Solution NMR Structure of the Barrier-to-Autointegration Factor-Emerin Complex

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The barrier-to-autointegration factor BAF binds to the LEM domain (EmLEM) of the nuclear envelope protein emerin and plays an essential role in the nuclear architecture of metazoan cells. In addition, the BAF2 dimer bridges and compacts double-stranded DNA nonspecifically via two symmetry-related DNA binding sites. In this article we present biophysical and structural studies on a complex of BAF2 and EmLEM. Light scattering, analytical ultracentrifugation, and NMR indicate a stoichiometry of one molecule of EmLEM bound per BAF2 dimer. The equilibrium dissociation constant (Kd) for the interaction of the BAF2 dimer and EmLEM, determined by isothermal titration calorimetry, is 0.59 ± 0.03 μM. Z-exchange spectroscopy between corresponding cross-peaks of the magnetically non-equivalent subunits of the BAF2 dimer in the complex yields a dissociation rate constant of 78 ± 2 s⁻¹. The solution NMR structure of the BAF2-EmLEM complex reveals that the LEM and DNA binding sites on BAF2 are non-overlapping and that both subunits of the BAF2 dimer contribute approximately equally to the EmLEM binding site. The relevance of the implications of the structural and biophysical data on the complex in the context of the interaction between the BAF2 dimer and EmLEM at the nuclear envelope is discussed.

The barrier to autointegration factor (BAF)⁴ (1) and the inner nuclear envelope LEM-domain protein emerin (2) are highly conserved cellular proteins throughout the metazoan kingdom that play an important role in nuclear architecture (3). BAF is an all-helical obligate dimer (4) that possesses two symmetry related DNA binding sites that permit BAF to bridge DNA chains and thereby compact DNA (5). Emerin is a member of the LEM (LAP2, Emerin, MAN1) family of nuclear proteins, and its loss is associated with the X-linked recessive form of Emery-Dreifuss muscular dystrophy (6). Emerin is a multidomain protein comprising an N-terminal globular LEM domain (EmLEM) of ~50 residues (7), followed by two polycyferase segments separated by a hydrophobic nuclear localization signal, and a C-terminal transmembrane region. EmLEM comprises three helices (8) and is very similar in structure to the LEM domain of the related nuclear envelope protein LAP2 (9). BAF binds to EmLEM (as well as to the LEM domain of LAP2; Ref. 9) and is required for assembly of emerin at the nuclear envelope (10). BAF prevents autointegration of Moloney murine leukemia virus pre-integration complexes in vitro (1), and BAF and emerin have been reported to promote engagement of the HIV-1 pre-integration complex with chromatin prior to integration (11). To further our understanding of the interaction between BAF and the LEM domain of emerin we have characterized the stoichiometry of the complex by NMR, light scattering, and analytical ultracentrifugation; determined the equilibrium constant by isothermal titration calorimetry (ITC) and the dissociation rate constant by z-exchange spectroscopy; and solved the three-dimensional structure of the complex in solution by multidimensional heteronuclear NMR spectroscopy.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—The LEM domain (residues 1–47) of human emerin (7), EmLEM, was subcloned into a modified pET-32a vector (12) to form a thioredoxin fusion protein with a His₆ tag and expressed in Escherichia coli strain BL21(DE3) (Novagen, La Jolla, CA). The construct was verified by DNA sequencing. E. coli transformed with the EmLEM vector was grown on either Luria Bertini or minimal medium (with ¹⁵NH₄Cl and ¹³C₆-glucose as the nitrogen and carbon sources, respectively), induced with 1 mM isopropyl D-thiogalactopyranoside at A₆0₀nm ~ 0.8, and harvested by centrifugation 3 h following induction. After harvesting, the cell pellet was resuspended in 50 ml (per liter of culture) of 50 mM Tris, pH 7.4, 100 mM NaCl, 10 mM imidazole, and 1 mM phenylmethylsulfonyl fluoride. The suspension was lysed by three passages through a microfluidizer and centrifuged at 10,000 × g for 20 min. The supernatant fraction was loaded onto a HisTrap HP column (5 ml; Amersham Biosciences), and the fusion protein was eluted with a 100-mM gradient of imidazole (25–500 mM). The fusion protein was then dialyzed against 20 mM Tris, pH 8.0, and 200 mM NaCl, and digested with thrombin (10 NIH units/mg of protein). Thrombin was removed by passage over a benzami-
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dine-Sepharose column (1 ml; Amersham Biosciences), followed by the addition of 1 mM phenylmethylsulfonyl fluoride. The cleaved His<sub>6</sub>-thioredoxin was removed by loading the digested proteins over a HiTrap HP column. Em<sub>LE</sub> was further purified by a Sephadex-75 gel filtration column (Amersham Biosciences) equilibrated with 50 mM potassium phosphate, pH 6.5, and 0.01% (w/v) sodium azide. This buffer was used for NMR studies on free Em<sub>LE</sub>.

