ABSTRACT: The calmodulin- and calcium-stimulated protein phosphatase calcineurin, PP2B, consists of two subunits: calcineurin B, which binds Ca$^{2+}$, and calcineurin A, which contains the catalytic site and a calmodulin binding site. Heteronuclear 3D and 4D NMR experiments were carried out on a recombinant human calcineurin B which is a 170-residue protein of molecular mass 19.3 kDa, uniformly labeled with $^{15}$N and $^{13}$C. The non-denaturing detergent CHAPS was used to obtain a monomeric form of calcineurin B. Three-dimensional triple resonance experiments yielded complete sequential assignment of the backbone nuclei ($^1$H, $^{13}$C, and $^{15}$N). This assignment was verified by a 4D HN(CO)CA(NH) experiment carried out with 50% randomly deuterated and uniformly $^{15}$N- and $^{13}$C-enriched calcineurin B. The secondary structure of calcineurin B has been determined on the basis of the $^{13}$Cα and $^{13}$Cβ secondary chemical shifts, J($^1$H$^1$H$^1$H$^1$H$^1$) couplings, and NOE connectivities obtained from 3D $^1$H-separated and 4D $^{13}$C/$^{15}$N-separated NOESY spectra. Calcineurin B has eight helices distributed in four EF-hand, helix-loop-helix [Kretsinger, R. H. (1980) CRC Crit. Rev. Biochem. 8, 119–174] calcium binding domains. The secondary structure of calcineurin B is highly homologous to that of calmodulin. In comparison to calmodulin, helices B and C are shorter while helix G is considerably longer. As was observed for calmodulin in solution, calcineurin B does not have a single long central helix; rather, helices D and E are separated by a six-residue sequence in a flexible nonhelical conformation.

Calcineurin is a calmodulin- and calcium-stimulated protein phosphatase present in all eukaryotic cells, including nerve cells and T cells (Klee et al., 1987). Calcineurin consists of two tightly bound subunits, calcineurin A and calcineurin B, with molecular masses of 59 and 19.3 kDa, respectively. The presence of the two calcium-binding proteins, calcineurin B and calmodulin, is essential for the phosphatase activity of calcineurin A, and they cannot replace each other (Klee et al., 1987).

Calcineurin has been identified as the intracellular target of the immunosuppressant–immunophilin complexes FKBP–FK506 and cyclophilin–cyclosporin A (Liu et al., 1991; Liu, 1993a,b; Schreiber & Crabtree, 1992; Schreiber, 1992). Recent findings suggest that during T-cell activation calcineurin is a rate-limiting signal-transducing protein in Ca$^{2+}$-dependent pathways (Liu, 1993a). Calcineurin activity was found to parallel the exocytosis of preformed granules from cytotoxic T lymphocytes in the presence of cycloheximide, an inhibitor of transcription (Dutz et al., 1993) and it was also seen to increase IL-2 promoter activity in vivo (Clipstone & Crabtree, 1992; Schreiber, 1992). Regarding this transcription-dependent activation pathway, current evidence indicates that calcineurin, when activated by calmodulin, forwards the signal to the nucleus by dephosphorylating the cytoplasmic subunit of NF-AT, NF-ATc (McCaffrey et al., 1993). NF-ATc (also known as NF-ATp), in turn, translocates to the nucleus to join with its nuclear counterpart, NF-ATn, and together they activate transcription of the T-cell growth factor, interleukin 2 (Flanagan et al., 1991). The translocation of NF-ATc is prevented by cyclosporin and FK506, which exert their immunosuppressive properties, after binding to their respective immunophilins cyclophilin and FKBP, by inhibiting calcineurin, thereby preventing dephosphorylation of NF-ATc. Cross-linking experiments between cyclophilin–cyclosporin

1$^1$H, $^{13}$C, $^{15}$N Nuclear Magnetic Resonance Backbone Assignments and Secondary Structure of Human Calcineurin B†

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A or FK506–FKBP and calcineurin (both A and B subunits) suggest that the immunophilin–immunosuppressant complexes may interact primarily with the calcineurin B subunit (Li & Handschumacher, 1993).

The amino acid sequence of calcineurin B suggests the presence of four calcium binding sites of the EF-hand helix-loop-helix (HLH) type (Aitken et al., 1984). The calcium binding loops share 54% sequence identity with the calcium binding loops of calmodulin (Guerini & Klee, 1991), but outside these four calcium binding loops the degree of sequence homology is only 20%. To better understand how calcineurin B regulates the activity of calcineurin A we have initiated NMR studies of the solution structure of calcineurin B.

