¹H NMR assignments of apo calyculin and comparative structural analysis with calbindin D_{9k} and S100β

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Abstract

The homodimeric S100 protein calyculin has been studied in the apo state by two-dimensional ¹H NMR spectroscopy. Using a combination of scalar correlation and NOE experiments, sequence-specific ¹H NMR assignments were obtained for all but one backbone and >90% of the side-chain resonances. To our knowledge, the 2 × 90 residue (20 kDa) calyculin dimer is the largest protein system for which such complete assignments have been made by purely homonuclear methods. Sequential and medium-range NOEs and slowly exchanging backbone amide protons identified directly the four helices and the short antiparallel β-type interaction between the two binding loops that comprise each subunit of the dimer. Further analysis of NOEs enabled the unambiguous assignment of 556 intrasubunit distance constraints, 24 intrasubunit hydrogen bonding constraints, and 2 × 26 intersubunit distance constraints. The conformation of the monomer subunit was refined by distance geometry and restrained molecular dynamics calculations using the intrasubunit constraints only. Calculation of the dimer structure starting from this conformational ensemble has been reported elsewhere. The extent of structural homology among the apo calyculin subunit, the monomer subunit of apo S100β, and monomeric apo calbindin D_{9k} has been examined in detail by comparing ¹H NMR chemical shifts and secondary structures. This analysis was extended to a comprehensive comparison of the three-dimensional structures of the calyculin monomer subunit and calbindin D_{9k}, which revealed greater similarity in the packing of their hydrophobic cores than was anticipated previously. Together, these results support the hypothesis that all members of the S100 family have similar core structures and similar modes of dimerization. Analysis of the amphiphilicity of Helix IV is used to explain why calbindin D_{9k} is monomeric, but full-length S100 proteins form homodimers.

Keywords: calyculin; chemical shift; EF-hand calcium-binding protein; homology; hydrophobic core; S100 protein

Calyculin is a small homodimeric calcium-binding protein (90 residues/subunit) that was first identified on the basis of the differential expression of its mRNA during the G₁ phase of the cell cycle (Hirschhorn et al., 1984; Calabretta et al., 1985, 1986). Subsequent studies have shown that the expression of the calyculin gene is

deregulated in certain malignant cells, including thick melanomas and *ras*-transformed metastatic NIH/3T3 cells (Guo et al., 1990; Weternan et al., 1992). Efforts directed toward the identification of calyculin's physiological targets have shown that the protein associates with glyceraldehyde-3-phosphate dehydrogenase (Zeng et al., 1993), sialic acid (Zeng & Gabius, 1991), and several annexins (Minami et al., 1992; Watanabe et al., 1993). A specific regulatory function for calyculin is suggested by its calcium-dependent binding to the regulatory domain of annexin XI (Minami et al., 1992; Tokumitsu et al., 1992, 1993). The calyculin-annexin XI interaction may involve two molecules of calyculin, in analogy to the calpactin complex (p11₂-annexinII₂), which contains two molecules of the homologous protein p11 (Gerke & Weber, 1985; Kube et al., 1992). This hypothesis is supported by the observation that calyculin forms homodimers in solution (Kuznicki et al., 1989; Pedrocchi et al., 1994; Wojda & Kuznicki, 1994; Potts et al., 1995; V.V. Krishnan, X. Jia, R.H. Ebright, and N. Assa-Munt, unpubl.).

Calyculin is a member of the S100 subfamily of helix-binding loop-helix (EF-hand) calcium-binding proteins (Kligman & Hilt,

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Abbreviations: COSY, correlation spectroscopy; R-COSY, relayed-COSY; DR-COSY, double relayed-COSY; NOESY, NOE spectroscopy; TOCSY, total correlation spectroscopy; 2Q, two quantum; 3Q, three quantum; MQ, multiple quantum; DG, distance geometry; rMD, restrained molecular dynamics; rEM, restrained energy minimization; RMSD, RMS deviation; 3-*spin*, the C^αH-C^βH₂ spin subsystem of serine, cysteine, aspartic acid, asparagine, and the aromatic residues; 5-*spin*, the C^αH-C^βH₂-C^γH₂ spin subsystem of glutamic acid, glutamine, and methionine residues.

1988; Hilt & Kligman, 1991). The basic functional unit for these proteins is a pair of EF-hands, which together constitute a single globular domain. The consensus binding loop coordinates calcium with three monodentate side-chain carboxyl oxygen atoms, one backbone carbonyl oxygen, and a bidentate glutamic acid carboxylate, as well as an additional water-mediated ligand, all within 12 contiguous residues (Strynadka & James, 1989). S100s are distinguished by a nonconsensus 14-residue binding loop in the N-terminal EF-hand, in which the three side-chain carboxyl oxygen atom ligands are replaced by backbone carbonyl oxygen atoms. The three-dimensional structure of one S100 protein, calbindin $\text{D}_{9\text{k}}$, has been solved by X-ray crystallography in the calcium-saturated state (Szebenyi & Moffat, 1986; Svensson et al., 1992) and by NMR 1 spectroscopy in the apo, $(\text{Cd}^{2+})_1$ 11 half-saturated, and calcium-saturated states (Kördel et al., 1993; Akke et al., 1995; Skelton et al., 1995). Although the core structure is expected to be representative of all S100 proteins, calbindin $\text{D}_{9\text{k}}$ is substantially smaller than all other S100s sequenced to date (75 versus ca. 90 amino acids), and the only one not implicated in some regulatory or effector activity. Furthermore, calbindin $\text{D}_{9\text{k}}$ is a monomer, whereas other S100s have a tendency to form dimers (Gerke & Weber, 1985; Teigelkamp et al., 1991; Barger et al., 1992; Dell'Angelica et al., 1994; Pedrocchi et al., 1994; Wojda & Kuznicki, 1994).

Our laboratory recently reported the three-dimensional structure of apo calyculin, revealing an integral dimer motif that is unique among the known EF-hand calcium-binding protein structures (Krebs et al., 1995; Potts et al., 1995). Based on extensive sequence homology with calyculin, we proposed that other full-length S100 proteins will exhibit a similar dimeric structure. The substantial differences in the organization of this motif versus that of the current calmodulin paradigm (Crivici & Ikura, 1995) suggest a novel mode for receptor binding by S100 proteins.

Our earlier report focused almost exclusively on the dimeric nature of the apo calyculin structure, and its biological implications. Structural homology within the globular domain was discussed briefly and preliminary results on comparisons between the structures of the apo calyculin subunit and monomeric apo calbindin $\text{D}_{9\text{k}}$ were presented. In this report, we describe an in-depth analysis of the extent of homology among the apo calyculin subunit, the monomer subunit of apo S100 β , and apo calbindin $\text{D}_{9\text{k}}$, including a comprehensive comparison of the three-dimensional structures of the calyculin monomer subunit and calbindin $\text{D}_{9\text{k}}$. We also include a description of the ^1H NMR studies that formed the foundation for determining the three-dimensional structure of the protein. ^1H NMR assignments are discussed in this particular case because the calyculin dimer represents the largest system for which such extensive assignments have been made by purely homonuclear methods. These resonance assignments provided the basis for directly identifying secondary structure and the global folding pattern, distinguishing intrasubunit and intersubunit proton-proton contacts, and calculating the three-dimensional structure. Specific comparisons of ^1H chemical shifts, amide proton exchange rates, NMR-determined elements of secondary structure, and three-dimensional structures are presented to highlight the evidence in support of our analysis of structural homology.

Results

For any Ca^{2+} -binding protein, comparisons of the apo and ion-bound states are essential to generating an understanding of virtually all aspects of protein function. A preliminary assessment of

the effects of calcium on calyculin's ^1H NMR spectrum was made on a sample of murine calyculin isolated from Ehrlich ascites tumor cells. A number of significant changes in chemical shifts was observed upon addition of calcium, along with a substantial increase in resonance linewidths. We note that similar observations have been reported for the homologous protein S100 β (Kilby et al., 1995; Smith et al., 1996). The changes in chemical shift are presumably due to conformational changes in response to calcium binding that expose new surfaces for interaction with specific target proteins. In typical EF-hand calcium-binding proteins such as calmodulin, this involves exposure of hydrophobic side chains (Finn et al., 1995; Kuboniwa et al., 1995; Zhang et al., 1995). In the absence of a receptor, exposure of hydrophobic residues can lead to self-aggregation, which would explain the substantial increase in resonance linewidths observed upon calcium binding. Although heteronuclear NMR could be applied to overcome such problems, this approach is not possible on a native protein sample of limited quantity. Thus, our initial studies focused on the protein in the apo state, for which the linewidths are somewhat narrower. Furthermore, because our first objective was to generate some idea of the global molecular architecture of the protein, the state of ion occupancy was not a critical factor.

Solution properties

^1H NMR spectra of the sample of apo rabbit lung calyculin exhibit resonance linewidths of ~ 11 Hz for methyl protons and ~ 15 Hz for backbone amide protons. These are approximately two times larger than the linewidths observed for calbindin $\text{D}_{9\text{k}}$ under similar conditions (6 Hz and 9 Hz, respectively), and similar to the linewidths of the ~ 20 -kDa proteins caltractin and cyclophilin A (Weber et al., 1994; C. Weber, pers. comm.). This suggests that the rotational correlation time for calyculin is ~ 2 times greater than that of monomeric calbindin $\text{D}_{9\text{k}}$, despite the fact that calyculin contains only 12 additional amino acid residues. This observation, together with the well-documented tendency for S100 proteins to form dimers (Gerke & Weber, 1985; Teigelkamp et al., 1991; Barger et al., 1992; Dell'Angelica et al., 1994; Pedrocchi et al., 1994), suggests that calyculin forms homodimers in solution. Equilibrium sedimentation experiments on recombinant human calyculin (K. Früh, pers. comm.), and pulsed field gradient NMR diffusion measurements (V.V. Krishnan, X. Jia, R.H. Ebricht, and N. Assa-Munt, unpubl.) and light scattering experiments on recombinant rabbit calyculin in our laboratory confirm that the apo protein is predominantly dimeric in solution, with a corresponding molecular weight of ~ 20 kDa. The presence of a single set of resonance lines indicates that the dimer is symmetric on the NMR chemical shift time scale.