Human BAF was expressed and purified as described (4). The following isotopically labeled samples were prepared: U-15N/13C-labeled, 10% 13C-labeled and unlabeled (natural isotopic abundance) Em<sub>LE</sub>; U-15N/13C-labeled, U-15N/13C/2H/[methyl-H]<sub>Val</sub>/Leu/Ile-labeled, 10% 13C-labeled and unlabeled BAF<sub>2</sub> dimer. NMR samples of the BAF<sub>2</sub>-Em<sub>LE</sub> complex were prepared in 50 mM potassium phosphate, pH 6.5, 200 mM NaCl, and 95% H<sub>2</sub>O, 5% D<sub>2</sub>O.

Light Scattering—Static light scattering data were obtained using an analytical Superdex-75 column (1.0 × 30 cm; GE HealthCare) with in-line multilayer light scattering (DAWN EOS, Wyatt Technology, Inc., Santa Barbara, CA) and refractive index detectors (OPTILAB DSP, Wyatt Technology Inc.). 145 μg of BAF<sub>2</sub> dimer mixed with or without Em<sub>LE</sub> in 125 μl of 50 mM potassium phosphate, pH 6.5, 200 mM NaCl was applied to the pre-equilibrated Superdex-75 column (1 × 30 cm; GE HealthCare) at a flow rate of 0.5 ml/min at room temperature and eluted in the same buffer.

Analytical Ultracentrifugation—Protein stocks purified in 50 mM potassium phosphate, pH 6.5, 200 mM NaCl, and 5 mM 2-mercaptoethanol were used to prepare the samples for sedimentation equilibrium experiments. Samples of the purified complex (eluted in a single peak by gel filtration chromatography) were studied at a loading concentration of 12 μM. Different stoichiometric BAF<sub>2</sub> dimer to Em<sub>LE</sub> mixtures were prepared at 3:1, 2:1, 1:1, 1:2, and 1:3 ratios and nominal BAF<sub>2</sub> concentrations of 20 μM. The 2:1, 1:1, and 1:2 mixtures were also studied at nominal BAF<sub>2</sub> concentrations of 13 μM. All samples were kept at 4 °C and loaded into pre-chilled cells.

Sedimentation equilibrium experiments were conducted at 4 °C on a Beckman Optima XL-A analytical ultracentrifuge. Samples of the complex and various BAF<sub>2</sub>/Em<sub>LE</sub> mixtures were studied at rotor speeds of 16,000, 20,000, 24,000, and 28,000 rpm. Data were acquired using 6-hole cells as an average of 4 absorbance measurements at 280 nm and a radial spacing of 0.001 cm. Sedimentation equilibrium was achieved within 48 h. Data collected at different speeds and different loading concentrations were analyzed globally in terms of various species analysis models using SEDPHAT 4.1b (13) to obtain the buoyant molecular mass M(1 − ν)p. A solution density ρ of 1.01310 cm<sup>3</sup>/g was measured at 20 °C on a Mettler-Toledo DE51 density meter and corrected to a value of 1.0149 cm<sup>3</sup>/g at 4 °C, which is the value used experimentally. Partial specific volumes (ν) for BAF<sub>2</sub> and Em<sub>LE</sub> (at 4 °C) were calculated based on the amino acid composition using SEDNTERP: the values are 0.7287 and 0.7184 cm<sup>3</sup>/g, respectively.

Isothermal Titration Calorimetry—ITC was performed using a high-precision VP-ITC calorimetry system (Microcal Inc.). BAF<sub>2</sub> dimer and Em<sub>LE</sub> were dialyzed against degassed 25 mM Tris-HCl buffer, pH 6.5, and 0.2 mM NaCl prior to the experiment. BAF<sub>2</sub>, dimer (31 μM) in the calorimetric cell at 30 °C was titrated with Em<sub>LE</sub> (at a concentration of 854 μM in the injection syringe). Analysis of the data were performed using the Origin software provided with the instrument.