At the concentration required for NMR observation, the calcium-saturated form of calcineurin B dissolved in water shows resonance line widths that are indicative of aggregation (Anglister et al., 1993). The line width could be influenced to some degree by optimizing temperature, pH, and salt concentrations (Wagner, 1993), but in the absence of detergent no conditions could be found where the protein behaves as a monomeric unit. In the presence of approximately a 10-fold excess of the zwitterionic detergent 3-[[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), resonance line widths are considerably narrower and are consistent with a mass of the protein plus attached detergent of ~25 kDa. Calcineurin (the complex of calcineurin A and B) retains a high degree of activity in the presence of 20 mM CHAPS. The presence of the nondeuterated CHAPS does not interfere with the isotope-directed NMR techniques employed, as the signals from protons not attached to 15N or 13C are removed by isotope filtering, purge pulses, and pulsed field gradients (Anglister et al., 1993).

The present NMR study deals with genetically engineered human calcineurin B, overexpressed and isotopically enriched in Escherichia coli and lacking the posttranslational myristoylation of natural calcineurin B at its amino-terminal glycine residue (Aitken et al., 1982). Residues Cys12 and Cys14 were mutated to alanine and lysine, respectively, to avoid aggregation due to disulfide bond formation. These substitutions correspond to the residue types found in yeast calcineurin B.

The recent cross-linking results with FKBP and cyclophilin (Li & Handschumacher, 1993) indicate that some structural rearrangement occurs upon complexation of calcineurin B with the A subunit. Such a rearrangement is unlikely to involve large changes in secondary structure, however. The present study is therefore restricted to the secondary structure, with future work directed toward determination of the 3D structures of complexes between calcineurin B and recently identified fragments of A that bind to the B subunit.

MATERIALS AND METHODS

Molecular Cloning. Restriction enzyme digestion, ligations, DNA gel electrophoresis, and other recombinant DNA techniques were performed essentially as described by Sambrook et al. (1989). Custom oligonucleotides were synthesized on a Model 8700 DNA synthesizer (Millipore, Bedford, MA).

Mutagenesis. The coding region of human calcineurin B cDNA subcloned in the vector pBE (Guerini et al., 1989) was amplified and mutated by the polymerase chain reaction. The sense oligonucleotide, BA12 (5'-C GGG ATC CCC ATG GGA AAT GAG GCA AGT TAT CCT TTG GAA ATG GCT TCA CAC TTT GAT GCG), corresponds to the 16 amino-terminal amino acids of calcineurin B except that Cys12 (TCG) was changed to Ala12 (GCT). Note that the residue numbering used in the present paper (Figure 4) starts with Met1, although this residue was present in only a 30% fraction of the protein. A NcoI site was introduced in front of the initiation codon ATG and a BamHI site at the 5'-end. The antisense oligonucleotide, BK154 (5'-CGC GGA TCC TCA CAT ATC CAT CAT CTT TTT GTG GAT ATC TAG GCC ACC TAC AAC AGC TTT GAA TTC TTC AAA GG), is complementary to the 21 carboxy-terminal amino acids of calcineurin B except that Cys14 (TGT) was changed to Lys14 (AAA). A BamHI site was introduced downstream of the termination codon to facilitate subcloning. The amplified fragment was digested with BamHI and ligated into the BamHI site of the pBK SK( -) vector (Stratagene, La Jolla, CA) to produce pBC-BAK. The sequence of the entire coding region of the calcineurin B mutant CnB(A12K154) was verified by the method of Sanger et al. (1977). The DNA fragment containing the entire coding sequence and the termination codon was excised from pBC-BAK by digestion with NcoI and BamHI and subcloned into the expression vector pET3d (Novagen, Madison, WI) under the control of the T7 RNA polymerase promoter (Studier et al., 1990).

Expression and Purification of Calcineurin B (A11K153). E. coli strain BL21 (DE3) plysS (Novagen, Madison, WI), a IDE3 lysogen containing the T7 RNA polymerase gene under the lacUV5 promoter, was transformed with pBAKE and grown at 37 °C in M9 minimal salt medium supplemented with 0.4% glucose, 0.1 mM CaCl2, 1 mM MgSO4, 2 mg/L thiamin, 2 mg/L biotin, 100 mg/L ampicillin, and 30 mg/L chloramphenicol. When the absorbance at 600 nm reached 0.7, the production of protein was induced by addition of isopropyl β-D-thiogalactoside to a final concentration of 1 mM. After 5 h, the cells were harvested by centrifugation, washed, and stored at -70 °C. Calcineurin B uniformly labeled with 15N and 13C was obtained by growing the cells in the same medium supplemented with 15NH4Cl and 13C-D-glucose (Isotec, Miamisburg, OH) as sole nitrogen and carbon sources. M9 medium prepared with 60% D2O/40% H2O was used to prepare a fractionally deuterated sample of calcineurin B.