Sequence-specific assignments and secondary structure

The procedure used to obtain the sequence-specific ^1H NMR resonance assignments is well-established (see Wüthrich, 1986). Amino acid spin systems were identified primarily by a strategy that emphasizes observation of relayed connectivities from the side-chain protons to the backbone amide proton (Chazin & Wright, 1987; Chazin et al., 1988). Relayed connectivities to C^αH and protons of the side-chain termini (e.g., leucine $\text{C}^\delta\text{H}_3$) provide supplementary relayed information along with multiple quantum and MQ-filtered COSY spectra, which unambiguously distinguish the protons of

Table 1. ^1H NMR chemical shifts of apo calyculin from rabbit lung cytosol^a

Residue	NH	Chemical shifts (ppm)				
		C $^{\alpha}\text{H}$	C $^{\beta}\text{H}$	C $^{\gamma}\text{H}$	C $^{\delta}\text{H}$	Other
M01		4.43	2.32 ^b , 2.57 ^b	—		2.03 (C $^{\epsilon}\text{H}_3$)
A02	8.39	4.46	1.42			
S03	9.30	4.88	4.01, 4.54			
P04		4.39	1.95, 2.51	2.31, —	3.98, 4.23	
L05	8.94	4.12	1.63, 1.76	1.74	0.83, 0.97	
D06	7.99	4.24	2.69, 3.08			
Q07	8.60	4.07	2.16, 2.26	2.46, (2.46)		6.79, 7.14 (N $^{\epsilon}\text{H}_2$)
A08	8.49	4.09	1.69			
I09	8.50	3.73	2.11	1.29, 1.69	0.82	0.88 (C $^{\gamma}\text{H}_3$)
G10	8.31	3.74, 4.00				
L11	8.26	4.31	1.48, 2.17	1.89	0.97, 0.97	
L12	8.23	4.07	1.52, 2.45	1.97	0.73, 0.77	
I13	8.74	3.60	1.80	0.06, 1.75	0.40	0.50 (C $^{\gamma}\text{H}_3$)
G14	8.36	3.94, 4.14				
I15	8.26	3.99	2.11	1.61, 1.68	0.90	1.16 (C $^{\gamma}\text{H}_3$)
F16	7.63	3.65	2.88, 3.17			6.13, 7.00, 7.35 (C $^{\delta}\text{H}$, C $^{\epsilon}\text{H}$, C $^{\zeta}\text{H}$)
H17	8.61	4.87	3.17, 3.20			7.22 (C $^{\delta}\text{H}$), 8.46 ^l (C $^{\epsilon}\text{H}$)
K18	8.02	3.93	1.98, 2.18	—	—	—
Y19	7.21	4.19	2.52, 2.93			7.55, 6.77 (C $^{\delta}\text{H}$, C $^{\epsilon}\text{H}$)
S20	—	4.76 ^c	3.80 ^c , 3.91 ^c			
G21	8.04	3.79, 3.99				
K22	7.70	4.09	1.78, 1.83	[1.33, 1.47, 1.59 ^d , 1.65]		2.91 ^j (C $^{\epsilon}\text{H}_2$)
E23	8.13	4.40	1.89, 2.07	2.18, (2.18)		
G24	8.31	3.74, 3.94				
D25	8.35	4.50	2.60, 2.84			
K26	8.23	4.23	1.60, 1.68	—	—	—
H27	8.88	5.02	3.31, 3.48			7.25 (C $^{\delta}\text{H}$), 8.56 ^l (C $^{\epsilon}\text{H}$)
T28	7.62	5.23	4.14	1.08		
L29	8.67	4.78 ^e	1.29, 1.49	1.36	0.50, 0.55	
S30	8.88	5.14	4.04, 4.42			
K31	8.58	3.81	1.58, 1.66 ^f	—	—	—
K32	8.52	4.11	1.81, —	—	—	—
E33	7.69	4.17	2.10, 2.41	2.33, (2.33)		
L34	8.56	3.96	1.19, 2.10	1.29	0.42, 1.07	
K35	8.02	3.55	1.74, 2.07	—	—	—
E36	7.22	3.98	2.18, 2.18	2.36, 2.43		
L37	8.16	3.07	0.86, 1.61	1.23	0.69, 0.72	
I38	8.20	3.36	1.98	1.43, 1.60	0.79	0.86 (C $^{\gamma}\text{H}_3$)
Q39	7.93	3.96	2.02, 2.24	2.65, (2.65)		6.84, 7.17 (N $^{\epsilon}\text{H}_2$)
K40	8.27	4.34	1.89 ^g , 2.11	[1.52, 1.61 ^h , 1.67 ^h , 1.79 ^h]		3.07 (C $^{\epsilon}\text{H}_2$)
E41	8.53	4.70 ^e	1.84 ⁱ , 2.45	2.70, (2.70)		
L42	7.84	4.74 ^e	1.35 ^f , 1.92	1.31	0.75, 0.84	
T43	9.87	4.30	4.30	1.17		
I44	7.93	3.79	1.54	0.44, 1.11	0.44	0.45 (C $^{\gamma}\text{H}_3$)
G45	8.44	3.74, 3.99				
S46	8.81 ^j	4.32	3.95, 4.04			
K47	7.87	4.32	1.77, 1.94	[1.54, 1.63, —, —]		3.09 (C $^{\epsilon}\text{H}_2$)
L48	7.61	4.00	1.58, 1.66	1.67	0.82, 0.92	
Q49	7.52	4.27	1.95, 2.31	—		6.77, 7.46 (N $^{\epsilon}\text{H}_2$)
D50	7.74	4.40	2.75, 2.75			
A51	8.89	3.96	1.49			
E52	8.95	4.11	2.07, 2.07	2.31, 2.40		
I53	7.52	3.77	2.26	1.15, 1.77	0.86	0.91 (C $^{\gamma}\text{H}_3$)
V54	8.47	3.50	1.98	0.91, 0.97		
K55	7.52	4.10	1.88, —	[1.47, 1.73 ^d , —, —]		2.98 ^d (C $^{\epsilon}\text{H}_2$)
L56	7.27	4.23	1.61, 1.94	1.80	0.69, 0.73	
M57	8.51	4.33	2.00, 2.32	2.68, 2.82		2.03 (C $^{\epsilon}\text{H}_3$)
D58	8.80	4.43	2.64, 2.87			
D59	7.58	4.34	2.83, 2.89			
L60	7.57	3.93	1.47, 2.02	2.04	0.48, 0.71	
D61	7.33	4.49	2.72, 2.74			

(continued)

Table 1. Continued

Residue	NH	Chemical shifts (ppm)				
		C $^\alpha$ H	C $^\beta$ H	C $^\gamma$ H	C $^\delta$ H	Other
R62	8.09	4.10	1.85, 1.87	1.63, 1.75	3.23 ^g	7.42 ^g (N $^\epsilon$ H)
N63	7.84	5.11	2.52, 3.03			6.90, 7.67 (N $^\delta$ H ₂)
K64	6.83	4.20	—	—	—	—
D65	8.31	4.83 ^e	2.59, 2.84			
Q66	7.98	4.17	2.08, 2.08	2.40, 2.55		6.84, 7.70 (N $^\epsilon$ H ₂)
E67	8.51	4.71 ^e	1.87, 1.93 ^k	2.13 ^d , 2.32 ^k		
V68	9.41	4.71 ^e	2.24	0.83, 0.89		
N69	8.91	5.28	2.98, 3.66			7.11, 7.47 (N $^\delta$ H ₂)
F70	9.13	3.28	2.44, 2.63			6.36, 6.92, 6.85 (C $^\delta$ H, C $^\epsilon$ H, C $^\zeta$ H)
Q71	7.68	3.67	1.98, 2.19	2.26, (2.26)		7.02, 7.16 (N $^\epsilon$ H ₂)
E72	8.55	3.93	1.97, 2.34	2.25, 2.49		
Y73	8.49	4.25	3.03, 3.15			6.72, 6.54 (C $^\delta$ H, C $^\epsilon$ H), 7.65 (OH)
I74	8.04	3.16	1.29	0.80, 0.91	0.41	0.59 (C $^\gamma$ H ₃)
T75	8.12	3.58	4.15	1.18		5.37 (OH)
F76	7.96	4.12	3.18, 3.23			7.11, 7.25, 7.09 (C $^\delta$ H, C $^\epsilon$ H, C $^\zeta$ H)
L77	7.97	3.86	1.07, 1.86	1.62	0.38, 0.70	
G78	8.38	3.54, 3.68				
A79	8.02	3.94	1.32			
L80	7.98	3.82	1.51, 1.72	1.52	0.74, 0.81	
A81	8.64	3.58	1.22			
M82	7.50	3.84	2.12, 2.14	2.56, 2.89		2.18 (C $^\epsilon$ H ₃)
I83	7.37	3.94	1.88	1.04, 1.63	0.73	0.81 (C $^\gamma$ H ₃)
Y84	7.91	4.67 ^e	2.52, 3.22			6.96, 6.68 (C $^\delta$ H, C $^\epsilon$ H)
N85	7.76	4.29	1.48, 2.50			6.48, 6.93 (N $^\delta$ H ₂)
E86	9.07	3.85	1.99, 2.11	2.33, 2.38		
A87	8.28	4.26	1.41			
L88	7.42	4.28	1.49, 1.57	0.97	-0.05, 0.33	
K89	7.28	4.27	1.75, 1.85	1.37, 1.42	1.60 ^f , 1.61 ^g	2.92 ^g (C $^\epsilon$ H ₂)
G90	7.92	3.73, 3.73				

^aChemical shifts are referenced to the H₂O signal at 4.75 ppm and are generally accurate to ± 0.01 ppm (± 0.03 ppm for geminal protons separated by < 0.1 ppm). The bulk of the chemical shifts listed are from a 60-ms NOESY experiment acquired at pH 7.1, 300 K. Additional entries were obtained from the following spectra as indicated: ^b2Q acquired in H₂O solution; ^c2QF COSY; ^dTOCSY (310 K); ^eNOESY ($\tau_m = 200$ ms, pH 7.0); ^f2Q acquired in ²H₂O solution; ^gTOCSY ($\tau_m = 67$ ms, pH 7.1); ^hTOCSY ($\tau_m = 80$ ms, pH 6.2); ⁱRelayed COSY; ^jTOCSY ($\tau_m = 64$ ms, pH 6.2); ^kTOCSY ($\tau_m = 99$ ms, pH 7.1); ^l1D spectrum acquired in ²H₂O solution (pH 6.2); the two His C $^\alpha$ H resonances were identified but not assigned specifically. Parentheses indicate that degeneracy is assumed. Brackets indicate that the specific position along the side chain could not be determined. Dashed lines indicate that the resonance frequency was not identified.

each side chain and identify instances of resonance degeneracy. As might be expected, it was not possible to identify all spin systems solely from these scalar correlated experiments due to the sensitivity limitations associated with the relatively large resonance linewidths and the limited quantity of sample available. A modest number of additional resonance assignments were obtained from NOESY spectra. In all, resonance assignments were made for all but one backbone proton and some of the side-chain protons of the proline, glutamine, methionine, and lysine residues (Table 1). To our knowledge, the 2×90 residue calcyclin homodimer is the largest protein system (20 kDa) for which such complete ^1H assignments have been obtained by homonuclear methods.

