 112D in the Receptor Binding Sites

1) The residues forming the two domain-type motions distinguished in the tube model are colored yellow and white. 2) This figure is the same as Fig. 8-1e.

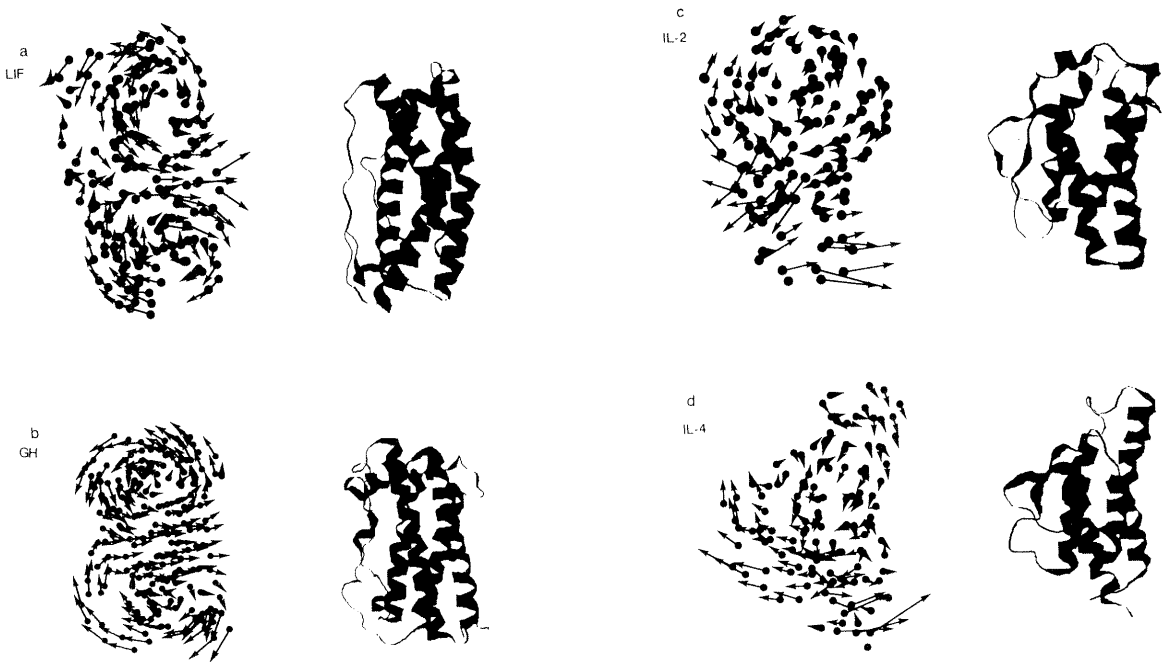

Fig. 11. Domain-Type Motions in Low Frequency Modes for Other Cytokines (LIF, GH, IL-2 and IL-4)

a, LIF; b, GH; both from the long chain group. Two domain-type motions similar to GCSF were found; c, IL-2; d, IL-4; both from the short chain group. Two domain-type motions similar to GCSF were not clearly detected. Wavenumber frequencies (cm^{-1}) are as follows. LIF, 15.9 cm^{-1}; GH, 17.9 cm^{-1}; IL-2, 13.2 cm^{-1}; IL-4, 19.5 cm^{-1}.

Normal mode vectors for Cα atoms in which the amplitude was multiplied by 400 are shown with arrows.

cytokines bundled into four helices has been confirmed dynamically from our studies of normal mode analysis.

Conclusion

Normal mode analysis was carried out on bovine GCSF. It was found that the fluctuation in the helical region is smaller than in the loop region, and that the smaller fluctuating residues belong to the large hydrophobic core region which is surrounded by the four helices bundled together. Normal mode calculations showed that bovine GCSF has two separate domain-type motions in low frequency normal modes. The same results were also

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Table 1. The Residue Numbers Separated into the Two Domain Regions with the Rotational Motions