The frozen cells were routinely processed in batches corresponding to 2.5 L of original bacterial culture. The thawed cells were dispersed in 40 mL of lysis buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2 mM MgCl2, 2 mM CaCl2, 10 mg/L N-tosyl-L-phenylalanine chloromethyl ketone, 10 mg/L N-tosyl-L-lysine chloromethyl ketone, and 75 mg/L phenylmethanesulfonyl fluoride) and disrupted with a W-375 cell disrupter (Heat Systems, Farmingdale, NY) by 10 1-min bursts of sonication at 2-min intervals. Cell debris was removed by centrifugation at 4 °C for 1 h at 30000g. To eliminate the nucleic acids present in the supernatant fluid, its NaCl concentration was adjusted to 0.3 M prior to loading onto a DEAE-Sepharose column which had been pre-equilibrated with 50 mM NaCl, 0.1 mM CaCl2, 50 mM Tris-HCl at pH 8, 2 mM MgCl2, 2 µg/mL aprotinin, 1 µg/mL leupeptin, and 0.02% NaN3. After the sample was loaded, the DEAE column was washed with this same solution and the eluent was monitored for absorption at 280 and 260 nm. The first fractions had the highest ratios of 280/260 absorption and 0.5 were collected for further purification on a phenyl-Sepharose CL-4B column (Pharmacia). This column was equilibrated with a loading buffer containing 0.5 M NaCl, 0.1 mM CaCl2, 50 mM Tris-HCl at pH 8, 2 µg/mL aprotinin, 1 µg/mL leupeptin, and 0.02% NaN3. The DEAE fractions were adjusted to 0.5 M NaCl and loaded onto the phenyl-Sepharose column, which was washed with the loading buffer until the absorbance at
280 nm decreased to 0.1, at which point the buffer was changed to a 0.1 mM CaCl₂ solution to wash out contaminants which bind phenyl-Sepharose. When the absorbance of the eluent reached 0.01, calcinurin B was eluted with 2 mM EGTA, pH 7.5. Purity in excess of 99% was determined bypolyacrylamide gel electrophoresis. The protein was concentrated using collodion bags with a molecular weight cutoff of 10 000. The calcinurin B concentration was determined by its absorbance at 276 nm using an extinction coefficient of ε276 = 2.41 (Tris-HCl at pH 7.5, 0.1 M NaCl, and 1 mM CaCl₂).

**Binding Experiments.** The binding of the peptide M13 (KRRWKKNFIAVSAANRFKKISSSGAL) to calcinurin B and calmodulin was assayed using the Hummel–Dryer technique (1962). For this purpose we used a 1- x 30-cm column packed with Sephadex G-25 fine. The column was equilibrated with the binding buffer, 100 mM KCl, 40 mM Tris-HCl at pH 8, 0.1 mM CaCl₂, and 2 μM M13. A 0.5-mL sample containing 20 μM either calmodulin or calcineurin B in the running buffer was loaded onto the column and 0.4-mL fractions were monitored for absorption at 290 nm. Binding is indicated by a first peak with strong absorbance above background followed by a peak with absorbance below background (Hummel & Dreyer, 1962). It should be noted that both calmodulin and calcineurin B do not contain tryptophan and thus the contribution of M13, which has a tryptophan, to the absorption at 290 nm could be studied.

**NMR Sample Preparation.** Four samples were prepared for the NMR experiments. One contained 2.3 mM calcineurin B uniformly labeled (>95%) with 15N and dissolved in 95% H₂O/5% D₂O, pH 4.95, 25 mM CHAPS, and 20 mM Ca²⁺.

The second sample contained 2.3 mM calcinurin B uniformly labeled (>95%) with 15N and dissolved in the same solution.

The third sample contained 1.6 mM calcineurin B uniformly labeled (>95%) with 15N and 13C dissolved in 99.99% D₂O at pH 4.75 (not corrected for isotope effect), 20 mM CHAPS, and 16 mM Ca²⁺.

The fourth sample contained 1.4 mM calcineurin B uniformly labeled (>95%) with 15N and 13C and randomly 2H-labeled to ~50%, dissolved in 95% H₂O/5% D₂O at pH 5.6, 20 mM CHAPS, and 20 mM Ca²⁺.

**Preparation of Calcinurin B Dissolved in D₂O.** A 2-mL sample of 0.4 mM calcineurin B in H₂O solution containing 4 mM CaCl₂, 5 mM CHAPS, and 0.01% NaN₃ was lyophilized and then dissolved in 0.7 mL of D₂O. The pH was adjusted to 7.8. This sample was kept at 30 °C for 3 days to allow exchange of the amide protons and then lyophilized a second time. The solid material was dissolved in D₂O and incubated for 24 h at 30 °C. The pH was then adjusted to 4.77 and once more lyophilized and dissolved in 0.5 mL of D₂O. To identify slowly exchanging amide protons, a 2D 1H-15N HSQC spectrum was recorded 24 h after this procedure.