The first stage of analysis involved identification of spin systems, starting from the fingerprint regions of the COSY spectrum. Examination of the backbone fingerprint (Fig. 1) reveals 81 of the 88 expected crosspeaks (90 residues minus one proline and the N-terminus) at pH 7.0 and 300 K. Three cases of near degeneracy were resolved by raising the temperature to 310 K (Glu 41/Glu 67) or lowering the pH (Ala 79/Lys 18; Leu 5/Glu 52). The Ser 46 backbone amide proton was observed only at pH values ≤ 6.2 .

C $^\alpha$ H-NH correlations for Lys 64 and Phe 70 were observed only in NOESY spectra, and the backbone amide proton resonance of Ser 20 could not be identified in any of the experiments. The nine methyl resonances from alanine and threonine residues, as well as all aromatic ring protons, are resolved in their corresponding fingerprint regions. Fifty discrete COSY crosspeaks are observed for the 54 methyl groups of the valine, leucine, and isoleucine residues. Subsequent analysis revealed that one of the Leu 5 C $^\delta$ H-C $^\gamma$ H crosspeaks is obscured due to general spectral crowding, and that some of the paired crosspeaks of the methyl resonances of Leu 11, Ile 15, and Ile 44 are not resolved.

The second step involved searching for relayed crosspeaks from the side chains to the corresponding backbone amide resonance. Although this protein system is rather large, coherence transfer in homonuclear ^1H NMR experiments was reasonably efficient. Although less than 50% of all possible correlations from C $^\beta$ H to backbone amide protons were observed at 300 K, this figure improved to 70% after analysis of the TOCSY acquired at 310 K. The correlations to C $^\beta$ H were verified and many additional resonances were identified (including cases of degenerate C $^\beta$ H resonances) in

Fig. 1. Backbone fingerprint region of the 600 MHz COSY spectrum of apo rabbit lung calyculin acquired at pH 7.0 and 300 K. Each crosspeak is labeled with its sequence-specific assignment. The crosspeaks of T43 and K64 are located outside the region shown at the $C^{\alpha}H$ frequencies of the arrows. Open circles indicate the position of crosspeaks observed at lower contour levels. Open boxes indicate the expected positions of crosspeaks that were not observed in the COSY spectrum. Filled boxes indicate crosspeaks that are not observed due to inadvertent saturation of the $C^{\alpha}H$ resonance. A diamond indicates the position of the S46 crosspeak observed only at pH 6.2.

2Q spectra, bringing the $C^{\beta}H$ assignments to 90% completion. Correlations from other side-chain protons to the backbone amide proton are discussed below on a per-residue basis.

The complete assignments of all seven glycines, the six alanines, and three threonines were obtained by standard methods. The 2Q spectra were critical for identifying the complete spin system for glycine residues and the degeneracy of the $C^{\alpha}H$ and $C^{\beta}H$ resonances of Thr 43. Twenty of 22 residues with 3-spin side chains were identified by standard methods, with a large percentage obtained directly from connectivities to the backbone amide proton. No scalar correlations from $C^{\beta}H$ to $C^{\alpha}H$ or NH were observed for Ser 30, but the resonances were ultimately assigned from NOESY spectra. The $C^{\alpha}H-C^{\beta}H-C^{\beta}H'$ spin subsystem of Ser 20 could be identified by scalar correlations, but its backbone NH resonance was never assigned. The 15 5-spin systems (excluding Met 1, discussed below) were assigned primarily by standard methods, with at least one $C^{\beta}H_2$ and one $C^{\gamma}H_2$ correlation to the backbone NH or $C^{\alpha}H$ for all residues except Glu 41 and Gln 49. The resonance assignments for Glu 41 were obtained from NOESY spectra, and the $C^{\gamma}H_2$ resonances for Gln 49 could not be assigned. The S-methyl groups of Met 57 and Met 82 were identified on the basis of NOEs to the C^{γ} protons.

The spin systems of all valines and leucines except Leu 60 were correlated to the backbone NH proton or to the $C^{\alpha}H$ resonances in TOCSY spectra. The complete Leu 60 spin system assignment could only be confirmed via analysis of NOESY spectra. The $C^{\gamma}H_3$ and C^{β} protons of the eight isoleucine residues showed correla-

tions to $C^{\alpha}H$ or the backbone NH in R-COSY or TOCSY spectra. The $C^{\delta}H_3-C^{\gamma}H_2$ spin subsystems were identified in COSY, 2QF-COSY, and MQ spectra. These two methyl-based spin subsystems were correlated in TOCSY spectra, except for Ile 15, Ile 38, and Ile 53, for which assignments could only be confirmed from the analysis of NOESY spectra. The complete Arg 62 spin system was identified via relayed coherence transfer to the backbone amide proton. Of the 11 lysine residues, complete side-chain proton assignments were obtained from correlations to the backbone amide only for Lys 89. Correspondence between relayed coherence transfer connectivities in backbone NH-based and $C^{\alpha}H_2$ -based spin subsystems (Chazin et al., 1987) provided assignments for Lys 40, Lys 47, Lys 55, and Lys 59. Side-chain assignments for the other six lysine residues were extended only to the C^{β} protons due to resonance degeneracies and limited coherence transfer.

The identification of Met 1 and Pro 4 was deferred until the last stages of analysis, when there remained few unassigned scalar correlated spin systems in the MQ and MQF-COSY spectra. The $C^{\alpha}H-C^{\beta}H_2$ portion of the Met 1 side chain could be identified at low contour level in 2Q and TOCSY spectra, but the C^{γ} protons could not be identified. The S-methyl resonance, degenerate with that of Met 57, was identified by a strong NOE to the Ala 2 backbone amide proton. The Pro 4 spin system was identified by correlations from the $C^{\alpha}H-C^{\beta}H_2$ and $C^{\delta}H-C^{\delta}H$ spin subsystems to the same $C^{\gamma}H$ resonance (2.31 ppm) in the TOCSY and 3Q spectra. The spin system could be assigned to Pro 4 based on the characteristic pattern of chemical shifts.

The sequence-specific assignments were obtained using the standard procedure (Billeter et al., 1982). The short-hand notation of Wüthrich and coworkers (Wüthrich et al., 1984) is adopted to specify proton-proton distances. A summary of the sequential connectivities is included in Figure 2. At least one $d_{NN}(i, i+1)$ or $d_{\alpha N}(i, i+1)$ connectivity was identified for each pair of consecutive residues with the following exceptions: Met 1/Ala 2, Ala 8/Ile 9, Tyr 19/Ser 20, Ser 20/Gly 21, Gly 45/Ser 46, Ser 46/Lys 47, and Phe 76/Leu 77. All but Ser 20/Gly 21 and Gly 45/Ser 46 are associated with multiple resonance degeneracies. In two of these instances (Ser 46/Lys 47 and Phe 76/Leu 77), the sequential assignment could be made on the basis of $d_{\beta N}$ connectivities. The lack of connectivities involving Ser 20 and Ser 46 reflects the inability to identify their backbone amide protons in NOESY spectra. The $d_{\alpha N}$ connectivity for Ser 20/Gly 21 may be lost due to the coincidence of the Ser 20 $C^{\alpha}H$ resonance with the solvent signal. The sequential NOE connectivities between Ser 3 and Pro 4 include correlations from the Ser 3 backbone NH and $C^{\alpha}H$ to the Pro 4 C^{δ} protons, indicating that the Pro 4 peptide bond has adopted the *trans* geometry.

The elements of secondary structure were identified on the basis of sequential and medium-range NOE connectivities and backbone amide exchange rates (reviewed in Wüthrich, 1986), as summarized in Figure 2. Supporting evidence was obtained from the $C^{\alpha}H$ chemical shift index (Wishart et al., 1992). Although backbone amide scalar coupling constants are also useful for this purpose, we were unable to obtain accurate measurements of $^3J_{NH\alpha}$ using either the method of Kim and Prestegard (1989) or line-fitting routines. The helical regions are distinguished by networks of sequential d_{NN} NOEs, medium-range $[(i, i+2), (i, i+3), (i, i+4)]$ NOEs, slow backbone amide proton exchange rates, and clusters of negative values for the $C^{\alpha}H$ chemical shift index. Four primary helices were identified: Ser 3-His 17, Glu 33-Leu 42, Ile 53-Asn 63, and Phe 70-Asn 85. An additional turn of helix is indicated just beyond

Fig. 2. Summary of sequential and medium-range NOE connectivities, backbone amide proton exchange rates, and chemical shift index of C^αH protons for apo calyculin. The one-letter amino acid sequence is given at the top. NOE connectivities summarize the correlations observed in a series of 2D NOESY spectra (see the Materials and methods). Very slowly exchanging backbone amide protons are indicated by hatched squares. The chemical shift index of each C^αH residue is indicated by “-”, “0”, or “+” symbols, corresponding to the indices -1, 0, or +1, respectively, described by Wishart et al. (1992). The location of elements of secondary structure deduced from the data is marked by coils for helices and two parallel lines for the short antiparallel β -type interaction. Fraying at the ends of helices is indicated by a broken coil. x, unassigned resonances; *, degenerate or near degenerate; #, Ile 53 and Lys 55 NH resonances are degenerate, therefore, the d_{NN} NOE can be assigned to either Ile 53/Val 54 or Val 54/Lys 55.

the C-terminus of Helix II, from Ile 44 to Leu 48. An antiparallel β -type interaction between the two binding loops has been identified on the basis of intense sequential $d_{\alpha\text{N}}$ NOEs, low-field shifted C^αH resonances, and cross-strand NOEs (Fig. 3). Well-defined extended conformation is clearly indicated for the polypeptide segments Thr 28–Ser 30 and Glu 67–Asn 69, with a pair of hydrogen bonds between Leu 29 and Val 68.