NMR Spectroscopy—Spectra were recorded at 30 °C on Bruker DMX500, DRX600, DRX750, and DRX800 spectrometers. Spectra were processed using the program NMRPipe (15), and analyzed using the programs PIPP, CAPP, and STAPP (16). Sequential assignment of H<sub>1</sub>, 15N, and 13C resonances was achieved by means of through-bond heteronuclear scalar correlations along the protein backbone and side chains (17, 18) using three-dimensional HNCO/CABC, HNCABC, (H)(CO)NH TOCSY, H(CCO)NH-TOCSY, and CCH-COSY experiments. Interproton distance restraints were derived from three-dimensional 15N- and 13C-separated NOE experiments. Stereospecific assignments of valine and leucine methyl groups were obtained from a 1H-13C NOSY correlation spectrum recorded on 10% 13C-labeled protein (19). Side chain rotamers were derived from 3J<sub>NCC</sub>(aromatic, methyl, and methylene) and 3J<sub>CC</sub> (aromatic, methyl, and methylene) scalar couplings measured by quantitative J correlation spectroscopy (20), in combination with data from a short mixing time (40 ms) three-dimensional 13C-separated NOE spectrum recorded in H<sub>2</sub>O (21). Intermolecular interproton distance restraints were derived from three-dimensional 13C-filtered/13C-separated NOE experiments recorded on complexes comprising either U-15N/13C or U-15N/13C/H/[methyl-H]<sub>Val</sub>/Leu/Ile BAF<sub>2</sub> dimer complexed to unlabeled Em<sub>LE</sub>, or U-15N/13C-labeled Em<sub>LE</sub> complexed to unlabeled BAF<sub>2</sub> dimer. Residual dipolar couplings (RDCs) were measured by taking the difference in J couplings between aligned (~15 and ~11 mg/ml phage pf1 (22) for free Em<sub>LE</sub> and the BAF<sub>2</sub>-Em<sub>LE</sub> complex, respectively) and isotropic media using well established procedures (23). For free Em<sub>LE</sub>, 1DNH, 1DNC, and 1DHC RDCs were obtained. For the BAF<sub>2</sub>-Em<sub>LE</sub> complex 1DNH RDCs were measured on complexes of 15N/13C-labeled BAF<sub>2</sub> dimer and unlabeled Em<sub>LE</sub>, and 15N/13C-labeled Em<sub>LE</sub> and unlabeled BAF<sub>2</sub> dimer (note only 1DNH RDCs are required for the structure determination of the complex because the backbones of the two proteins are treated as rigid bodies, see below; Ref. 24). The magnitudes of the axial (D<sub>az</sub> (NH)) and rhombic (η) components of the alignment tensor for free Em<sub>LE</sub> were obtained from a histogram of the distribution of the normalized RDCs (25). For the complex, D<sub>az</sub> (NH) and η were obtained by singular value decomposition using the coordinates of the free proteins (23).

Z-exchange spectroscopy was carried out using the pulse sequence described previously (26, 27) using U-15N/13C/H/[methyl-H]<sub>Val</sub>/Leu/Ile-labeled BAF<sub>2</sub> in the presence of 2, 3, and 4 eq of unlabeled Em<sub>LE</sub>. The auto- and exchange-peak intensities as a function of mixing time were fitted by numerically integrating the appropriate McConnell (28) differential equations and optimizing the unknown parameters (dissociation rate constant, spin-lattice relaxation rate, and scale factors) using the program FACSIMILE (29), as described previously (27, 30).

Structure Calculations—Interproton distance restraints were derived from the NOE spectra and classified into generous approximate distance ranges corresponding to strong, medium, weak, and very weak NOE cross-peak intensities (21).
Nonstereospecifically assigned methyl, methylene, and aromatic protons and ambiguous intermolecular NOEs were represented by a $\Sigma^{16}$ sum (31). $\phi/\psi$ torsion angle restraints for free $\text{Em}^{\text{LEM}}$ were derived from backbone (N, C', Ca, Cβ, Hα) chemical shifts using the program TALOS (32). Side chain $\chi$ torsion angle restraints were derived from $\gamma$ heteronuclear couplings and short mixing time NOE experiments using standard procedures (21). The minimum range for the torsion angle restraints was ±20°.

