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The SCAN Domain of ZNF174 Is a Dimer

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Abstract

The SCAN domain is a conserved region of 84 residues found predominantly in zinc finger DNA-binding proteins in vertebrates. The SCAN domain appears to control the association of SCAN domain containing proteins into noncovalent complexes and may be the primary mechanism underlying partner choice in the oligomerization of these transcription factors. Here we have overexpressed, purified, and characterized the isolated SCAN domain (amino acids 37–132) from ZNF174. Both size exclusion chromatography and equilibrium sedimentation analysis demonstrate that the ZNF174 SCAN domain forms a homodimer. Circular dichroism shows that the isolated SCAN domain dimer has ~42% α-helix. Thermal denaturation experiments indicate that the SCAN domain undergoes a single reversible unfolding transition with a T_m of over 70 °C. The midpoint of the equilibrium unfolding transition increases with increasing protein concentration, consistent with a two-state unfolding transition in which folded dimer is in equilibrium with unfolded monomer. These findings demonstrate that the isolated SCAN domain forms a stable dimer and support a model in which the SCAN domain is capable of mediating the selective dimerization of a large family of vertebrate-specific, zinc finger-containing transcription factors.

Transcription factors are composed of modular elements that include a DNA-binding domain and one or more separable effector domains that activate or repress transcription. Other modules within these factors regulate subcellular localization and gene expression by mediating selective association of the transcription factors with each other, or with other cellular components. Identification of these domains often provides a conceptual framework for understanding the function of the transcription factor.

The SCAN[1] domain is a highly conserved vertebrate-specific protein domain found in ~60 genes in the human genome (1,2). Only a handful of these gene products have been characterized thus far, and they appear to control a wide range of biological processes including development, cell differentiation, and lipid metabolism (reviewed in Refs. 3 and 4). The name for the SCAN domain was derived from the first letters of the names of four proteins initially found to contain this domain (SRE-ZBP, CTfin51, AW-1 (ZNF174), and Number 18 cDNA or ZNF197) (5). Alternatively, this domain has been referred to as LeR for leucine-rich domain (6). The SCAN domain consists of an 84-residue, leucine-rich region and is predicted to contain a high degree of α-helix. It is located at the N terminus when it is part of a zinc finger-containing transcription factor. The primary amino acid sequence of the domain does not resemble any of the other zinc finger-associated domains, such as the Kruppel-associated box (KRAB) or the poxvirus and zinc finger (POZ) domain, which is also known as the BTB (Broad-complex, Tramtrack, and Bric-a-brac) domain (7–9). Members of the SCAN domain family are broadly

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[1]The abbreviations used are: SCAN, SRE-ZBP, CTfin51, AW-1 (ZNF174), and Number 18 cDNA or ZNF197; DTT, dithiothreitol; GST, glutathione *S*-transferase; MALDI-MS, matrix-assisted laser desorption mass spectrometry.

expressed and appear to function as either activators or repressors of transcription. The SCAN domain in isolation generally does not affect transcription.

In previous studies we and others (10–12) demonstrated that the SCAN domain functions as an oligomerization domain, mediating self-association or association with other proteins bearing SCAN domains. In part of those studies, a fragment of the N terminus of either ZNF174 or ZNF202 that encompassed the SCAN domain behaved as an oligomeric species. However, in those studies the definitive subunit stoichiometry of the oligomeric SCAN domain was not determined. Here we have utilized the sequences of the 60 SCAN domains in the human genome to more completely define the limits of the domain. We have overexpressed, purified, and characterized the isolated SCAN domain from ZNF174. Gel filtration, sedimentation equilibrium, and thermal denaturation studies demonstrate that the isolated SCAN domain forms a stable homodimer. These findings support a model in which the SCAN domain is capable of mediating the selective dimerization of a large family of vertebrate-specific, zinc finger-containing transcription factors.

EXPERIMENTAL PROCEDURES

Plasmid Construction

The DNA segment containing residues 37–132 of ZNF174 was amplified from a full-length clone of ZNF174 using polymerase chain reaction. This amplified fragment was then subcloned into the *Bam*HI site of pGEX-2T (Amersham Biosciences, Inc.). Automated dideoxynucleotide sequencing of the SCAN domain insert verified the correct orientation and authenticity of the insert. The predicted protein product consists of the SCAN domain insert fused with glutathione *S*-transferase (GST). The amino acid sequence of the final protein product after cleavage from GST with thrombin consists of the 96-residue sequence illustrated in Fig. 1, preceded by glycine-serine. This 98-residue protein has a calculated isoelectric point of 8.7 and a molecular mass of 11.56 kDa.