Fig. 3. Schematic diagram of the NOEs indicating the cross-strand β -type interaction between the two binding loops in apo rabbit lung calyculin. Single asterisks indicate ambiguity in an NOE assignment as a result of the degeneracy of C^αH resonances (E67/V68). Double asterisks indicate that the $d_{\alpha\alpha}$ NOE between Thr 28 and Asn 69 could not be identified due to resonance degeneracy.

Distinguishing intramolecular NOEs from intermolecular NOEs

The folding pattern of the four helical elements of apo calyculin was apparent from intramolecular long-range NOEs between side chains, especially those at the end of the helices. The identification of these NOEs was complicated because they had to be distinguished from intermolecular contacts across the dimer interface.

Fortunately, many of the intermolecular NOEs could be identified as a subset of NOEs that are inconsistent with the elements of regular secondary structure summarized in Figure 2. For example, these data indicate the presence of a regular helix that extends from Phe 70 to Asn 85 but there are well-resolved NOEs between the δ -methyl protons of Leu 88 and the aromatic ring protons of Phe 70. Clearly, NOEs from opposite ends of a helix would be inconsistent with their assignment as intramolecular contacts. However, these could be assigned as intermolecular contacts by aligning Helix IV of one subunit antiparallel to Helix IV of the second subunit in a dimer. A second important set of NOEs, for example, those between Met 82 and Leu 88, indicated that the residues beyond Asn 85 loop back and make contact with the C-terminal region of Helix IV. These and NOEs such as Phe 70/Ala 81, Phe 70/Met 82, Gln 71/Leu 88, and Tyr 73/Leu 88, further support the antiparallel packing of Helices IV and IV' indicated in Figure 4. Additional NOEs were assigned as intermolecular contacts based on comparisons with the characteristic packing of helices in EF-hand calcium-binding proteins. For example, in calbindin $\text{D}_{9\text{k}}$, Phe 10 and Phe 63 of helices I and IV are in contact and make important contributions to the hydrophobic core of the protein (Skelton et al., 1995). NOEs between the analogous residues in calyculin, Phe 16 and Phe 70, imply a similar packing within the calyculin monomer. Because the Phe 70/Leu 88 contacts are intermolecular, it follows that the NOEs between Phe 16 and Leu 88 are also intermolecular. Only those NOEs that could be unambiguously assigned as strictly intermolecular were used for the subsequent calculations of the dimer structure. We note that other homodimeric protein and peptide systems, for which only homonuclear ^1H NMR data were available, have been analyzed in a similar manner (e.g., Breg et al., 1989; Kay et al., 1991; Shaw et al., 1992).

There are a number of intermolecular contacts present in the dimer that could not be assigned due to ambiguities at the center of

Fig. 4. A selection of intramolecular contacts (solid arrows) and intermolecular contacts (dashed arrows) illustrating the assignment of NOEs in the region of the dimer interface. One subunit is shown in red and the other in blue.

Helix IV, particularly from residues that are near each other both in the amino acid sequence and across the dimer interface, e.g., Ile 74 and Thr 75 (Fig. 4). These potentially ambiguous NOEs were assigned as intramolecular constraints, and the upper bounds were adjusted to the highest category. NOE back-calculations were used to verify the assignments of these NOEs. Although this strategy resulted in the absence of intermolecular distance constraints at the very center of the dimer interface, the number of assigned intermolecular constraints was sufficient to generate an ensemble of converged dimer structures.

Structure calculations

A two-stage procedure was utilized to calculate the three-dimensional structure of apo calcyclin. The first step involved consideration of only the intramolecular constraints. Once these input data were refined fully, an ensemble of monomer subunit structures was calculated using a standard protocol of several cycles of distance geometry, restrained energy minimization, restrained molecular dynamics calculations, and relaxation matrix back calculation of the NOEs (e.g., Skelton et al., 1995). The second stage involved docking pairs of subunit structures and refining, using the full complement of intra- and intermolecular constraints, and has been described elsewhere (Potts et al., 1995).

Eight iterations of this protocol were used to generate the final ensemble of converged subunit structures. The final round of calculations included 106 intraresidue and 450 interresidue (123 sequential, 172 medium-range, and 155 long-range) NOE-derived distance constraints, along with 24 distance constraints for 12 hydrogen bonds. Although dihedral angle constraints could not be derived from $^3J_{\text{HN}\alpha}$ (see above), loose backbone dihedral angle constraints were included for 56 residues in the final round of calculations. These additional restraints on the conformational sampling improved the precision of the structures in a manner reminiscent of the REDAC procedure (Güntert & Wüthrich, 1991). The validity of this approach was confirmed on the basis of the negligible effect on the restraint violation energies, while achieving significant improvement (reduction) in total molecular energies. An ensemble of six structures was selected for subsequent calculation of the dimer structures and for all further analysis in this report. These structures of the monomer subunit have no distance violations greater than 0.3 Å, and an average pairwise RMSD from the mean structure of 1.6 Å for the backbone atoms in the helices and 3.32 Å for all heavy atoms of all residues.

Discussion

NMR analysis shows that apo calcyclin forms a typical, folded globular calcium-binding protein domain consisting of a pair of EF-hand motifs: four sequentially antiparallel helices and an antiparallel β -type interaction between the two putative Ca^{2+} binding loops (Fig. 5). Helices I and II are joined by the first Ca^{2+} -binding loop and together, these comprise the N-terminal EF-hand. Similarly, helices III and IV, joined by the second Ca^{2+} -binding loop, form the C-terminal EF-hand. The two EF-hands are connected by a poorly defined 10-residue linker loop extending from the C-terminus of Helix II to the N-terminus of Helix III, and by the short antiparallel β -type interaction mediated by two hydrogen bonds. As anticipated from sequence homology, the global fold of apo calcyclin is analogous to that of other EF-hand proteins whose structures have been determined (Strynadka & James, 1989).

Beyond the generalized similarity of the apo calcyclin structure to other members of the EF-hand calcium-binding protein family, we have specifically analyzed the extent of structural homology with the apo states of two other S100 proteins, S100 β and calbindin D_{9k} . NMR analyses of calcyclin (this report), S100 β (Amburgey et al., 1995; Kilby et al., 1995), and calbindin D_{9k} (Skelton et al., 1992) indicate a very close correspondence in secondary structure: all three proteins contain four major helices of similar length and alignment in the sequence, and a cross-strand hydrogen bonding interaction between the two putative calcium-binding loops. An additional turn of helix in the linker loop that joins the two EF-hands is found in both calcyclin and bovine S100 β (Kilby et al., 1995). This helical element is not present in rat S100 β (Amburgey et al., 1995) or in the calbindin D_{9k} solution structure (Skelton et al., 1995), although a helical turn has been reported in a similar location in the calbindin D_{9k} crystal structure (Szebenyi & Moffat, 1986).

Calcyclin (90 residues) is longer than calbindin D_{9k} (75 residues) by six residues at the N-terminus, eight residues at the C-terminus, and one residue in the linker loop. Helices I and IV are extended by one full helical turn at their N- and C-termini, respec-

Fig. 5. Ribbon diagram of the apo rabbit lung calcyclin monomer. Prepared with the MOLSCRIPT program (Kraulis, 1991) using the coordinates of the structure with the lowest residual restraint violation energy.

tively, as compared to calbindin D_{9k} (Skelton et al., 1995). The N-terminal extension includes an apparent helix capping box from Ser 3 to Asp 6. The presence of the capping box is indicated by unique NOEs (Lyu et al., 1993) involving Ser 3 and Asp 6 [$d_{\text{NN}}(i, i + 3)$, $d_{\text{NB}}(i, i + 3)$, $d_{\text{BN}}(i, i + 3)$], and is supported by the known preference for serine at the Ncap position and aspartic acid at the N3 position of helix capping boxes (Harper & Rose, 1993).

A comparative analysis of backbone amide proton exchange rates provides additional insight into S100 protein structure. For calcyclin, even at the level of our current qualitative analysis, amide exchange is found to be more rapid in Helix III than in helices I, II, and IV (Fig. 2), a pattern also observed for apo calbindin D_{9k} and bovine apo S100β (Skelton et al., 1992; Kilby et al., 1995). Given this similarity in the apo state, we expect that the marked lowering of the rates of backbone amide proton exchange of Helix III in calbindin D_{9k} upon binding of ions (Skelton et al., 1992) will also be observed for calcyclin (and S100β). We also note that in the calcyclin structures, Helix III appears to be less well-packed than the other helices, an observation that correlates with the NMR structures of apo calbindin D_{9k} and the apo TR₁C fragment of troponin C (Findlay et al., 1994; Skelton et al., 1994, 1995).

The extent of structural homology among the apo states of calcyclin, S100β, and calbindin D_{9k} has also been assessed by the highly sensitive ¹H NMR chemical shift parameter. Although the chemical shift is difficult to interpret directly at atomic resolution, very close similarity of chemical shifts of a series of residues is now well accepted as an indicator of very extensive similarity between structures. Due to differences in amino acid sequence, secondary chemical shifts (observed chemical shift minus the random coil chemical shift) are commonly used for making comparisons between different proteins. The alignment of the sequences is also important. For the three proteins being compared here, the helices and binding loops are aligned easily, whereas the linker loops are more difficult because they have different lengths and very little sequence homology. An additional complication in comparing the chemical shifts is significant differences in the experimental conditions for the three proteins: calcyclin data were acquired at pH 7.0, 300 K, bovine S100β at pH 6.3, 310 K, and calbindin D_{9k} at pH 5.3, 300 K. Corrections have been made for the temperature differences, but not for the differences in pH.