Residue No.	Human,Bovine,Canine	Helix	U/L	Core
1	T			
2	P			
3	L			
4	Q			
5	P			
6	A,A,T			
7	S,R,G			
8	A,S,P		L	
9	L		L	
10	P		L	
11	Q	A	L	
12	S	A	L	
13	F	A	L	Core
14	L	A	L	Core
15	L	A	L	
16	K	A	L	
17	C	A	L	Core
18	L	A	L	
19	E	A	L	
20	Q	A	L	
21	V,V,M	A	L	Core
22	R	A	L	
23	K	A	L	
24	I,I,V	A	L	Core
25	Q	A	L	
26	G,A,A	A	U	
27	D	A	U	
28	G	A	U	
29	A,A,T	A	U	
30	A,E,A	A	U	
31	L	A	U	Core
32	Q	A	U	
33	E	A	U	
34	K,R,T	A	U	
35	L	A	U	Core
36	C	A	U	
37	A	A	U	
38	T,A,T	A	U	
39	Y,H,H	A	U	
40	K,K,Q		U	
41	L		U	
42	C		U	
43	H		U	
44	P	E	U	
45	E	E	U	
46	E	E	U	
47	L	E	U	
48	V,M,V	E	U	
49	L		U	
50	L		U	
51	G,R,G		U	
52	H		U	
53	S,S,A		U	
54	L		U	
55	G		U	
56	I		U	
57	P		U	
58	W,Q,Q		L	
59	A,A,P		L	
60	P		L	
61	L		L	
62	S		L	
63	S		L	
64	C		L	
65	P,S,S		L	
66	S		L	
67	Q		L	
68	A,S,A		L	
69	L		L	
70	Q		L	
71	L	B	L	Core
72	A,R,M	B	L	
73	G	B	L	
74	C	B	L	Core
75	L	B	L	Core
76	S,N,R	B	L	
77	Q	B	L	
78	L	B	L	Core
79	H	B	L	
80	S,G,S	B	L	
81	G	B	L	
82	L	B	L	Core
83	F	B	U	
84	L	B	U	
85	Y	B	U	Core
86	Q	B	U	
87	G	B	U	
88	L	B	U	
89	L	B	U	Core
90	Q	B	U	
91	A	B	U	
92	L		U	
93	E,A,A		U	
94	G		U	
95	I		U	
96	S		U	
97	P		U	
98	E		U	
99	L	C	U	Core
100	G,A,A	C	U	
101	P	C	U	
102	T	C	U	Core
103	L	C	U	Core
104	D	C	U	
105	T	C	U	
106	L	C	U	Core
107	Q	C	U	
108	L	C	U	
109	D	C	U	
110	V,V,T	C	L	Core
111	A,T,T	C	L	
112	D	C	L	
113	F	C	L	Core
114	A	C	L	Core
115	T,T,I	C	L	
116	T,N,N	C	L	
117	I	C	L	Core
118	W	C	L	Core
119	Q,L,Q	C	L	
120	Q	C	L	Core
121	M	C	L	Core
122	E	C	L	
123	E,D,D	C	L	
124	L	C	L	Core
125	G		L	
126	M,A,M		L	
127	A		L	
128	P		L	
129	A		L	
130	L,V,V		U	
131	Q,Q,P		U	
132	P		U	
133	T		U	
134	Q		U	
135	G		U	
136	A,A,T		U	
137	M		U	
138	P		U	
139	A,T,A		U	
140	F		U	
141	A,T,T		U	
142	S		U	
143	A	D	U	
144	F	D	U	Core
145	Q	D	U	
146	R	D	U	
147	R	D	U	
148	A	D	U	Core
149	G	D	U	
150	G	D	U	
151	V	D	U	
152	L	D	U	
153	V	D	U	
154	A	D	U	
155	S	D	U	
156	H,Q,N	D	U	
157	L	D	U	Core
158	Q,H,Q	D	L	
159	S,R,S	D	L	
160	F	D	L	
161	L	D	L	Core
162	E	D	L	
163	V,L,L	D	L	
164	S,A,A	D	L	
165	Y	D	L	Core
166	R	D	L	
167	V,G,A	D	L	
168	L	D	L	Core
169	R	D	L	
170	H,Y,H		L	
171	L,L,F		L	
172	A		L	
173	Q,R,K			
174	P			

Residue number, human, bovine and canine amino acid residues, helices A, B, C, D, and E, the two domains (U, upper domain; L, lower domain) and the large hydrophobic core surrounded by the four main helices bundled together are all indicated. The classification of amino acid residues into U and L was performed from the results in Fig. 9. The amino acid residues 1—8 and 173—174 were not included in the normal mode calculations due to the lack of coordinates.

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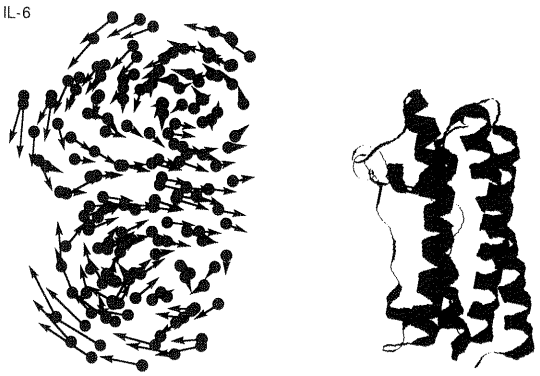

Fig. 12. Two Domain-Type Motions in Low Frequency Modes for the Model of IL-6

IL-6 was also found to have two domain-type motion. The wavenumber freqency (cm^{-1}) was 15.3 cm^{-1}. Normal mode vectors for Cα atoms in which the amplitude was multiplied by 400 are shown with arrows.

obtained with both human and canine GCSFs. In addition, similar two domain-type motions were found with the other cytokines in the long chain group namely LIF, GH, and IL-6. However, they were not present with those in the short chain group, IL-2 and IL-4.

Recently, a new experimental method with high resolution NMR spectroscopy has been able to monitor low frequency modes in proteins. It is called the residual dipole coupling method as proposed by Bax *et al.*[24] and Prestegard *et al.*[25] Hopefully, in the near future, such two domain-type motions will be observed with long chain group proteins and our results, which we believe are important for a better understanding of the biological activity of these cytokines, will be confirmed.

References and Notes

1) Abbreviations: IL-6, interleukin-6; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; GCSF, granulocyte colony-stimulating factor; LIF, leukemia inhibitory factor; CNTF, ciliary neurotrophic factor; BIOCES[E], BIOchemical Expart System.
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