All structure calculations were carried out using Xplor-NIH (33, 34) and the IVM (35) module for torsion angle and rigid body dynamics. The structure of the free $\text{Em}^{\text{LEM}}$ domain was calculated by simulated annealing in torsion angle space (35). The structure determination of the BAF$_2$-$\text{Em}^{\text{LEM}}$ complex was carried out using conjoined rigid body/torsion angle dynamics (24, 35). The target function for simulated annealing comprises: square well potentials for interproton distance and torsion angle restraints (36), harmonic potentials for $^{13}$Cα/$^{13}$Cβ chemical shift restraints (37), RDC restraints (38), and covalent geometry; and a quartic van der Waals repulsion potential (39), mean force (41), and a radius of gyration term (42) to represent a weak overall packing potential and the target value is the mean force (41), and a radius of gyration term (42) to represent the non-bonded contacts. The radius of gyration term represents a weak overall packing potential and the target value is given by 2.2N$^{0.38}$, where $N$ is the overall number of residues (42).

Structures were displayed using the VMD-XPLOR software (43). Reweighted atomic probability density maps used to represent the conformational space sampled by the interfacial side chains within the complex were calculated and displayed as described previously (44).

RESULTS AND DISCUSSION

Structure Determination of the Free $\text{Em}^{\text{LEM}}$ Domain—The structure of the free $\text{Em}^{\text{LEM}}$ domain was determined on the basis of 820 experimental NMR restraints, including 110 backbone RDCs. A summary of the structural statistics is provided in Table 1, a stereoview of the superposition of the ensemble of 180 simulated annealing structures is shown in Fig. 1A, and a ribbon diagram is provided in Fig. 1B. The structure comprises a 3–10 helix (residues 2–6) and two α-helices (residues 9–19 and 28–46) oriented at an angle of 43° to one another. The structure of $\text{Em}^{\text{LEM}}$ is very similar to our previously published structure of the BAF binding LEM domain of LAP2 (9) with a Ca atomic r.m.s. difference of 1.3 Å for 44 atoms (residues 2–46 of $\text{Em}^{\text{LEM}}$ and 111–154 of LAP2; percentage sequence identity of 36%). The Ca atomic r.m.s. difference between the current $\text{Em}^{\text{LEM}}$ structure and the NMR structure previously published by Wolff et al. (8) is 2.4 Å for residues 2–46 and 1.5 Å for residues 3–44. Although the fold and topology of the two $\text{Em}^{\text{LEM}}$ structures are obviously the same, there are clearly differences in detail, which are significant when one wants to use the coordinates of the free $\text{Em}^{\text{LEM}}$ domain to solve the structure of the BAF$_2$-$\text{Em}^{\text{LEM}}$ complex using conjoined rigid body/torsion angle dynamics. In this regard, we note that the agreement of the $\text{Em}^{\text{LEM}}$ coordinates of Wolff et al. (8) with the $^{1}$D$_{\text{NH}}$ RDCs measured for $\text{Em}^{\text{LEM}}$ both free and bound to the BAF$_2$ dimer is rather poor with RDC $R$-factors (46) of 49 and 61%, respectively, determined by singular value decomposition. In contrast, the present structure of the $\text{Em}^{\text{LEM}}$ domains agrees extremely well with the $^{1}$D$_{\text{NH}}$ RDCs measured on the BAF$_2$-$\text{Em}^{\text{LEM}}$ complex with an RDC $R$-factor of 14.8%. The latter value is comparable with the value one would expect for a 1.5–2 Å resolution crystal structure (23, 47, 48). Note that the overall orientation of the alignment tensors of free $\text{Em}^{\text{LEM}}$ and the BAF$_2$-$\text{Em}^{\text{LEM}}$ complex differ by 128°; hence the RDCs measured for $\text{Em}^{\text{LEM}}$ in the BAF$_2$-$\text{Em}^{\text{LEM}}$ complex provide a good cross-validation measure of the quality of the coordinates of free $\text{Em}^{\text{LEM}}$.