Despite the complications noted above, there are striking similarities in the chemical shifts of these three proteins. A summary of the differences in backbone NH and C^αH resonances is provided in Table 2, as the percentage of residues with similar chemical shifts, using three different thresholds. This approach for the qualitative analysis of chemical shifts required here has been selected on the basis of an extensive analysis of chemical shift homology in more than 50 proteins (B.C.M. Potts & W.J. Chazin, in prep.). We find that 21% of the backbone NH and 38% of the C^α proton chemical shifts of S100β fall within 0.1 ppm of the values of calcyclin, rising to 55% and 74% using a threshold of 0.3 ppm. In comparing calbindin D_{9k} and calcyclin, 13% of backbone NH and 36% of C^α proton chemical shifts are within 0.1 ppm of each other, and the corresponding values increase to 51% and 75% using the 0.3 ppm threshold. In considering only residues with sequence identity, the similarities are even greater. Thus, the comparison of ¹H chemical shifts of the backbone protons of calcyclin, S100β, and calbindin D_{9k} in their apo states strongly suggests substantial structural homology among these three S100 proteins.

To further probe the structural homology among calcyclin, S100β, and calbindin D_{9k}, the chemical shift comparison has been ex-

Table 2. Secondary chemical shift comparison for backbone protons of the apo states of calcyclin, S100β, and calbindin D_{9k}^a

Threshold (ppm)	Cacyclin vs. S100β			Cacyclin vs. calbindin D _{9k}		
	All residues ^b		Conserved ^c	All residues ^d		Conserved ^e
	NH	C ^α H	C ^α H	NH	C ^α H	C ^α H
0.1	21	38	52	13	36	46
0.2	40	60	76	31	63	68
0.3	55	74	91	51	75	86

^aPercentage of secondary chemical shift differences that fall within the threshold limits. As a result of unassigned resonances and/or mismatches in sequence alignment (e.g., proline for a backbone NH-containing residue), it was not possible to compare chemical shifts for all residues. The maximum number of possible residues was compared in each case (see footnotes b–e below).

^b82 residues.

^c33 residues with sequence identity.

^d71 backbone NH and 76 C^αH resonances.

^e28 residues with sequence identity.

tended to the side chains of conserved residues. Among the side-chain resonance assignments available for bovine S100β (Kilby et al., 1995), the chemical shifts of several residues within the hydrophobic core (Leu 12 [Leu 10 in S100β], Ile 3 [Ile 11], Leu 29 [Leu 27], and Phe 16 [Phe 14]) are close to the corresponding values for calcyclin. A more extensive comparison can be made with calbindin D_{9k}, and we find half of the 26 conserved residues have side-chain proton chemical shifts within 0.23 ppm of those in calcyclin. Striking examples include Asp 25, Ser 30, and Glu 67 (Asp 19, Ser 24, Glu 60 in calbindin D_{9k}) in the calcium-binding loops, for which chemical shift differences are <0.1 ppm. The unique side-chain chemical shifts in apo calcyclin reflect not only packing in the hydrophobic core, but also packing of side chains at the dimer interface. For example, the side chain of Leu 88 in one subunit is in close proximity to the aromatic rings of both Phe 70 and Phe 16 of the second subunit. This arrangement causes the Leu 88 δ-methyl protons to experience a significant ring current effect, which shifts the ¹H resonances far upfield to 0.33 and –0.05 ppm. These marked upfield chemical shifts observed for Leu 88 appear to be diagnostic for monitoring packing at the dimer interface (B.C.M. Potts, M. Sastry, M. Lubienski, & W.J. Chazin, unpubl. results). Leu 88 is substituted by phenylalanine in S100β, precluding a direct comparison, but, if this protein adopts an analogous fold to that of calcyclin, one can anticipate a corresponding clustering of aromatic residues (Phe 14, Phe 70, and Phe 88) and unique chemical shift values.

The relationship between sequence homology and the conservation of structure in S100 proteins can be explored more directly by comparing the low-resolution three-dimensional structure of the apo calcyclin monomer subunit and the high-resolution structure of apo calbindin D_{9k}. In our preliminary analysis, we reported a comparison of interhelical angles for these two proteins (Potts et al., 1995). Here we extend and amplify the analysis by an in-depth comparison of the packing of side chains in the hydrophobic core. An overview of the structures and the disposition of key residues in the hydrophobic core of each protein is shown in Figure 6A. These key residues include Leu 12, Phe 16, Leu 29,

Fig. 6. Comparison of the three-dimensional structures of the apo calmodulin monomer subunit and apo calmodulin D_{9k} (accession #1CLB, Brookhaven Protein Data Bank). **A:** Overview of calmodulin D_{9k} (left) and apo calmodulin monomer (right), highlighting the conserved residues in the hydrophobic core (magenta). The selected structures have the lowest residual restraint violation energies within the final structural ensembles. The two structures were first overlaid by best-fit superposition of the backbone atoms of helices I, II, and IV, then separated to facilitate comparison. **B:** Close up view of the conserved hydrophobic residues at the interface between helices I and IV, showing the similarity in the packing of the corresponding side chains. The structures (same as in A) were overlaid by best-fit superposition of the backbone atoms of helices I and IV. Calmodulin D_{9k} in turquoise, calmodulin in yellow, and side chains labeled for calmodulin only. Prepared using INSIGHT (MSI Inc., San Diego, California). **C:** Close-up view of the extended hydrophobic network in the calmodulin dimer structure. Ribbon trace of backbone atoms shown in blue for one subunit and yellow for the other subunit of the dimer. The conserved hydrophobic core (same as in B, with side chains shown in white) is extended by Ile 13 and Ile 74 from the same subunit (turquoise) and Met 82 and Leu 88' from the second subunit (yellow). The selected dimer structure is closest to the calculated mean dimer structure. Prepared using INSIGHT. **D:** Calmodulin dimer structure (same as in C), with one subunit shown in blue, the other in yellow. Key hydrophobic residues (highlighted in C) and their symmetry-related residues are shown with CPK space filling, and together comprise a continuous hydrophobic network at the core of the dimer structure. Prepared using INSIGHT.

Val 68, Phe 70, Tyr 73, and Leu 77 in calmodulin, which correspond to Leu 6, Phe 10, Leu 23, Val 61, Phe 63, Phe 66, and Val 70 in calmodulin D_{9k} , respectively. Figure 6B shows a close-up of the side-chain packing at the interface between helices I and IV. There is clearly a very extensive level of homology in this region: the RMSD over all heavy atoms is only 0.96 Å.

The hydrophobic packing arrangement common to calmodulin D_{9k} and the monomer subunit of calmodulin is clearly maintained in the calmodulin dimer structure, and is extended by direct contact with several conserved residues at the dimer interface (Ile 13, Ile 74 from the same subunit, and Met 82 and Leu 88 from the second subunit; Fig. 6C). This extended hydrophobic network has a symmetry-related counterpart in the symmetric homodimer. Al-

though knowledge of the precise packing of the interior residues in the dimer is limited by the current level of structural resolution, the two symmetry-related networks appear to form a continuous hydrophobic core (Fig. 6D), implying a very high affinity between the two subunits. The fact that many of these hydrophobic residues are identical or conservatively substituted throughout the S100 protein subfamily supports our hypothesis that an analogous packing arrangement should be observed for other full-length S100 proteins, such as S100 β .

To obtain insight into the difference between the dimeric S100 proteins and monomeric calmodulin D_{9k} we compare the amphiphilicity of Helix IV of calmodulin with that of calmodulin D_{9k} because Helices IV and IV' make a significant contribution to the calmodulin

dimer interface (Potts et al., 1995). A helical wheel diagram delineates a definitive amphipathic helix in the case of calbindin D_{9k} (Fig. 7). In the solution structure of apo calbindin D_{9k} (Skelton et al., 1995), the hydrophobic residues of Helix IV project toward the interior of the protein, whereas the hydrophilic residues face the solvent. In calcyclin, the hydrophobic face of Helix IV is retained and extended by three residues, Ile 74, Ala 81, and Ile 83. The remaining surface is somewhat hydrophilic, albeit less so than the analogous surface of calbindin D_{9k} . For example, Lys 71 and Lys 72 of calbindin D_{9k} are substituted by Gly 78 and Ala 79 in calcyclin. In addition, the hydrophilic face of Helix IV in calcyclin is interrupted by a methionine residue that makes key hydrophobic contacts at the dimer interface. The less definitive nature of the amphiphilicity of calcyclin is reflected in its per-residue hydrophobic moment (Eisenberg, 1984), which is up to five times less than that of calbindin D_{9k} . The apparent dotting of the hydrophilic surface with hydrophobic residues in calcyclin (and by analogy all of the highly homologous full-length S100 proteins including S100 β) is consistent with the burial of Helix IV by dimerization. The continuous hydrophilic surface of Helix IV in calbindin D_{9k} is consistent with its monomeric state, in which one side must remain exposed to solvent.

Materials and methods

Calcyclin was purified from rabbit lung cytosol as described previously (Tokumitsu et al., 1991). A single 8-mg sample was utilized for all NMR experiments. In addition, a few preliminary experiments were conducted on a sample of murine calcyclin isolated from Ehrlich ascites tumor cells (kindly provided by Dr.

Jacek Kuznicki, Nencki Institute, Warsaw, Poland). Light scattering experiments were performed on a solution (15 mg protein/mL, 50 mM Tris buffer, pH 7.5) of recombinant wild-type rabbit calcyclin (M. Lubienski, J. Glasser, Z. Hu, K. Okazaki, H. Hidaka, & W.J. Chazin, unpubl.) in the apo state using a Biotage DynaPro-801 Molecular Size Detector.