**NMR Spectroscopy.** All NMR experiments were carried out at 37 °C on a Bruker 600-MHz AMX spectrometer equipped with an external class A/B 100-W power amplifier for the 13C channel. The following 3D spectra were recorded using the 13C/15N-labeled sample dissolved in H₂O with pulse sequences reported previously. Unless noted otherwise, a 1.8-ms transfer interval to suppress unwanted water and detergent magnetization. The number of complex points and acquisition times in each experiment are as follows: CT-HNCO (Grzesiek & Bax, 1992a), 15N (F₁) 32, 24.0 ms, 13C0 (F₂) 100, 53.9 ms, 1HN (F₃) 512, 55.3 ms (64 scans/hypercomplex t₁, t₂ increment); CT-HNCA (Grzesiek & Bax, 1992a), 15N (F₁) 32, 19.8 ms, 13CA (F₂) 53, 11.0 ms, 1HN (F₃) 512, 55.3 ms (128 scans/hypercomplex t₁, t₂ increment); HBHA(CO)NH (Grzesiek & Bax, 1993a), using a 0.79-s water presaturation pulse (γB2/2π = 10 Hz) and a 1.0-ms water scrambling pulse, 13J(N-H) (F₁) 62, 15.5 ms, 15N (F₂) 32, 19.8 ms, 1HN (F₃) 512, 55.3 ms (128 scans/hypercomplex t₁, t₂ increment); CBCA(CO)NH (Grzesiek & Bax, 1992c), 13C₀-13Ca (F₁) 52, 6.2 ms, 15N (F₂) 32, 19.8 ms, 1HN (F₃) 512, 55.3 ms (52 scans/hypercomplex t₁, t₂ increment); CBCANH (Grzesiek & Bax, 1992b), 13C₀-13Ca (F₁) 52, 6.2 ms, 15N (F₂) 32, 19.8 ms, 1HN (F₃) 512, 55.3 ms (52 scans/hypercomplex t₁, t₂ increment); C(CO)NH (Grzesiek et al., 1993a), 13C (F₁) 57, 6.2 ms, 15N (F₂) 32, 19.8 ms, and 1HN (F₃) 512, 55.3 ms (128 scans/hypercomplex t₁, t₂ increment); H(CC0)NH (Grzesiek et al., 1993a), 1H (F₁) 68, 10.1 ms, 15N (F₂) 32, 19.8 ms, 1HN (F₃) 512, 55.3 ms (128 scans/hypercomplex t₁, t₂ increment). A 13C/15N-separated 4D NOESY was recorded using the pulse sequence described by Kay et al. (1990) with a 60 ms mixing time, water presaturation during the delay between scans and a "soft-hard" read pulse (Sklinar & Bax, 1987) at the end of the NOESY mixing period to further suppress the H₂O resonance. The number of complex data points collected and acquisition times are 1H (F₁) 40, 12.0 ms, 13C (F₂) 8, 2.7 ms, 15N (F₃) 16, 12.8 ms, 1HN (F₄) 384, 41.4 ms (64 scans for each set of t₁, t₂, and t₃ values).

A 4D HN(COCA)NH experiment (Grzesiek et al., 1993b) was carried out with randomly 50% deuterated 15N/13C-labeled calcineurin with the following number of complex data points and acquisition times: 1HN (F₁) 22, 14.5 ms, 15N (F₂) 20, 22.0 ms, 13C (F₃) 24, 26.4 ms, 1HN (F₄) 512, 55.3 ms (64 scans for each set of t₁, t₂, and t₃ values).

The following experiments were recorded using the 15N-labeled sample: A 15N-separated 3D HOHAHA spectrum (Marion et al., 1989a) was recorded with a 35-ns mixing period using the DIPS2-2 mixing scheme (Shaka et al., 1988) and a 1.5-ns trim pulse (but no solvent presaturation) (Messier et al., 1989) to suppress the water signal. Number of data points and acquisition times used for each dimension are 1H (F₁) 100, 20.0 ms, 15N (F₂) 35, 26.2 ms, 1HN (F₃) 512, 55.3 ms (64 scans/hypercomplex t₁, t₂ increment). The 35-ns mixing period was followed by a 17-ns NOE period (Marion et al., 1989a) to offset the positive NOE occurring during isotropic mixing. A 15N-separated 3D NOESY-HSQC was recorded using a 75-ns mixing period and presaturation of the H₂O resonance during a 0.8-s delay between the scans. A 1.5-ns trim pulse was used to further suppress the water resonance and to eliminate resonances of the detergent.