Bacterial Expression and Purification of the SCAN Domain

Escherichia coli BL21 bacteria transformed with the pGEX-SCAN fusion construct were grown to an A_{600} of 0.6 at 37 °C and then induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside for 1–2 h. The cells were pelleted at 4000 × *g* for 30 min. The cells were then suspended in phosphate-buffered saline (67 mM phosphate, 150 mM NaCl, 5 mM DTT, pH 7.4, or phosphate-buffered saline, 30 ml/1 liter of culture) containing 0.1% Nonidet-P40 (Roche Molecular Biochemicals) and Complete protease inhibitor mixture (1 tablet/50 ml, Roche Molecular Biochemicals). The cells were lysed by sonication, and the homogenate was centrifuged at 3000 × *g* for 30 min. Glutathione-Sepharose 4B (Amersham Biosciences, Inc.) was added to the resulting supernatant (0.75 ml resin/1 liter of culture). The supernatant was then agitated on a rocker for 20 min and then centrifuged at 500 × *g* for 5 min. The resulting supernatant was removed, and the resin was placed in a chromatography column and washed with 2 bed volumes of phosphate-buffered saline. The resin was then incubated as a 50% slurry with thrombin (80 units/0.75 ml of resin) at 25 °C overnight. The resin was then washed with one bed volume of phosphate-buffered saline, and the eluate was diluted 1:2 with water.

All subsequent steps were performed at 4 °C using a Biologic HR chromatography system (Bio-Rad). The sample was applied at 1 ml/min to an S5 cation exchange column (Bio-Rad). The column was washed with 34 mM phosphate, 75 mM NaCl, and 5 mM DTT, pH 7.4, and then the protein was eluted with a linear NaCl gradient running from 75 mM to 1 M in 34 mM phosphate, 5 mM DTT, pH 7.4. The sample was then concentrated to 0.5 ml with a Centricon YM-3 (Amicon, 3000 Da MWCO) at 3000 × *g*. The sample was then applied to a Bio-Prep S.E.-100/17

size exclusion chromatography column (8 × 300 mm, Bio-Rad) equilibrated in 25 mM phosphate, 200 mM NaCl, 1 mM DTT, pH 6.5, at a flow rate of 0.2 ml/min.

Analytical Size Exclusion Chromatography

Recombinant purified SCAN domain (225 μM subunit) in 25 mM phosphate, 200 mM NaCl, 1 mM DTT, pH 6.5 was applied a Bio-Sil SEC 250-5 analytical gel filtration column (Bio-Rad) at 0.7 ml/min with a back-pressure of 800 p.s.i. using a Biologic HR chromatography system (Bio-Rad). The column was calibrated under the same conditions with the following protein standards (thyroglobulin, molecular weight 670,000; IgG, 158,000; ovalbumin, 44,000; myoglobin, 17,000).

Sedimentation Equilibrium

The isolated SCAN domain was dialyzed overnight against 25 mM phosphate, 200 mM NaCl, 1 mM DTT, pH 6.5. Sedimentation equilibrium was performed at 20 °C in a Beckman XL-A Analytical Ultracentrifuge using three rotor speeds (16,000, 21,000, and 26,000 rpm) and at three different protein concentrations (5, 15, and 50 μM subunit). After samples had reached equilibrium (typically 18 h), they were scanned in triplicate at 280, 236, and 229 nm. Data were analyzed with a linear least squares fitting program (KaleidaGraph) using a partial specific volume of 0.738 calculated using WEBTools.[2]

CD Spectroscopy

CD spectra were recorded at 4 °C on an Aviv 62DS spectropolarimeter equipped with a thermoelectric temperature controller. Samples of recombinant ZNF174-isolated SCAN domain (25 μM subunit) were prepared in 25 mM phosphate, 200 mM NaCl, 0.2 mM DTT, pH 6.5. Spectra representing the average of five scans from 260 to 195 nm were measured in a 1-mm path length cuvette, using a step size of 1 nm and a 3-s signal-averaging time. All spectra were corrected for the base line obtained with the buffer alone.