NMR samples were prepared by dissolving the protein in 0.44 mL calcium-free $^1\text{H}_2\text{O}$ containing 5% (v/v) $^2\text{H}_2\text{O}$ for the spectrometer lock circuitry, 10 mM NaCl, and ~ 2 mM EDTA. The final protein concentration of the rabbit lung calcyclin sample used for the detailed NMR analysis was 1.8 mM (0.9 mM in dimer). For experiments performed in $^2\text{H}_2\text{O}$ solution, the H_2O sample was lyophilized repeatedly and redissolved in 100% $^2\text{H}_2\text{O}$ (99.996%, Isotec) under an atmosphere of Ar. The pH was adjusted by addition of microliter quantities of 0.1 M NaOH, HCl, NaO^2H , or ^2HCl calcium-free solutions. The sample was maintained at pH 7.0 for the majority of experiments acquired in H_2O solution. Thereafter, the pH was adjusted in order to acquire a series of TOCSY spectra over a range of pH values (pH 7.1–5.3) and a NOESY spectrum at pH 6.2. After acquisition of a TOCSY experiment at 310 K (pH 6.7, $\tau_m = 63$ ms) in H_2O solution, the sample began to degrade, as evidenced by new peaks in the NMR spectra. Sodium azide was then added to the sample to a final concentration of 0.05 mM, the pH was adjusted to 6.2, and the sample was exchanged into $^2\text{H}_2\text{O}$. Because the new signals could be distinguished by their uniformly low intensity, $^2\text{H}_2\text{O}$ experiments were acquired on this partially degraded sample.

All NMR experiments were performed on a Bruker AMX-600 spectrometer at 300 K unless otherwise indicated. Standard pulse sequences and phase cycling were utilized to record phase-sensitive COSY (Marion & Wüthrich, 1983), R-COSY/DR-COSY (Wagner, 1983), 2Q/3Q (Braunschweiler et al., 1983), and 2QF-COSY (Rance et al., 1983). The coherence transfer delays for R-COSY ($\tau = 38$ ms) and DR-COSY ($\tau_1 = 22$ ms, $\tau_2 = 38$ ms) were optimized according to Chazin & Wüthrich (1987), and in a similar manner for 2Q ($\tau = 30$ ms) and 3Q ($\tau = 22$ ms). A NOESY spectrum ($\tau_m = 60$ ms) was acquired with the standard pulse sequence (Kumar et al., 1980; Macura & Ernst, 1980) to derive quantitative distance restraints for structure calculations. A second NOESY spectrum ($\tau_m = 200$ ms) was recorded 8 days after the H_2O sample was lyophilized and redissolved in $^2\text{H}_2\text{O}$; this spectrum provided the qualitative backbone amide proton exchange data for Figure 2. Additional NOESY spectra ($\tau_m = 200$ ms) were acquired without presaturation by replacing the observation pulse with a jump-and-return sequence (Plateau & Guéron, 1982). TOCSY experiments were acquired by the modified z-filter technique (Rance, 1987) with 50–100-ms mixing periods using DIPSI-2 spin-locking (Shaka et al., 1988). For NOESY and TOCSY experiments, baseline distortions in the ω_2 dimension were minimized by incorporating a short Hahn-echo period after the observe pulse (Rance & Byrd, 1983; Davis, 1989) and by digitally oversampling by a factor of two (Delsuc & Lallemand, 1986).

All spectra were recorded in phase-sensitive mode. Quadrature detection in the ω_1 dimension was achieved by using time-proportional phase incrementation (Marion & Wüthrich, 1983). The residual solvent resonance was attenuated by coherent low-power irradiation for 1.2 s preceding the pulse sequence and during the mixing time of the NOESY spectra. Total recycle delay for the 60-ms NOESY experiment was set to 2 s. The carrier was set to the solvent frequency except in the 2Q, 3Q, and TOCSY experiments in $^2\text{H}_2\text{O}$ solution. The 2Q and 3Q spectra in $^2\text{H}_2\text{O}$ were acquired

Fig. 7. Helical wheels for Helix IV of calbindin D_{9k} (top) and calcyclin (bottom). Black areas highlight the hydrophobic surfaces; dashed lines indicate hydrophobic residues incorporated within the hydrophilic face of the helix.

with no quadrature detection in ω_1 ; the carrier was set downfield of the $\text{C}^{\alpha}\text{H}$ resonances ($\delta = 5.75$ ppm) and the low-field portion of the spectrum was folded in ω_1 . For the $^2\text{H}_2\text{O}$ TOCSY experiments, the transmitter frequency was set on the solvent resonance for the presaturation period, then shifted to a point of low signal intensity in the upfield region of the spectrum (~ 2.8 ppm) for both the excitation and spin-lock pulses. Sixty-four or 96 transients/ t_1 value were recorded for most experiments, with additional transients for TOCSY with $\tau_m \geq 90$ ms (128), DR-COSY (192), and 3Q (192). For each FID, a total of 2,048 complex data points were collected in simultaneous acquisition mode over 7,246 Hz for COSY-type experiments, or 4,096 complex points over 12,500 Hz for TOCSY and NOESY experiments. The number of t_1 values acquired was adjusted to give values of $t_{1\text{max}}$ in the range of 20–30 ms for DR-COSY, 2Q, and 3Q, 30–40 ms for NOESY, 30–50 ms for R-COSY and TOCSY, and 80 ms for the COSY spectrum.

The raw data were processed on a SUN SPARCstation LX or a SUN SPARCstation 20 computer with FELIX 2.1 or 2.3 (Biosym, Inc.). Application of window functions and baseline corrections were performed as described by Chazin et al. (1988), with the exception that, in NOESY and TOCSY spectra, the data were treated with Lorentzian–Gaussian window functions in ω_2 prior to Fourier transformation. The NOESY spectrum ($\tau_m = 60$ ms) from which quantitative distance constraints were derived for structure calculations (see below) was processed with a weak Lorentzian–Gaussian window function in both dimensions. Cosine-modulated experiments were treated routinely for suppression of ridges by halving the intensity of the first data point (Otting et al., 1986). Typically, the final 2D matrix size was $2,048 \times 2,048$.

Distance and dihedral angle constraints

The assignment of distance constraints was made on the basis of crosspeaks in the $\omega_2 = \text{backbone NH}$, aromatic, and methyl regions of NOESY spectra, as well as from specific $d_{\alpha\beta}(i, i + 3)$ NOEs. Crosspeaks were picked manually and their volumes measured within FELIX. Assignment of crosspeaks was facilitated by a series of short programs written in AWK (Aho et al., 1988) that compared all NOESY crosspeak frequencies with a master frequency list of the assigned proton resonances. This automated comparison provided potential assignments for each NOESY crosspeak. The integrated volumes of the crosspeaks in the 60-ms NOESY spectrum recorded in H_2O solution were translated into three distance classes with upper bounds of 3.4, 4.2, and 5.5 Å. This classification was based on an empirical multiple point calibration scheme. The crosspeaks utilized for calibration included those corresponding to fixed distances between vicinal protons in aromatic rings and characteristic distances in regions of regular secondary structure [$d_{\text{N}\alpha}$, $d_{\alpha\text{N}}$, d_{NN} , $d_{\alpha\text{N}}(i, i + 3)$, $i, i + 4$] and β -sheet-type cross-strand NOEs (Billeter et al., 1982; Wüthrich et al., 1984). An additional distance category was created with a loose upper bound of 6.5 Å for crosspeaks that were present in the 200-ms NOESY spectrum but absent in the 60-ms NOESY spectrum. Crosspeak volumes derived from NOEs involving methyl and degenerate methylene groups were scaled according to the number of protons in the group prior to the volume-to-distance conversion (Yip, 1990).

In DG calculations, the standard pseudoatom representation (Wüthrich et al., 1983) was used for methylene protons and methyl groups. For cases where NOE connectivities were observed from a single proton to both diastereotopic methylene protons, the corresponding upper bound to the pseudoatom was modified as de-

scribed by Güntert et al. (1991) and Akke et al. (1992); the resulting value was used if it was lower than the value obtained from the standard pseudoatom correction. For constraints involving an alanine, threonine, or isoleucine methyl group, the pseudoatom correction was set to 0.5 Å (Tropp, 1980; Kline et al., 1988). The lower distance bound was set to the sum of the van der Waals radii, except for the backbone amide and backbone carbonyl pairs, for which the lower bound was set to 1.8 Å to allow potential hydrogen bonds to form. All sequential and intraresidue distance constraints that were less restraining than elementary sterically imposed constraints (Wüthrich et al., 1983) were filtered out prior to the DG calculations.

No backbone dihedral angle restraints were initially included in the structure calculations because measurement of $^3J_{\text{NH}\alpha}$ scalar couplings from COSY spectra were precluded by the large resonance linewidths associated with this 20-kDa dimer. However, loose backbone dihedral angle restraints were included in the *final round of calculations* for 50 residues within elements of secondary structure that are clearly defined by the collective data in Figure 2, and that were consistently observed in the penultimate ensemble of structures. Restraints were set to $\phi = -60 \pm 30^\circ$, $\psi = -40 \pm 60^\circ$ for helices, and $\phi = -120 \pm 30^\circ$, $\psi = 120 \pm 60^\circ$ for extended conformation. Negative ϕ angles were enforced for six additional residues based on the ratio of intraresidue to sequential $d_{\alpha\text{N}}$ crosspeaks being greater than 1 (Ludvigsen & Poulsen, 1992).

Hydrogen-bonding constraints were used in the distance geometry calculations only. Twelve hydrogen bonds were identified on the basis of characteristic NOEs indicative of regular secondary structure. An $(i, i - 4)$ hydrogen bond was assigned for residues in helices if both the corresponding $d_{\alpha\text{N}}(i, i + 3)$ and $d_{\alpha\text{N}}(i, i + 4)$ connectivities were observed. A pair of hydrogen bonds between Leu 29 and Val 68 was assigned on the basis of characteristic cross-strand NOEs for a β -type interaction between the two empty calcium binding loops. For each hydrogen bond, the HN–O distance was constrained to 1.8–2.0 Å and the N–O distance to 2.7–3.0 Å.

Structure calculations for the monomer subunit

Starting structures were generated using an extensively modified version of the distance geometry program DISGEO (Havel & Wüthrich, 1984). These calculations made use of the refinement procedures available in DISGEO for chirality and planarity of peptide bonds. A total of 40 structures were calculated at each iteration. Initially, only a subset of atoms was embedded. For substructures that converged ($\geq 75\%$ in each case), further embedding was at the all atom level. These DG structures were subsequently subjected to restrained energy minimization and restrained molecular dynamics in the SANDER module of AMBER 4.0 (Pearlman et al., 1991a, 1991b) in order to more completely sample conformational space in the vicinity of the starting structure, as well as to refine the geometry and energies of the structures. The rMD simulations were performed in vacuo, essentially as described previously (Kördel et al., 1993), except that chirality constraints were imposed for chiral and prochiral atoms with a force constant of $10 \text{ kcal}\cdot\text{mol}^{-1}$.