The number of complex data points and acquisition times used per dimension are 1H (F₁) 128, 20.5 ms, 15N (F₂) 40, 29.9 ms, 1HN (F₃) 512, 53.3 ms (64 scans/hypercomplex t₁, t₂ increment). A 2D 15N-1H HSQC spectrum (Bodenhausen & Ruben, 1980; Bax et al., 1990), with a 1.5-ns trim pulse (Messier et al., 1989) at the end of the first INEPT transfer for water and detergent signal suppression, was recorded using the following number of complex data points and acquisition times: 15N (F₂) 256 131.5 ms, 1HN (F₃) 512, 60.4 ms (16 scans/corresponding t₁ increment) A 15N-separated HHN spectrum (Vuister & Bax, 1993) was recorded for measuring JHN(HH) couplings. The homonuclear dephasing/repulsing delays was set to 20 ms, which includes the 4.5 ms JHN dephasing/repulsing delays. The number of complex data points and acquisition times used per dimension are 15N (F₁) 36, 29 ms, 1H (F₂) 52, 11.4 ms, 1HN (F₃) 512, 60 ms (64 scans/hypercomplex t₁, t₂ increment). For residues Met¹–His¹⁴ and Asp¹⁶¹–Val¹⁷⁰ the J values derived from the resonance intensities in the HHN spectrum were multiplied...
due to a second conformation of calcineurin as a result of degeneracies were resolved in the 3D HNCO experiment, however, observed in the 3D HNCO spectrum due to the dispersion of the chemical shifts of the carbonyl group of intraresidue cross peaks [(Tyr₆, Gln₁₁₄); (Asp₁₈, Glu¹⁰); (Leu³, Glu¹⁰), Met¹¹, Ala¹₂, Ser¹³, and His¹₄]; these are marked by asterisks in Figure 1.

**Sequential Assignment Procedure.** The sequential assignment of resonances was achieved in several steps, primarily using in-house software (Grzesiek, unpublished work). Initially, possible amino acid spin systems were identified as follows: Chemical shift values for amide ¹H and ¹⁵N resonances were taken from the HNCO spectrum. For each amide cross peak in the HNCO spectrum, the program identified cross peaks in the CBCA(CO)NH, CBCANH, HCNA, HBHA(CO)NH, HOHAHA, and NOESY spectra which showed the same amide ¹H/¹⁵N chemical shift values to within a specified tolerance. In this manner, connections between amide ¹⁵N/¹H and intraresidue Ca (HNCA) groups were explored. Cross peaks marked by asterisks correspond to a second conformation of Glu⁴-His¹⁴, presumably caused by cis-trans isomerization of Pro⁸. Residues assigned to overlapping cross peaks are given in parentheses.

Amide ¹⁵N-¹H Correlations. Figure 1 shows the most crowded region of the 2D ¹⁵N-¹H HSQC spectrum of uniformly ¹⁵N-labeled calcineurin B. The spectrum illustrates the severe overlap in the ¹⁵N and ¹H dimensions for this protein: Eight correlations are actually composites of overlapping pairs of ¹⁵N-¹H cross peaks [(Phe¹⁵ and Val¹⁵); (Met¹¹*, Ile¹³⁷); (Ala³, Ala¹²*); (Phe¹⁵ and Val¹⁵)]. Two correlations are 3-fold degenerate [His¹₄**, Ile²₀, Gln⁶⁰]. Asp¹⁸, Ala¹²*, Lys¹⁴, and Asn side-chain NH₂ groups. Cross peaks marked by asterisks correspond to a second conformation of Glu⁴-His¹⁴, presumably caused by cis-trans isomerization of Pro⁸. Residues assigned to overlapping cross peaks are given in parentheses.

by 1.1 to account for ¹H spin flips during the homonuclear de- and rephasing delays (Vuister & Bax, 1993).

**RESULTS**

**Secondary Structure of Calcineurin B**

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**Figure 1:** Most crowded region of the 2D ¹⁵N-¹H HSQC spectrum of calcineurin B, uniformly labeled with ¹⁵N (>95%), recorded at 600 MHz. Cross peaks connected by dotted lines correspond to Gln and Asn side-chain NH₂ groups. Cross peaks marked by asterisks correspond to a second conformation of Glu⁴-His¹⁴, presumably caused by cis-trans isomerization of Pro⁸. Residues assigned to overlapping cross peaks are given in parentheses.