Thermal Denaturation

Thermal denaturation was performed by monitoring the molar ellipticity at 222 nm of the SCAN domain at various temperatures. Experiments were performed with protein concentrations of 2 and 5 μM subunit in a cuvette with a 1-cm path length. Measurements were made with a 2-min equilibration time and a 2 °C step interval. The melting temperature (T_m) for each protein concentration was determined from the peak position on the plot of $-d\Theta_{222\ nm}/d(1/T)$ *versus T*.

Secondary Structure Predictions

The predicted secondary structure for the isolated SCAN domain was determined using the following on-line prediction programs: PredictProtein, PREDATOR, nnPredict, Hierarchical Neural Network, Prof, Jpred, and SSpro.[3]

Miscellaneous Methods

The purity of the protein was assessed by SDS-polyacrylamide electrophoresis using an XCell Surelock electrophoresis cell (Novex) and 4–12% precast gradient gels. N-terminal sequencing and matrix-assisted laser desorption mass spectrometry (MALDI-MS) of the purified SCAN domain was performed by the University of Michigan, Protein Core Facility, Ann Arbor, MI.

[2]Found on the Web at bmb.psu.edu/nixon/WEBTools/mwtvbar.htm.

[3]Found on the Web, respectively, at: dodo.cpmc.columbia.edu/predictprotein/; embl-heidelberg.de/cgi/predatorserv.pl; cmpharm.ucsf.edu/~nomi/nnpredict.html; npsa-pbil.ibcp.fr/cgi-bin/npsaautomat.pl? page=npsann.html; aber.ac.uk/~phiwww/prof/; jura.ebi.ac.uk:8888/; promoter.ics.uci.edu/BRNN-PRED/.

Protein concentrations were determined using the Bradford microassay (Bio-Rad) with bovine serum albumin as the standard. The SCAN domain sequences were aligned using the ClustalW alignment program in MacVector, version 6.5.3.

RESULTS

Defining the Limits of the SCAN Domain

Analysis of the Celera human genome data base has revealed the presence of ~60 genes containing the SCAN domain (1–3).[4] An alignment of a select number of these members is illustrated in Fig. 1 (5,12–19). There is clear homology starting with E-43 of ZNF174 and extending to R-126 of ZNF174. Below the sequences is a compilation of the predicted secondary structure based on several publicly available programs, with "H" corresponding to a residue predicted to be in an α-helix by all of the programs, and "h" corresponding to a residue predicted to be in an α-helix by some but not all of the programs. The secondary structure prediction algorithms predict three to five α-helices within the SCAN domain. Based on the sequence alignment, the secondary structure prediction, and the biochemical properties of the recombinant protein detailed below, the SCAN domain is being defined as 84 residues, beginning with E-43 and ending with R-126 of ZNF174. In addition, there are frequently one or more proline residues just before and after the domain.

Purification of the Isolated SCAN Domain from ZNF174

In initial studies we examined the behavior of an N-terminal region of ZNF174 that contained the SCAN domain (10). Collectively, these previous studies, as well as work by others with the SCAN domain of ZNF202 (11), demonstrate that the SCAN domain behaves as an oligomeric species under near physiologic conditions. However, the precise subunit stoichiometry of the oligomeric SCAN complexes was not determined. To better define the nature of the SCAN domain, we over-expressed and purified to homogeneity only the isolated SCAN domain (amino acids 37–132) from ZNF174 (Fig. 2). The 98-residue recombinant protein consists of the 96 residues illustrated in Fig. 1, preceded by glycine-serine. The protein was prepared as a GST fusion, affinity-purified, cleaved with thrombin to remove the GST, and then subjected to cation exchange and size exclusion chromatographies (Fig. 2*A*). The protein preparation yields about 0.3 mg/liter of bacterial cell culture. The composition of the recombinant protein was verified by both N-terminal sequencing (GSKN) and MALDI-MS (predicted 11560 Da; observed 11562 Da).

The SCAN Domain Forms Dimers

The recombinant SCAN domain was subjected to analytical size exclusion chromatography. The protein eluted as a single peak with a retention time of 13.8 min (Fig. 2*B*). The following molecular mass standards were employed: myoglobin, 17 kDa, 14.5 min; ovalbumin, 44 kDa, 12.5 min; IgG, 158 kDa, 11.2 min; and thyroglobulin, 670 kDa, 8.9 min. Based on a plot of the logarithm of the molecular mass of the protein standards *versus* the retention time, the molecular mass of the recombinant SCAN domain was determined to be 23.6 kDa. Because the subunit molecular mass is 11.56 kDa, the result indicates that the recombinant SCAN domain is a dimer.