Stoichiometry of the BAF$_2$-$\text{Em}^{\text{LEM}}$ Complex by NMR—The BAF$_2$-$\text{Em}^{\text{LEM}}$ complex is in slow exchange on the chemical shift scale and portions of the $^{1}$H/$^{15}$N HSQC spectra recorded as a function of various ratios of $\text{Em}^{\text{LEM}}$ to BAF$_2$ dimer are shown in Fig. 2A. The binding of $\text{Em}^{\text{LEM}}$ to the BAF$_2$ dimer disrupts the symmetry of the dimer such that the chemical shifts of many equivalent residues of the two subunits (about 55% of the $^{1}$H/$^{15}$N cross-peaks for BAF) are no longer identical in the complex. The precision of the coordinates is defined as the average atomic r.m.s. difference if the vectors were randomly distributed, given by $\Sigma_r^{1/6}$ sum (31).
plex. Under the conditions of the NMR experiment (concentration of BAF2 dimer of ∼130 μM), binding of EmLEMD is stoichiometric with one molecule of EmLEMD bound per BAF2 dimer (Fig. 2B). Increasing the ratio of EmLEMD to BAF2 dimer above 1:1 results in no change in the intensity of the bound BAF2 cross-peaks (Fig. 2, A and B).

Because a single molecule of EmLEMD binds to the BAF2 dimer, the chemical environments of the two subunits of BAF2 are necessarily no longer identical. The backbone of the two subunits of BAF2, however, remains identical within coordinate errors as judged from RDC measurements (i.e. the values of the $^{1}D_{NN}$ RDCs for the two subunits of BAF2 are identical in the complex). It should also be noted that if two molecules of EmLEMD bound the BAF2 dimer symmetrically, the chemical environment and hence the chemical shifts of the two subunits of BAF2 would be identical in the complex.

Stoichiometry of the BAF2-EmLEMD Complex by Light Scattering—The calculated molecular mass of the BAF2 dimer and the EmLEMD domains are 20,116 and 5,572 Da, respectively. The BAF2 dimer elutes as a single peak with a molecular mass of 21.3 ± 0.2 kDa determined from light scattering and the refractive index data (Fig. 3A). A 1:1 mixture of BAF2 dimer and EmLEMD results in a shift of the BAF2 peak to a lower retention volume with a molecular mass of 25.9 ± 0.2 kDa (Fig. 3B). Increasing the ratio of EmLEMD to BAF2 does not change the position of the latter peak and the molecular mass obtained at a ratio of BAF2 dimer to EmLEMD of 1:2, 1:3, and 1:4 is 24.7 ± 0.2, 24.8 ± 0.2, and 27.4 ± 0.2 kDa, respectively. The peak eluting at ∼15.4 ml with a molecular mass of 6.48 ± 0.02, 6.45 ± 0.01, and 6.76 ± 0.01 kDa shown in Fig. 3, C-E, respectively, corresponds to free EmLEMD. These results clearly indicate that the BAF2-EmLEMD complex comprises one BAF2 dimer and one molecule of EmLEMD. The concentration of complex upon elution is ∼12 μM. The observation that the position of the peak corresponding to the complex does not change upon increasing concentration of EmLEMD indicates that the equilibrium dissociation constant for the complex is $\leq 1$ μM.

Stoichiometry of the BAF2-EmLEMD Complex by Analytical Ultracentrifugation—Sedimentation equilibrium experiments on the BAF2-EmLEMD complex purified by size-exclusion gel filtration chromatography were carried out at rotor speeds of 16,000 to 28,000 rpm and analyzed in terms of a single ideal solute. Excellent data fits were obtained (Fig. 4) returning a molecular mass of 26.8 ± 0.4 kDa. Based on the amino acid sequence and solution density, the BAF2 dimer and EmLEMD monomer have calculated molecular masses of 20,116 and 5,572 Da, respectively, indicating that the complex has a 2:1 BAF:EmLEMD stoichiometry ($n = 1.04 ± 0.02$): that is one molecule of EmLEMD for two subunits of BAF monomer. To confirm the stoichiometry of the complex, sedimentation equilibrium experiments were carried out on a 1:1 loading mixture of BAF2 dimer and EmLEMD at concentrations of 13 and 20 μM BAF2 dimer. A global data analysis in terms of a single ideal solute returned excellent fits with an experimental molecular mass of 25.8 ± 0.3 kDa (Fig. 4), confirming the formation of a 2:1 BAF:EmLEMD complex ($n = 1.00 ± 0.01$).
Sedimentation equilibrium experiments carried out in the presence of excess Em<sup>LEM</sup>, namely 1:2 and 1:3 BAF<sub>2</sub> dimer to Em<sup>LEM</sup> mixtures, could not be modeled adequately in terms of a single ideal solute. Accordingly, data were analyzed in terms of two ideal solutes, of which one represents the free Em<sup>LEM</sup> domain. Fixing the molecular mass of the smaller species to 5,572 Da, a 1:2 BAF<sub>2</sub> dimer to Em<sup>LEM</sup> mixture returns a molecular mass of 27.0 ± 0.95 kDa for the second species with excellent data fits (data not shown). These data are consistent with the sole formation of a 2:1 BAF-Em<sup>LEM</sup> complex (n = 1.05 ± 0.04). A 1:3 BAF<sub>2</sub> dimer to Em<sup>LEM</sup> mixture containing 18.4 μM BAF<sub>2</sub> dimer and 55.2 μM Em<sup>LEM</sup> returns a molecular mass of 29.2 ± 1.5 kDa (n = 1.14 ± 0.06) (data not shown). Thus the stoichiometry of the BAF<sub>2</sub>-Em<sup>LEM</sup> complex, comprising one molecule of Em<sup>LEM</sup> bound per BAF<sub>2</sub> dimer, is unambiguously confirmed by three independent techniques covering a range of concentrations and molar ratios.