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The sequential assignment procedure involves determination of the amino acid type for each ¹⁵N-¹H chemical shift pair. On the basis of the correlated Ca and Cα chemical shifts, a probability for the different amino acid types can be calculated (Grzesiek & Bax, 1993a). When longer stretches of sequentially connected Ca and Cα have been determined, the computer tries to match these stretches of possible amino acid types to locations in the protein on the basis of the known primary sequence (Grzesiek, 1993a). The results of this automatic identification procedure are then inspected manually by the computer.
Fast exchanging residues are found near the amino and carboxy termini and, most interestingly, for residues Val$^{94}$--Glu$^{99}$, which are homologous with Lys$^{77}$--Glu$^{82}$ in calmodulin. Although in the X-ray crystal structure of calmodulin these residues are in an $\alpha$-helical conformation (Babu et al., 1985; Taylor et al., 1991), in solution this region is highly flexible, nonhelical, and subject to rapid hydrogen exchange (Barbato et al., 1992; Spera et al., 1991). For a number of other calcineurin B residues, NOE and ROE cross peaks to water are of opposite sign (Figure 4, open circles) (Grzesiek & Bax, 1993b). This indicates that for these amides the magnetization exchange with water is dominated by NOE/ROE effects, either to water molecules directly or to protein protons that are in rapid exchange with water (Otting et al., 1989). Most likely, NOE/ROE interactions with water provide evidence for tightly bound water molecules in calcineurin B. More precisely, the inverse of the exchange rates with bulk water for water molecules that may be present in the protein structure, and which have protons less than 3 Å from a backbone amide, must be considerably shorter than 10 ns at the temperature where the experiments were conducted (37°C).

Secondary Structure Determination. Secondary structure elements in proteins display distinct NOE connectivity patterns (Wüthrich, 1986). For example, $\alpha$-helices are characterized by the coexistence of strong $d_{\text{NN}}(i, i+1)$ (sequential amide proton) connectivities and $d_{\text{NN}}(i, i+3)$ connectivities ($\text{H}^\alpha$ of residue $i$ to $\text{H}^\text{N}$ of $i + 3$). On the other hand, $\beta$-sheets are characterized by strong $d_{\text{NN}}(i, i+1)$ signals and by the absence of the connectivities that characterize $\alpha$-helices. In addition to connectivity patterns there is a very good correlation between the deviations of $\text{C}^\alpha$ chemical shifts from their random-coil values, i.e., secondary shifts, and the secondary structure of proteins (Spera & Bax, 1991; Wishart et al., 1991; Ikura et al., 1991; Grzesiek et al., 1992). $\alpha$-Helices are characterized by positive $\text{C}^\alpha$ secondary shifts of ca. 3 ppb and $\text{C}^\beta$ chemical shifts close to the random coil value, whereas $\beta$-strands are characterized by negative $\text{C}^\alpha$ secondary shifts and positive $\text{C}^\beta$ secondary shifts. Finally, $\alpha$-helical structures are characterized by small ($\leq5$ Hz) $\text{H}^\alpha$--$\text{H}^\beta$ J couplings, whereas in $\beta$-strands large ($>7$ Hz) values are typically observed.

The $d_{\text{NN}}(i, i+1)$, $d_{\text{NN}}(i, i+3)$, and $d_{\text{NN}}(i, i+4)$ connectivities together with the $\text{C}^\alpha$ and $\text{C}^\beta$ secondary chemical shifts and $\text{H}^\alpha$--$\text{H}^\beta$ J coupling data are presented in Figure 4. The precise values of $\text{H}^\alpha$--$\text{H}^\beta$ J couplings are listed in Table I (supplementary material). The data in Figure 4 identify the location of eight helices and four short $\beta$-strands. The helices span the regions (A) Ala$^{17}$--Leu$^{30}$, (B) Val$^{40}$--Ser$^{45}$, (C) Val$^{55}$--Phe$^{59}$, (D) Phe$^{62}$--Gln$^{66}$, (E) Lys$^{72}$--Tyr$^{79}$, (F) Asp$^{90}$--Met$^{119}$, (G) Asp$^{126}$--Ala$^{140}$, and (H) Phe$^{190}$--Gly$^{198}$. Except for helix C, the beginnings of the helices are marked by a strong $\text{C}^\alpha$ secondary shift following a sequence of at least two residues with small or negative $\text{C}^\beta$ secondary shifts; the ends of all eight helices are marked by negative or positive $\text{C}^\alpha$ secondary shifts.
FIGURE 4: Summary of the calcineurin B data on sequential and medium-range NOEs involving the HN and Hα protons, amide exchange data, J(HNHa) couplings, and 13Cα and 13Cβ secondary shifts (Δ13Cα and Δ13Cβ) observed for calcineurin B, together with the secondary structure deduced from these data. For the J(HNHa) data, open boxes refer to upper limits for the J coupling, based on a cross-peak intensity in the HNHA spectrum that is below the signal-to-noise threshold. Shaded boxes refer to amides for which the HN-15N correlation is partially overlapping, resulting in a less precise measurement of J(HNHa). J values for glycine residues are reported in the supplementary material only. Residues with amide protons subject to fast (>0.3 s⁻¹) hydrogen exchange (HX) are marked by filled circles. Amide protons which exchange magnetization with water via an (indirect) NOE effect are marked by open circles. Amide protons subject to extremely slow hydrogen exchange are marked by filled stars. NOE connectivities that could not be established unambiguously because of overlap are marked by open boxes (dαN and dαN) or dashed lines (dαN).

very small Cα secondary shifts, increases in J(HNHa) values, and the absence of dαN(i, i + 3) connectivities. Except for Arg57, which displays a small positive Cα secondary shift, all residues within the helices indeed exhibit strong positive Cα secondary shifts and small J(HNHa) couplings. Also, strong dαN(i, i + 1) connectivities are observed together with numerous dαN(i, i + 3) and several weak dαN(i, i + 4) connectivities. The latter type of connectivity is only expected in α-helices (Wüthrich, 1986).