Given that knowledge of the precise subunit stoichiometry of oligomeric SCAN is critical for understanding its function, the native molecular mass of the recombinant SCAN domain was further assessed by sedimentation equilibrium. Sedimentation equilibrium is generally considered the definitive method for determining the native molecular mass of an oligomeric

[4]T. Sander, J. R. Stone, J. L. Maki, and T. Collins, unpublished observations.

protein in solution, as it assesses the protein in solution at equilibrium (20). Unlike gel filtration and velocity-sedimentation based measurements, sedimentation equilibrium analysis is independent of the shape of the molecule. The defining characteristic of sedimentation equilibrium is the concentration gradient that forms at equilibrium as the flux of sedimenting protein is exactly balanced by the flux of diffusing molecules. The analysis was performed at three rotor speeds (16,000, 21,000, and 26,000 rpm) and three different protein concentrations (5, 15, and 50 μM). Absorbances were recorded at three wavelengths (229, 236, and 280 nm) in triplicate. When the natural log of the absorbance is plotted against the square of the radius, a linear plot is obtained, the slope of which allows for the calculation of the native molecular mass of the oligomer. A representative plot at a rotor speed of 16,000 rpm and at a protein concentration of 50 μM is shown in Fig. 3*A*. Also on this plot are the calculated lines that would result for a protein with a subunit mass of 11.56 kDa and with oligomeric subunit stoichiometries of 1, 2, or 3. The residuals from a best fit of the plotted data are shown in Fig. 3*B*. The calculated subunit stoichiometry for each protein concentration is depicted in Fig. 3*C*. The values were averaged over the three acquisitions and for the three different rotor speeds. The sedimentation equilibrium results clearly demonstrate that the isolated SCAN domain is a dimer.

The SCAN Dimer Has High Thermodynamic Stability

CD spectra of the recombinant isolated SCAN domain shows that the protein has a high degree of secondary structure (Fig. 4*A*). Analysis of the spectra reveals that the SCAN domain has about 42% α-helix. These values are in good agreement with secondary structure predictions described above, which yield estimations of helical content ranging from 36 to 63%.

Thermal denaturation of the isolated SCAN domain was performed to investigate the stability of the SCAN dimer. The loss of negative ellipticity at 222 nm was monitored by CD spectroscopy as the temperature was increased. This analysis shows that unfolding is sigmoidal and single phase (Fig. 4*B*). The protein is surprisingly stable, with a melting temperature (T_m) of 75 °C at 5 μM subunit. The thermal denaturation was reversible with the same T_m as for unfolding and with 94% of the negative ellipticity at 222 nm recovered upon stepwise lowering of the temperature. As a consequence of the dimeric structure of the SCAN domain, the apparent stability should depend upon the protein concentration. In fact, as the protein concentration is decreased, the T_m is shifted to a lower temperature (72 °C with 2 μM subunit). This trend is consistent with a two-state model in which folded dimer is in equilibrium with unfolded monomer.

DISCUSSION

To minimize the possible proaggregatory effects of residues outside the actual SCAN domain, sequences of the 60 SCAN domains from the human genome were aligned to more clearly define the limits of the domain. In addition, secondary structure predictions were used to help predict where structural elements may begin and end. Based on this analysis the domain is defined as 84 residues extending from E-43 to R-126 in ZNF174. In many sequences, there are proline residues both before and after the domain, which helps to delineate the boundaries of the predicted secondary structural elements. The secondary structure prediction algorithms predict the presence of three to five α-helices in the domain. The limits for the domain described here are in good agreement with those listed in two on-line domain data bases.[5] In Prosite the domain is defined as ZNF174 residues 46 –128, and in Pfam the domain is defined as ZNF174 residues 40 –135. The third on-line domain data base, SMART (which refers to the domain as

[5]Prosite is found at expasy.ch/cgi-bin/nicesite.pl?PS50804, Pfam at sanger.ac.uk/cgi-bin/Pfam/getacc?PF02023, and SMART at smart.embl-heidelberg.de/.

LER), currently defines the domain as ZNF174 residues 42–154. However, the additional ~25 residues at the C terminus show much less homology than those within the domain as defined here. These additional residues are clearly not required for the formation of a stable recombinant protein, as they are lacking in the construct employed here. Thus, it is concluded that the SCAN domain extends from E-43 to R-126 (in ZNF174) and is frequently flanked by one or more proline residues on both ends.