**Equilibrium and Kinetic Characteristics of the BAF<sub>2</sub>-Em<sup>LEM</sup> Complex**—ITC was used to determine the equilibrium and thermodynamic properties of the interaction of Em<sup>LEM</sup> with the BAF<sub>2</sub> dimer.

To show that these species only form a 2:1 BAF:Em<sup>LEM</sup> complex, various BAF<sub>2</sub> dimer and Em<sup>LEM</sup> mixtures were studied. In the presence of excess BAF<sub>2</sub>, namely the 3:1 and 2:1 BAF<sub>2</sub>:Em<sup>LEM</sup> loading ratios, free BAF<sub>2</sub> dimer (molecular mass of 20,116 Da) and the 2:1 BAF:Em<sup>LEM</sup> complex (molecular mass of 25,688 Da) are the only species expected. As the molecular masses are too similar to distinguish by sedimentation equilibrium, a mixture of these two species represents a so-called paucidisperse system and an analysis in terms of a single ideal solute should return a weight average molecular mass of 25,688 Da) are the only species expected. As the molecular masses are too similar to distinguish by sedimentation equilibrium, a mixture of these two species represents a so-called paucidisperse system and an analysis in terms of a single ideal solute. The 3:1 BAF<sub>2</sub> dimer to Em<sup>LEM</sup> loading mixture were also consistent with a 2:1 BAF-Em<sup>LEM</sup> complex stoichiometry, within the error of the method.

The experimental ITC binding curve obtained upon addition of Em<sup>LEM</sup> to a 31 μM solution of BAF<sub>2</sub> dimer is shown in Fig. 5A. A best-fit to the experimental ITC data with a stoichiometry of one Em<sup>LEM</sup> molecule bound per BAF<sub>2</sub> dimer yields an equilibrium dissociation constant (K<sub>d</sub>) of 0.59 ± 0.03 μM, corresponding to a binding free energy (ΔG<sup>0</sup>) of −8.63 ± 0.03 kcal mol<sup>−1</sup>, and an enthalpy (ΔH<sup>0</sup>) of −5.70 ± 0.05 kcal mol<sup>−1</sup>. Thus, the interaction of Em<sup>LEM</sup> with BAF<sub>2</sub> is entropically favored with ΔS = 9.7 cal mol<sup>−1</sup> K<sup>−1</sup>, where ΔS = (ΔH − ΔG)/T, and T is the temperature in Kelvin. The increase in entropy upon complex formation arises from the hydrophobic effect (49) as a consequence of the displacement of ordered water at the binding interfaces of the two proteins, and suggests that the interaction between Em<sup>LEM</sup> and BAF<sub>2</sub> is predominantly stabilized by hydrophobic interactions.

The kinetics of the interaction of unlabelled Em<sup>LEM</sup> and U-<sup>15</sup>N/13C/[methyl-<sup>1</sup>H]<sup>2</sup>Val/Leu/Ile BAF<sub>2</sub> were studied by <sup>1</sup>H-exchange spectroscopy (26, 27), which revealed the presence of chemical exchange cross-peaks between the two sets of shifts for the BAF<sub>2</sub> dimer in the BAF<sub>2</sub>-Em<sup>LEM</sup> complex (Fig. 5B). This arises from the fact that Em<sup>LEM</sup> can bind to the BAF<sub>2</sub> dimer in two chemically equivalent ways related by a 180° rotation about
the C2 symmetry axis of the BAF2 dimer (Fig. 6). Thus cross-peaks corresponding to the two magnetically inequivalent sub-units of BAF2 in the complex are simply interchanged in the two bound states. The exchange process occurs via dissociation followed by reassociation (and note both orientations are equally probable because the two possible complexes are chemically equivalent) (Fig. 5C). The McConnell (28) differential equations describing the evolution of magnetization as a function of mixing time for the scheme shown in Fig. 5C are as follows,}