Ca²⁺ Binding Loops. Each calcium binding site consists of 12 residues and is flanked by two α-helices, forming a HLH EF-hand motif. There are four Ca²⁺ binding sites in calcineurin B, each made of a six-residue loop (Asp51–Gly56, Asp93–Gly98, Asp100–Gly105, and Asp141–Gly146) followed by three residues in a β-sheet conformation (Ser57–Ser59, Glu89–Asp1, Tyr106–Ser108, and Arg147–Ser149), which are in turn followed by three residues which form the beginning of the successive helix (Val140–Glu42, Phe72–Glu74, Asn109–Glu111, and Phe150–Glu152). The boundaries of a β-strand are more easily determined on the basis of strong dαN(i, i + 1) and weak or absent dαN(i, i + 1) connectivities and negative Cα and positive Cβ secondary shifts. Long-range NOEs between the backbone protons on different strands are needed to establish which strand is adjacent to which other strand and to determine whether the β-sheet is parallel or antiparallel. The 15N-separated 3D NOESY-HSQC reveals medium-intensity NOE connectivities between the amide protons of Leu38 and Val70 and between Ile107 and Ile148, while the 13C/15N-separated 4D NOESY reveals weak NOE connectivities between the amide proton of Ile148 and Hα of Ser108 and between the amide proton of Leu38 and the Ha of Asp51. These NOE connectivities indicate that strand Ser57–Ser59 forms an antiparallel sheet with Phe72–Glu74, whereas Asn109–Glu111 forms an antiparallel sheet with Phe150–Glu152. This pairing of the β-strands is similar to that observed in calmodulin (Babu et al., 1988).

Connecting Loops. Calcineurin B contains two other loops (Leu58–Pro53 and Val120–Lys125) which connect the first and second EF-hand in each of the protein's two domains. None of the amide protons of residues Leu46–Glu51 in the first loop are in fast exchange with solvent; for the last residues in this loop, Asn52 and Leu44, the absence of exchange cannot be established unambiguously because of resonance overlap. Strong positive secondary shifts for the Cα of three residues within this loop (Pro41, Glu44, and Glu50) are observed, together
with weak \( d_{\alpha N}(i, i + 1) \) connectivities for Glu\(^{48}\)-Gln\(^{50}\) and two \( d_{\alpha N}(i, i + 3) \) connectivities. This suggest the possible presence of multiple turns. However, the current data do not allow a more precise characterization of the structure of this loop. The second loop is shorter and presumably more extended, based on the presence of strong \( d_{\alpha N}(i, i + 1) \) connectivities and large \( J(H^N H^N) \) values. Rapid hydrogen exchange is observed for three residues in this loop and for the first three residues of helix G, following this loop (Grzesiek and Bax, 1993b).

Flexible Segments. Rapid amide hydrogen exchange, relatively narrow resonances, near random coil chemical shifts, strong \( d_{\alpha N}(i, i + 1) \) connectivities, and most importantly, the absence of intermediate-range \((i, i + 3) \) and \((i, i + 4) \) NOE connectivities are observed for three regions: the amino terminus (Met\(^1\)-Asp\(^9\)), the carboxy terminus (Gly\(^{159}\)-Val\(^{170}\)), and the linker between helix D and E (Phe\(^{42}\)-Asp\(^{47}\)). These data suggest that these three regions are subject to rapid conformational exchange.