SCAN domain proteins have been shown by *in vitro* co-expression studies to form homo- and hetero-oliogomers (10–12). Previous studies on recombinant polypeptides containing SCAN domains have also shown these proteins to be oligomers (10,11). However, in both of these studies, the precise oligomeric subunit stoichiometry was not clear, as the recombinant proteins demonstrated a retention time on gel filtration significantly shorter than would be expected for a simple dimeric species. However, in contrast to these previous studies, the protein employed here was a stable dimer not only on gel filtration but also by definitive sedimentation equilibrium analysis. This construct was designed to minimize nonspecific interactions (*i.e.* aggregation) and thus has allowed for the definitive determination of the subunit stoichiometry. Furthermore, the molecule used here was well behaved biophysically as evidenced by reversible thermal denaturation. The high degree of stability of the SCAN dimer, as indicated by the T_m of over 70 °C, is ideal for a protein that may serve as a dimerization domain, as is the case with BTB/POZ (21) and with the leucine zippers of GCN4, FOS, and JUN (22,23).

These studies indicate that the isolated SCAN domain forms dimers and suggests that the SCAN domain is utilized as a dimerization domain by multidomain zinc finger-containing transcription factors. However, most of the proteins in the SCAN family contain multiple zinc fingers, which could presumably enable them to bind DNA as monomers. Thus, a key question concerns why these transcription factors may need to dimerize for proper biological function. Analysis of the human genome indicates that there are ~60 SCAN-containing transcription factors present (1–3).[4] Furthermore, SCAN appears to be a vertebrate-specific (if not mammal-specific) domain. Given that there are 60 SCAN domain-containing proteins in humans and that the SCAN domain can dimerize, it then follows that there are 1830 possible dimeric complexes that could result from these 60 human genes (not accounting for alternatively spliced forms). However, because *in vitro* co-expression studies have suggested that there is selectivity in homo- and heterodimer formation (10–12), not all of the 1830 possible combinations may actually exist. Nonetheless, the introduction of the SCAN domain within a defined set of zinc finger-containing transcription factors allows for a potentially large diversity in the number of transcription factors available to the species. Complex organisms may require this diversity in transcription factors for development and homeostasis. Investigations to determine the structural characteristics regulating selective homo- and heterodimer formation are in progress.

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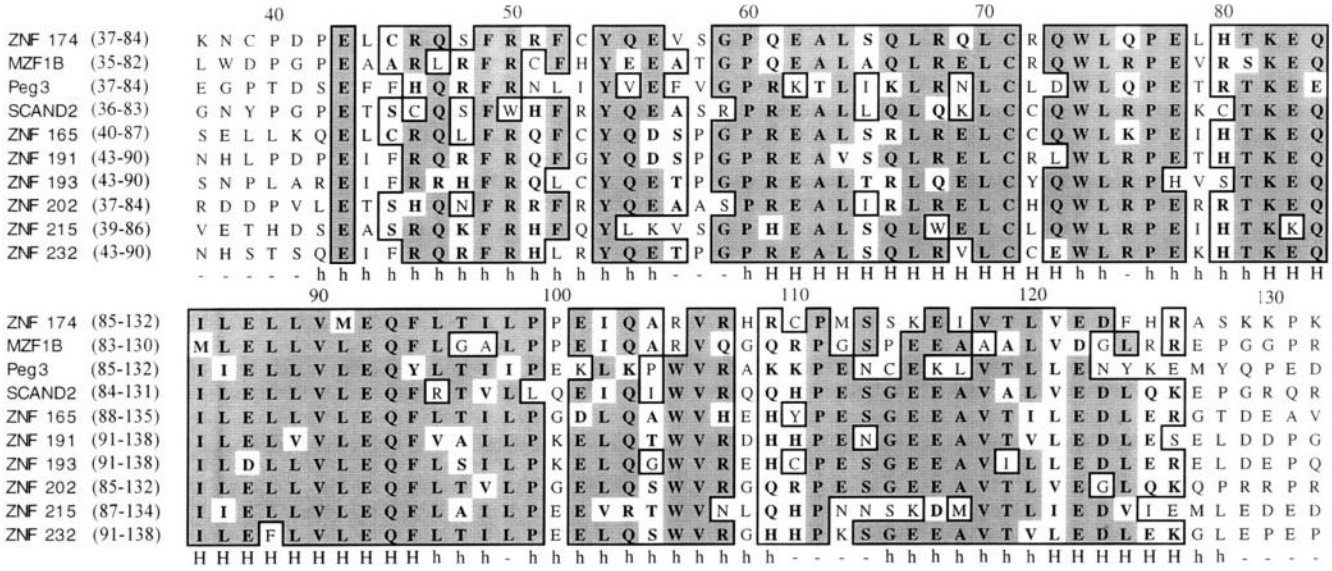

Fig. 1. The SCAN domain.