\[
\begin{align*}
\frac{dM_g}{dt} &= M_f \cdot k_{\text{on free}} - M_g (k_{\text{off}} + \rho_g) \\
\frac{dM_b}{dt} &= M_f \cdot k_{\text{on free}} - M_b (k_{\text{off}} + \rho_b) \\
\frac{dM_i}{dt} &= -M_i (p_i + 2k_{\text{on free}}) + k_{\text{off}} (M_g + M_b)
\end{align*}
\]

where \(M_g\) is the magnetization of free BAF2, and \(M_f\) and \(M_b\) are the magnetizations for the two species of BAF2 in the complex related by the 180° rotation of bound EmLEM; \(k_{\text{on}}\) and \(k_{\text{off}}\) are the association and dissociation rate constants, respectively; \(\rho_f\) and \(\rho_b\) are the spin-lattice relaxation rates for BAF2 in the free and bound states, respectively; \([\text{EmLEM}]_{\text{free}}\) is the concentration of free EmLEM, and \(k_{\text{on}}[\text{EmLEM}]_{\text{free}}\) is a pseudo-first order rate constant because the concentration of free EmLEM is not perturbed during the experiment. The intensity of a given auto-peak and its associated exchange-peaks as a function of mixing time are obtained by numerical integration of Equations 1–3 with the magnetization of the species corresponding to the auto-peak set to 1 and the magnetization of the species corresponding to the exchange-peaks set to zero.
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Z-exchange experiments were carried out at three different concentrations of free \( \text{Em}^{13\text{EM}} \), 0.39, 0.68, and 0.89 mM. The evolution of the intensities of the normalized auto- and exchange-peaks as a function of mixing time was found to be concentration independent (Fig. 5D). This is as expected because \( k_{\text{on}}[\text{Em}^{13\text{EM}}]_{\text{free}} \gg k_{\text{off}} \) so that the apparent rate of interconversion between the magnetizations \( M_B \) and \( M_a \) is \( k_{\text{off}}/2 \) in each direction. Note also that the calculated maximum magnetization of the exchange-peak for \( M_r \) is less than \( 10^{-3} \), and hence no exchange-peak corresponding to free BAF2 is observed. Simultaneous best-fitting of the time courses of the intensities of the auto- and exchange-peaks (Fig. 5D) yields a value of \( k_{\text{off}} = 78 \pm 2 \text{ s}^{-1} \). Given the value of \( K_d \) determined by ITC, the association rate constant \( (k_{\text{on}}) \) is calculated to be \( \approx 1.3 \times 10^8 \text{ M}^{-1} \text{ s}^{-1} \), typical of a diffusion-controlled protein-protein association reaction (50).

It should be noted that the exchange process observed by \( z \)-exchange spectroscopy is a phenomenon that can only be observed by NMR and is of no functional significance because the two binding orientations are chemically equivalent and therefore functionally identical. (It is, of course, of biophysical significance because it enables one to determine the value of the dissociation rate constant.) The existence of the exchange process does, however, have implications for the NMR structure determination of the complex. In particular, all intermolecular NOEs must be treated as ambiguous (\( \Sigma \sigma^r - \sigma^s \)) sums (31) because no distinction can be made \textit{a priori} as to which BAF subunit is involved in a given intermolecular NOE. This situation is exactly analogous to the situation that pertains to the \( \delta \) and \( \epsilon \) protons of Phe and Tyr residues undergoing 180° ring flips. It should also be noted that because of chemical exchange between the two binding orientations (related by a 180° rotation), an NOE cross-peak from a residue of \( \text{Em}^{13\text{EM}} \) to a residue on

![Figure 5](image_url)
one subunit of BAF will be transferred by chemical exchange to the corresponding residue on the other BAF subunit. This is clearly evident in some of the strips taken from a three-dimensional 12C-filtered/13C-separated NOE spectrum shown in Fig. 5E.