**DISCUSSION**

The data presented above provide the first experimental evidence that calcineurin B is highly homologous in its secondary structure to calmodulin. The four calcium binding sites of this protein display the typical EF-hand conformation characterized by a helix-loop-helix motif. The calcium binding loops of calcineurin B are highly homologous to those of calmodulin. They all contain the invariant asparagine, glycine, and glutamate residues at loop positions 1, 6, and 12, respectively, and follow the consensus sequence for \( Ca^{2+} \) binding sites (Szepenyi & Moffat, 1986; Strynadka & James, 1989). In all four calcium binding sites the second helix begins at position 10 of the calcium binding loop, immediately following the three-residue \( \beta \)-strand. The helices preceding and following the calcium binding sites vary in length from 6 to 15 residues. Helix B (Val\(^{140}\)-Ser\(^{45}\), the shortest of these, is four residues shorter than the corresponding helix in calmodulin, presumably due to the presence of a Pro in position 47 of calcineurin B. Helix C (Leu\(^{54}\)-Val\(^{55}\)-Phe\(^{62}\)) is also several residues shorter than helix C of calmodulin, which may be attributed to the absence of these codons in the gene of calcineurin B as compared to the calmodulin gene. Conversely, helix G of calcineurin is four residues longer than the corresponding helix in calmodulin, which corresponds to four additional amino acids in the calcineurin sequence relative to that of calmodulin. These differences in the lengths of the helices may have implications on the interaction of calcineurin A with calcineurin B (and with calmodulin). Other differences are observed in the regions connecting helices B and C and helices F and G. In calmodulin, these loops were found to be flexible and subject to rapid hydrogen exchange (Barbato et al., 1992; Spera et al., 1991). In contrast, the connecting region between helices B and C in calcineurin B does not exhibit rapid hydrogen exchange. Many of the residues in the loop connecting helices F and G exchange rapidly with solvent. In contrast to calmodulin, significant secondary \( C^\alpha \) shifts are found for residues in these two regions of calcineurin B, suggesting that they adopt a well-ordered conformation. In addition to these differences, calcineurin B has 9 and 10 additional residues relative to calmodulin at the amino and carboxy termini, respectively. The importance of the N-terminus of calcineurin B is implicated by the fact that although myristoylation of the N-terminal glycine increases the affinity of calcineurin B for calcineurin A only 3-fold, it is essential for the phosphatase activity of calcineurin (Ren & Klee, 1993).

Although our present results for free calcineurin B indicate that the N-terminal residues of calcineurin B are disordered, it is likely that this region in myristoylated calcineurin B adopts a well-defined structure upon interaction with calcineurin A. However, considering that the genetically engineered non-myristoylated form of calcineurin B, used in our study, retains very tight binding to the A subunit, it is unlikely that myristoylation has significant effects on the structure in other regions of the B subunit.

NOE interactions between the \( \beta \)-strands of calcium binding loops I and II and, similarly, calcium loops III and IV indicate that the first and second EF-hands and, similarly, the third and fourth EF-hands form small globular domains, each containing two calcium binding sites. These globular domains are connected by helix D, a flexible connection region (Val\(^{144}\)-Lys\(^{88}\)), and helix E. The NMR characteristics of the connection region are similar to those observed in the solution structure of calmodulin (Ikura et al., 1991; Barbato et al., 1992), where a flexible stretch of six residues disrupts the 27-residue \( \alpha \)-helix observed in the crystalline state (Babu et al., 1985; Taylor et al., 1991). However, at the present time it is not clear whether the two domains of calcineurin B are connected by a flexible linker, as observed for free calmodulin in solution (Barbato et al., 1992), or whether the two domains interact to form a globular structure similar to that of calmodulin when it is complexed with its target peptides (Ikura et al., 1992; Meador et al., 1992).

The differences in structure between calcineurin B and calmodulin manifest themselves in several other properties: First, while calmodulin tightly binds M13, no significant binding of M13 to calcineurin B was observed at 2 \( \mu \)M concentration of M13 (data not shown). Second, calcineurin B, at concentrations up to \( 10^{-4} \) M, neither activates the phosphatase activity of calcineurin A (3 \( \times \) \( 10^{-4} \) M) nor inhibits its stimulation by 3 \( \times \) \( 10^{-8} \) M calmodulin; calcineurin B was also unable to reverse the inhibition of calmodulin stimulation by 6 \( \times \) \( 10^{-4} \) M of the M13 peptide. Third, unlike calmodulin, calcineurin B aggregates in solution and monomeric molecules suitable for NMR studies could be obtained only in the presence of the detergent CHAPS. This suggests the presence of exposed hydrophobic surfaces which promote aggregation of the protein. Recently, a 28 amino acid peptide from calcineurin A has been identified which binds calcineurin B with a dissociation constant of 3 \( \mu \)M (P. Stemmer, M. H., Krinks, and C. B. Klee, manuscript in preparation). Our further structural studies of calcineurin are aimed at addressing the mode of interaction between calcineurin B and calcineurin A and at comparing this interaction to that observed in complexes of calmodulin with its peptide ligands.

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**SUPPLEMENTARY MATERIAL AVAILABLE**

One table containing the \( H^N \), \( H^\alpha \), \( H^\delta \), \( ^{13}N \), \( ^{13}CO \), \( ^{13}C^\alpha \), and \( ^{13}C^\delta \) chemical shifts and \( J(H^N H^N) \) coupling constant data (8 pages). Ordering information is given on any current masthead page.
REFERENCES