Ten of the 60 SCAN domains present in humans have been aligned with *dark gray* and *light gray backgrounds* used to indicate identical or conserved residues, respectively. The *numbers* at the *top* of the alignment indicate the amino acid positions in ZNF174. *Underneath* the sequences is the predicted secondary structure, with "*H*" representing a residue predicted to be in an α-helix by all of the programs used and "*h*" representing a residue predicted to be in an α-helix by some but not all of the programs used. The SCAN domain is defined as 84 residues extending from E-43 to R-126 in ZNF174.

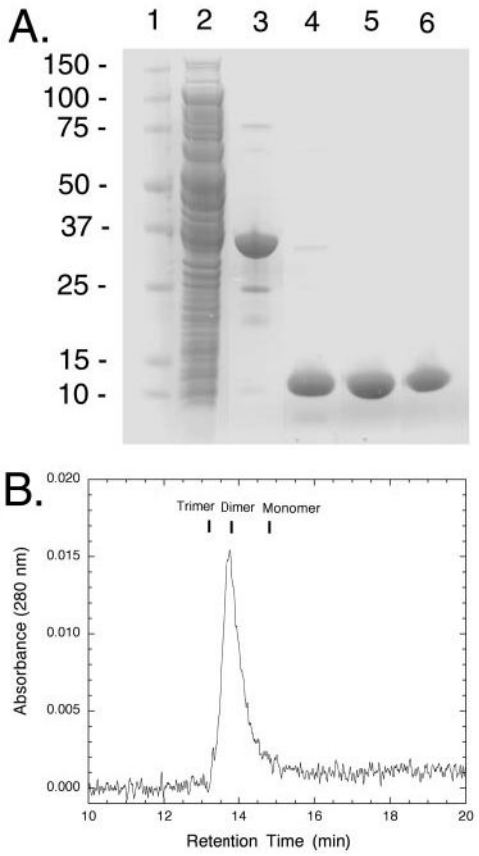

Fig. 2. Purification of recombinant SCAN domain.

A, 4 –12% gradient SDS-polyacrylamide gel. *Lane 1*, protein standards; *lane 2*, supernatant; *lane 3*, GST-Sepharose affinity-purified GST-SCAN domain fusion protein before thrombin cleavage; *lane 4*, eluate containing SCAN domain after cleavage with thrombin; *lane 5*, SCAN domain after cation exchange chromatography; *lane 6*, SCAN domain after size exclusion chromatography. *B*, analytical size exclusion chromatography of the purified SCAN domain. Recombinant purified SCAN domain (225 μm subunit) in 25 mm phosphate, 200 mm NaCl, 1 mm DTT, pH 6.5, was applied to an analytical gel filtration column. The column was calibrated under the same conditions with the following protein standards: thyroglobulin, 670,000 Da, 8.9 min; IgG, 158,000 Da, 11.2 min; ovalbumin, 44,000 Da, 12.5 min; myoglobin 17,000 Da, 14.5 min. The indicated positions for monomer, dimer, and trimer were determined from a linear regression of the standards using a subunit molecular mass of 11.56 kDa for the SCAN domain.