Eight iterations of the structure calculation protocol outlined above were conducted. The initial round of calculations used only unambiguously identified NOEs. At an intermediate stage, when 406 distance constraints had been assigned, a round of complete relaxation matrix analysis back-calculations using the CORMA program (Keepers & James, 1984; Borgias & James, 1990) was

performed on the eight structures with lowest residual violation and molecular energies. The mean theoretical NOE volume and standard deviation for each proton–proton pair was calculated, and then used to facilitate the assignment of NOEs that have multiple assignment possibilities based on resonance frequency alone (Skelton et al., 1995). This strategy brought the total number of distance restraints to 556, the number used in the last two rounds of calculations. In the final round of calculations, these distance constraints were supplemented with loose backbone dihedral angle restraints (see above).

Thirty-two of the 40 DG structures converged in the final round of calculations; however, two mirror-image families were observed. The 23 structures with the correct handedness (right-handed helices with a global folding pattern analogous to that of other EF-hand calcium-binding proteins) were further refined by rMD. The six structures with the lowest residual violation energies were utilized for all subsequent analysis.

Calculations of hydrophobic moment and secondary chemical shifts

The per-residue hydrophobic moment was calculated by the method of Eisenberg (1984) using three different normalized hydrophobicity scales (Consensus, Janin, and Kyte). For this analysis, α -helix is assumed for Helix IV of calyculin so that it can be aligned to calbindin D_{9k} , which is known to be α -helical in the apo state (Skelton et al., 1995).

The secondary chemical shifts (observed chemical shift minus random coil chemical shift) were calculated with the program "ssshift" (G. Gippert, unpubl.) using the C^α proton random coil chemical shifts (Wishart et al., 1991) (averaged C^αH for glycines) and the backbone amide random coil chemical shifts and temperature coefficients from Merutka et al. (1995). The secondary chemical shifts for bovine S100 β and calbindin D_{9k} were calculated using the ^1H chemical shifts reported by Kilby et al. (1995) and Skelton et al. (1990), respectively. The chemical shifts were corrected according to Wishart et al. (1995) in order to reference the data to a single, standard reference compound.

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References

- Aho AV, Kernighan BW, Weinberger PJ. 1988. *The AWK programming language*. Reading, Massachusetts: Addison-Wesley.
- Akke M, Drakenberg T, Chazin WJ. 1992. Three-dimensional solution structure of Ca^{2+} -loaded porcine calbindin D_{9k} determined by nuclear magnetic resonance spectroscopy. *Biochemistry* 31:1011–1020.
- Akke M, Forsén S, Chazin WJ. 1995. Solution structure of $(\text{Cd}^{2+})_1$ -calbindin D_{9k} reveals details of the stepwise structural changes along apo $\rightarrow (\text{Ca}^{2+})_1$ $\rightarrow (\text{Ca}^{2+})_2$ binding pathway. *J Mol Biol* 252:102–121.
- Amburgey JC, Abildgaard F, Starich MR, Shah S, Hilt DC, Weber DJ. 1995. ^1H , ^{13}C and ^{15}N NMR assignments and solution secondary structure of rat Apo-S100 β . *J Biomol NMR* 6:171–179.
- Barger SW, Wolchok SR, van Eldik LJ. 1992. Disulfide-linked S100 β dimers and signal transduction. *Biochim Biophys Acta* 1160:105–112.
- Billeter M, Braun W, Wüthrich K. 1982. Sequential resonance assignments in protein ^1H nuclear magnetic resonance spectra. *J Mol Biol* 155:321–346.
- Borgias BA, James TL. 1990. MARDIGRAS—A procedure for matrix analysis of relaxation for discerning geometry of an aqueous structure. *J Magn Reson* 87:475–487.
- Braunschweiler L, Bodenhausen G, Ernst RR. 1983. Analysis of networks of coupled spins by multiple quantum NMR. *Mol Phys* 48:535–560.
- Breg JN, Boelens R, George AVE, Kaptein R. 1989. Sequence-specific ^1H NMR assignment and secondary structure of the Arc repressor of bacteriophage P22, as determined by two-dimensional ^1H NMR spectroscopy. *Biochemistry* 28:9826–9833.
- Calabretta B, Kaczmarek L, Mars W, Ochoa D, Gibson CW, Hirschhorn RR, Baserga R. 1985. Cell-cycle-specific genes differentially expressed in human leukemias. *Proc Natl Acad Sci USA* 82:4463–4467.
- Calabretta B, Venturelli D, Kaczmarek L, Narni F, Talpaz M, Anderson B, Beran M, Baserga R. 1986. Altered expression of G_1 -specific genes in human malignant myeloid cells. *Proc Natl Acad Sci USA* 83:1495–1498.
- Chazin WJ, Rance M, Wright PE. 1987. Complete assignment of lysine resonances in ^1H NMR spectra of proteins as probes of surface structure and dynamics. *FEBS Lett* 222:109–114.
- Chazin WJ, Rance M, Wright PE. 1988. Complete assignment of the ^1H nuclear magnetic resonance spectrum of French bean plastocyanin. Application of an integrated approach to spin system identification in proteins. *J Mol Biol* 202:603–622.
- Chazin WJ, Wright PE. 1987. A modified strategy for identification of ^1H spin systems in proteins. *Biopolymers* 26:973–977.
- Chazin WJ, Wüthrich K. 1987. Optimization of homonuclear relayed coherence transfer experiments with proteins in H_2O solution. *J Magn Reson* 72:358–363.
- Crivici A, Ikura M. 1995. Molecular and structural basis of target recognition by calmodulin. *Annu Rev Biophys Biomol Struct* 24:86–116.
- Davis DG. 1989. Elimination of baseline distortions and minimization of artifacts from phased 2D NMR spectra. *J Magn Reson* 81:603–607.
- Dell'Angelica EC, Schleicher CH, Santome JA. 1994. Primary structure and binding properties of calgranulin C, a novel S100-like calcium-binding protein from pig granulocytes. *J Biol Chem* 269:28929–28936.
- Delsuc MA, Lallemand JY. 1986. Improvement of dynamic range in NMR by oversampling. *J Magn Reson* 69:504–507.
- Eisenberg D. 1984. Three-dimensional structure of membrane and surface proteins. *Annu Rev Biochem* 53:595–623.
- Findlay WA, Sonnichsen FD, Sykes BD. 1994. Solution structure of the TR1C fragment of skeletal muscle troponin-C. *J Biol Chem* 269:6773–6778.
- Finn BE, Evenäs J, Drakenberg T, Waltho JP, Thulin E, Forsén S. 1995. Calcium-induced structural changes and domain autonomy in calmodulin. *Nature Struct Biol* 2:777–783.
- Gerke V, Weber K. 1985. Calcium-dependent conformational changes in the 36-kDa subunit of intestinal protein I related to the cellular 36-kDa target of rous sarcoma virus tyrosine kinase. *J Biol Chem* 260:1688–1695.
- Güntert P, Braun W, Wüthrich K. 1991. Efficient computation of three-dimensional protein structures in solution from nuclear magnetic resonance data using the program DIANA and the supporting programs CALIBA, HABAS and GLOSM. *J Mol Biol* 217:517–530.
- Güntert P, Wüthrich K. 1991. Improved efficiency of protein structure calculations from NMR data using the program DIANA with redundant dihedral angle constraints. *J Biomol NMR* 1:447–456.
- Guo X, Chambers AF, Parfett CLJ, Waterhouse P, Murphy LC, Reid RE, Craig AM, Edwards DR, Denhardt DT. 1990. Identification of a serum-inducible messenger RNA (5B10) as the mouse homologue of calyculin: Tissue distribution and expression in metastatic, ras-transformed NIH 3T3 cells. *Cell Growth Diff* 1:333–338.
- Harper ET, Rose GD. 1993. Helix stop signals in proteins and peptides: The capping box. *Biochemistry* 32:7605–7609.
- Havel T, Wüthrich K. 1984. A distance geometry program for determining the structures of small proteins and other macromolecules from nuclear magnetic resonance measurements of intramolecular ^1H - ^1H proximities in solution. *Bull Math Biol* 46:673–698.
- Hilt DC, Kligman D. 1991. *The S-100 protein family: A biochemical and functional overview*. New York: Springer-Verlag.
- Hirschhorn RR, Aller P, Yuan Z, Gibson CW, Baserga R. 1984. Cell-cycle-specific cDNAs from mammalian cells temperature sensitive for growth. *Proc Natl Acad Sci USA* 81:6004–6008.
- Kay LE, Forman-Kay JD, McCubbin WD, Kay CM. 1991. Solution structure of a polypeptide dimer comprising the fourth Ca^{2+} -binding site of troponin C by nuclear magnetic resonance spectroscopy. *Biochemistry* 30:4323–4333.
- Keepers JW, James TL. 1984. A theoretical study of distance determinations from NMR. Two-dimensional nuclear Overhauser effect spectra. *J Magn Reson* 57:404–426.