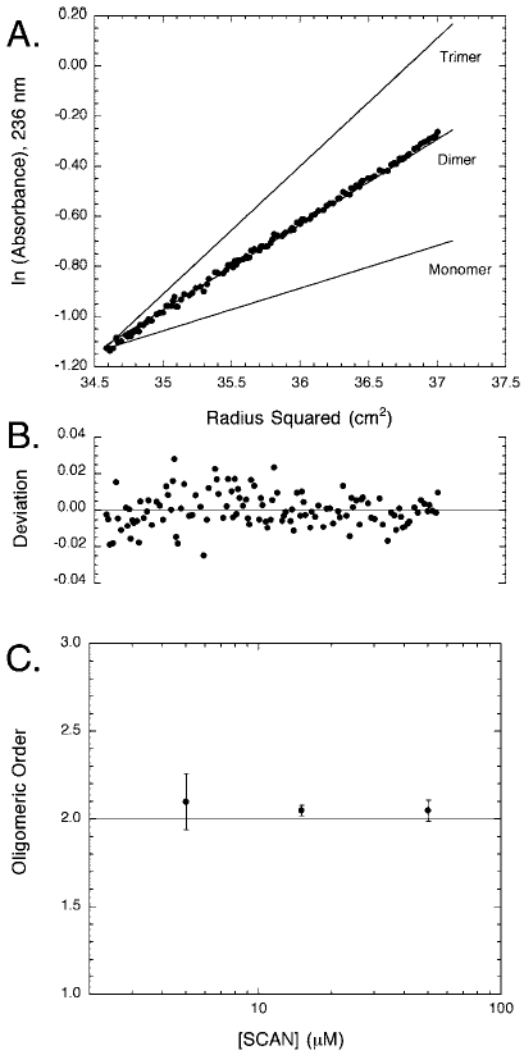

Fig. 3. Sedimentation equilibrium analysis of the SCAN domain.

Sedimentation equilibrium analysis of the recombinant SCAN domain from ZNF174 was performed using a Beckman XL-A Analytical Ultracentrifuge with three different protein concentrations in 25 mm phosphate, 200 mm NaCl, 1 mm DTT, pH 6.5, at 20 °C. *A*, a representative log plot from a sample containing 50 μm (subunit) SCAN centrifuged at 16,000 rpm. Shown with the data are the calculated lines that would result for a protein with a subunit mass of 11.56 kDa and with oligomeric subunit stoichiometries of 1, 2, or 3. *B*, the residuals from a linear regression of the data shown in *A* above. *C*, the calculated subunit stoichiometry for each protein concentration. The values were averaged over three separate acquisitions for each of three different rotor speeds. The *error bars* represent two standard deviations.

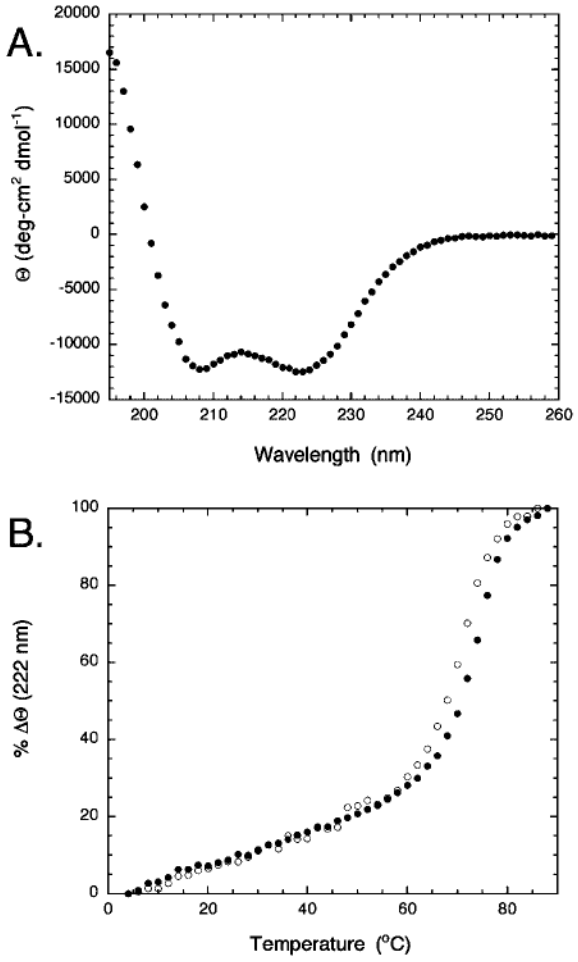

Fig. 4. CD spectroscopy of the SCAN domain.

CD spectroscopy of the purified SCAN domain was performed in 25 mm phosphate, 200 mm NaCl, 0.2 mm DTT, pH 6.5. *A*, CD spectrum of the isolated SCAN domain of ZNF174 (25 μm subunit) at 4 °C. *B*, thermal denaturation of the SCAN domain of ZNF174. The molar ellipticity at 222 nm at increasing temperatures was determined for the SCAN domain at either 5 μm subunit (*closed circles*) or 2 μm subunit (*open circles*).