- Kilby PM, van Eldik LJ, Roberts GCK. 1995. Nuclear magnetic resonance assignments and secondary structure of bovine S100 β protein. *FEBS Lett* 363:90–96.
- Kim Y, Prestegard JH. 1989. Measurement of vicinal couplings from cross peaks in COSY spectra. *J Magn Reson* 84:9–13.
- Kligman D, Hilt DC. 1988. The S100 protein family. *Trends Biochem Sci* 13:437–443.
- Kline AD, Braun W, Wüthrich K. 1988. Determination of the complete three-dimensional structure of the α -amylase inhibitor tendamistat in aqueous solution by nuclear magnetic resonance and distance geometry. *J Mol Biol* 204:675–724.
- Kördel J, Skelton NJ, Akke M, Chazin WJ. 1993. High-resolution solution structure of calcium-loaded calbindin D_{9k}. *J Mol Biol* 231:711–734.
- Kraulis PJ. 1991. MOLSCRIPT: A program to produce both detailed and schematic plots of protein structures. *J Appl Crystallogr* 24:946–950.
- Krebs J, Quadroni M, van Eldik LJ. 1995. Dance of the dimers. *Nature Struct Biol* 2:711–714.
- Kube E, Becker T, Weber K, Gerke V. 1992. Protein–protein interaction studied by site-directed mutagenesis. *J Biol Chem* 267:14175–14182.
- Kuboniwa H, Tjandra N, Grzesiek S, Ren H, Klee CB, Bax A. 1995. Solution structure of calcium-free calmodulin. *Nature Struct Biol* 2:768–776.
- Kumar A, Ernst RR, Wüthrich K. 1980. A two-dimensional nuclear overhauser enhancement (2D NOE) experiment for the elucidation of complete proton–proton cross-relaxation networks in biological macromolecules. *Biochem Biophys Res Commun* 95:1–6.
- Kuznicki J, Filippek A, Hunziker PE, Huber S, Heizmann CW. 1989. Calcium-binding protein from mouse Ehrlich ascites-tumor cells is homologous to human calyculin. *Biochem J* 263:951–956.
- Ludvigsen S, Poulsen FM. 1992. Positive ϕ -angles in proteins by nuclear magnetic resonance spectroscopy. *J Biomol NMR* 2:227–233.
- Lyu PC, Wemmer DE, Zhou HX, Pinker RJ, Kallenbach NR. 1993. Capping interactions in isolated α -helices: Position-dependent substitution effects and structure of a serine-capped peptide helix. *Biochemistry* 32:421–425.
- Macura S, Ernst RR. 1980. Elucidation of cross relaxation in liquids by two-dimensional NMR spectroscopy. *Mol Phys* 41:95–117.
- Marion D, Wüthrich K. 1983. Application of phase sensitive two-dimensional correlated spectroscopy (COSY) for measurement of ^1H - ^1H spin-spin coupling constants in proteins. *Biochem Biophys Res Commun* 113:967–974.
- Merutka G, Dyson HJ, Wright PE. 1995. "Random coil" ^1H chemical shifts obtained as a function of temperature and trifluoroethanol concentration for the peptide series GGXGG. *J Biomol NMR* 5:14–24.
- Minami H, Tokumitsu H, Mizutani A, Watanabe Y, Watanabe M, Hidaka H. 1992. Specific binding of CAP-50 to calyculin. *FEBS Lett* 305:217–219.
- Otting G, Widmer H, Wagner G, Wüthrich K. 1986. Origin of t_1 and t_2 ridges in 2D NMR spectra and procedures for suppression. *J Magn Reson* 66:187–193.
- Pearlman DA, Case DA, Caldwell JC, Seibel GL, Singh UC, Weiner P, Kollman PA. 1991a. SANDER/AMBER 4.0. San Francisco, California: University of California.
- Pearlman DA, Case DA, Yip P. 1991b. SANDER/AMBER 4.0. San Francisco, California: University of California.
- Pedrocchi M, Schafer BW, Durussel I, Cox JA, Heizmann CW. 1994. Purification and characterization of the recombinant human calcium-binding S100 protein CAPL and CACY. *Biochemistry* 33:6732–6738.
- Plateau P, Guéron M. 1982. Exchangeable proton NMR without base-line distortion, using new strong-pulse sequences. *J Am Chem Soc* 104:7310–7311.
- Potts BCM, Smith J, Akke M, Macke TJ, Okazaki K, Hidaka H, Case DA, Chazin WJ. 1995. The structure of calyculin reveals a novel homodimeric fold for S100 Ca^{2+} -binding proteins. *Nature Struct Biol* 2:790–796.
- Rance M. 1987. Improved techniques for homonuclear rotating-frame and isotropic mixing experiments. *J Magn Reson* 74:557–564.
- Rance M, Byrd RA. 1983. Obtaining high-fidelity spin-1/2 powder spectra in anisotropic media: Phase-cycled Hahn echo spectroscopy. *J Magn Reson* 52:221–240.
- Rance M, Sørensen OW, Bodenhausen G, Wagner G, Ernst RR, Wüthrich K. 1983. Improved spectral resolution in COSY ^1H NMR spectra of proteins via double quantum filtering. *Biochem Biophys Res Commun* 117:479–485.
- Shaka AJ, Lee CJ, Pines A. 1988. Iterative schemes for bilinear operators; applications to spin decoupling. *J Magn Reson* 77:274–293.
- Shaw GS, Hodges RS, Sykes BD. 1992. Determination of the solution structure of a synthetic two-site calcium-binding homodimeric protein domain by NMR spectroscopy. *Biochemistry* 31:9572–9580.
- Skelton NJ, Forsén S, Chazin WJ. 1990. ^1H NMR resonance assignments, secondary structure, and global fold of apo bovine calbindin D_{9k}. *Biochemistry* 29:5752–5761.
- Skelton NJ, Kördel J, Akke M, Chazin WJ. 1992. Nuclear magnetic resonance studies of the internal dynamics in apo, (Ca^{2+})₁ and (Ca^{2+})₂ calbindin D_{9k}. *J Mol Biol* 227:1100–1117.
- Skelton NJ, Kördel J, Akke M, Forsén S, Chazin WJ. 1994. Signal transduction versus buffering activity in Ca^{2+} -binding proteins. *Nature Struct Biol* 1:239–245.
- Skelton NJ, Kördel J, Chazin WJ. 1995. Determination of the solution structure of apo calbindin D_{9k} by NMR spectroscopy. *J Mol Biol* 249:441–462.
- Smith SP, Barber KR, Dunn SD, Shaw GS. 1996. Structural influence of cation binding to recombinant human brain S100b: Evidence for calcium-induced exposure of a hydrophobic surface. *Biochemistry* 35:8805–8814.
- Strynadka NCJ, James MNG. 1989. Crystal structures of the helix-loop-helix calcium-binding proteins. *Annu Rev Biochem* 58:951–998.
- Svensson LA, Thulin E, Forsén S. 1992. Proline *cis-trans* isomers in calbindin D_{9k} observed by X-ray crystallography. *J Mol Biol* 223:601–606.
- Szebenyi DME, Moffat K. 1986. The refined structure of vitamin D-dependent calcium-binding protein from bovine intestine. *J Biol Chem* 261:8761–8777.
- Teigelkamp S, Bhardwaj RS, Roth J, Meinardus-Hager G, Karas M, Sorg C. 1991. Calcium-dependent complex assembly of the myeloid differentiation proteins MRP-8 and MRP-14. *J Biol Chem* 266:13462–13467.
- Tokumitsu H, Kobayashi R, Hidaka H. 1991. A calcium-binding protein from rabbit lung cytosol identified as the product of growth-regulated gene (2A9) and its binding proteins. *Arch Biochem Biophys* 288:202–207.
- Tokumitsu H, Mizutani A, Hidaka H. 1993. Calyculin-binding site located on the NH_2 -terminal domain of rabbit CAP-50 (annexin XI): Functional expression of CAP-50 in *Escherichia coli*. *Arch Biochem Biophys* 303:302–306.
- Tokumitsu H, Mizutani A, Minami H, Kobayashi R, Hidaka H. 1992. A calyculin-associated protein is a newly identified member of the Ca^{2+} /phospholipid-binding proteins, annexin family. *J Biol Chem* 267:8919–8924.
- Tropp J. 1980. Dipolar relaxation and nuclear Overhauser effects in nonrigid molecules: The effect of fluctuating internuclear distances. *J Chem Phys* 72:6035–6043.
- Wagner G. 1983. Two-dimensional relayed coherence transfer spectroscopy of a protein. *J Magn Reson* 55:151–156.
- Watanabe M, Ando Y, Tokumitsu H, Hidaka H. 1993. Binding site of annexin XI on the calyculin molecule. *Biochem Biophys Res Commun* 196:1376–1382.
- Weber C, Lee VD, Chazin WJ, Huang B. 1994. High level expression in *Escherichia coli* and characterization of the EF-hand calcium-binding protein caltractin. *J Biol Chem* 269:15795–15802.
- Weternman MAJ, Stoopen GM, Muijen GNPV, Kuznicki J, Ruiter DJ, Bloemers HPI. 1992. Expression of calyculin in human melanoma cell lines correlates with metastatic behavior in nude mice. *Cancer Res* 52:1291–1296.
- Wishart DS, Bigam CG, Yao J, Abildgaard F, Dyson HJ, Oldfield E, Markley JL, Sykes BD. 1995. H-1, C-13 and N-15 chemical shift referencing in biomolecular NMR. *J Biomol NMR* 6:135–140.
- Wishart DS, Sykes BD, Richards FM. 1991. Relationship between nuclear magnetic resonance chemical shift and protein secondary structure. *J Mol Biol* 222:311–333.
- Wishart DS, Sykes BD, Richards FM. 1992. The chemical shift index: A fast and simple method for the assignment of protein secondary structure through NMR spectroscopy. *Biochemistry* 31:1647–1651.
- Wojda U, Kuznicki J. 1994. Calyculin from mouse Ehrlich ascites tumor cells and rabbit lung form non-covalent dimers. *Biochim Biophys Acta* 1209:248–252.
- Wüthrich K. 1986. *NMR of proteins and nucleic acids*. New York: John Wiley & Sons.
- Wüthrich K, Billeter M, Braun W. 1983. Pseudo-structures for the 20 common amino acids for use in studies of protein conformations by measurements of intramolecular proton–proton distance constraints with nuclear magnetic resonance. *J Mol Biol* 169:949–961.
- Wüthrich K, Billeter M, Braun W. 1984. Polypeptide secondary structure determination by nuclear magnetic resonance observation of short proton–proton distances. *J Mol Biol* 180:715–740.
- Yip PF. 1990. Scaling NOESY cross peaks involving methyl protons. *J Magn Reson* 90:382–383.
- Zeng F, Gabius H. 1991. Carbohydrate-binding specificity of calyculin and its expression in human tissues and leukemic cells. *Arch Biochem Biophys* 289:137–144.
- Zeng F, Gerke V, Gabius H. 1993. Identification of annexin II, annexin VI and glyceraldehyde-3-phosphate dehydrogenase as calyculin-binding proteins in bovine heart. *Int J Biochem* 25:1019–1027.
- Zhang M, Tanaka T, Ikura M. 1995. Calcium-induced conformational transition revealed by the solution structure of apo calmodulin. *Nature Struct Biol* 2:758–767.