Structure Determination of the BAF2-EmLEM Complex—The structure of the BAF2-EmLEM complex was solved by conjoined rigid body/torsion angle dynamics (24, 35) on the basis of 308 experimental NMR restraints including 140 backbone 1DNH RDCs that yield precise and accurate orientational restraints related to the relative positions of BAF2 and EmLEM within the complex, and 31 intermolecular interproton distance restraints derived from three-dimensional 12C-filtered/13C-separated NOE spectroscopy that provide translational, as well as orientational, information. In these calculations the backbone and non-interfacial side chains for the two proteins are treated as rigid bodies, whereas the interfacial side chains are given full torsional degrees of freedom (24, 35): the backbone of the BAF2 dimer is held fixed, EmLEM is free to rotate and translate, and the single RDC alignment tensor is free to rotate. The starting coordinates employed for the complex comprise the published NMR structure of the BAF2 dimer (Protein Data Bank code 1QCK; Ref. 42) and the present NMR structure of the EmLEM domain (the restrained regularized mean coordinates are shown in green and purple). The structure shown represents the restrained regularized mean coordinates derived from an ensemble of 180 simulated annealing structures calculated using conjoined rigid body/torsion angle dynamics (see Fig. 7).

![Diagram](image)

**EmLEM** binds to the BAF2 dimer in two chemically equivalent orientations related by a 180° rotation about the C₂ axis of symmetry of the BAF2 dimer. The two subunits of the BAFdimer are shown in red and blue; and the two orientations of EmLEM are shown in green and purple. The structure shown represents the restrained regularized mean coordinates derived from an ensemble of 180 simulated annealing structures calculated using conjoined rigid body/torsion angle dynamics (see Fig. 7).

### TABLE 2
Structural statistics for the BAF2-EmLEM complex

The notation is the same as that in Table 1. The number of experimental restraints for the various terms is given in parentheses.

| Measure of structure quality | (SA)_r | (SA)_i |
|-----------------------------|--------|--------|
| Interproton distances (Å)   | 0.007 ± 0.002 | 0.016  |
| Side chain torsion angles (°) | 0.033 ± 0.046 | 0.025  |
| Overall RDC R-factors*      | 15.2    | 14.8   |
| 1DNH BAF (54 × 2)           | 15.3 ± 0.003 | 14.8 ± 0.002 |
| 1DNH EmLEM (32)             | 3.9 ± 3.3 | 1.6    |
| Intermolecular repulsion energy (kcal mol⁻¹) | −14.8 ± 3.3 | −4.2 |

* The interproton distance restraints comprise 31 intermolecular interproton distances, 30 intramolecular distances related to the interfacial side chains of BAF (4 intra-residue, 9 i – j = 1 sequential, 13 i < j ≤ 5 medium range and 4 |i – j| > 5 long range inter-residue); and 61 intramolecular distances related to the interface side chains of EmLEM (3 intra-residue, and 22 sequential, 29 medium range and 7 long range inter-residue). The intermolecular repulsion energy is given by the value of the quartic van der Waals repulsion term calculated with a force constant of 4 kcal mol⁻¹ Å⁻⁴ and a van der Waals radius scale factor of 0.78. The intermolecular Lennard-Jones energy is calculated using the CHARMM19/20 parameters and is not included in the target function used to calculate the structures. The percentage of residues present in the most favorable region of the Ramachandran map for the NMR structure of free BAF and EmLEM is comparable with values expected for 1.5–2 Å resolution crystal structures (47, 48).

**FIGURE 6**. The notation is the same as that in Table 1. The number of experimental restraints for the various terms is given in parentheses.

| Coordinate precision of the complex (Å) |
|-----------------------------------------|
| Complete backbone (N, Cα, C', O) atoms  | 0.13 ± 0.06 |
| Interfacial side chain heavy atoms      | 1.02 ± 0.02 |

* Defined as the average r.m.s. difference between the final 180 conjoined rigid body/torsion angle dynamics simulated annealing structures and the mean coordinate positions. The value quoted for the complete backbone provides only a measure of the precision with which the orientation and translation of the BAF2 dimer and the EmLEM domain have been determined relative to each other and does not take into account the accuracy of the NMR coordinates of free BAF2 and EmLEM. The excellent agreement of the RDCs measured on the complex with the coordinates of free BAF2 and EmLEM, however, indicates good accuracy (23, 47, 48).
The RDC R-factors for BAF₂ and Em¹⁰⁰ in the refined complex (that is using a single alignment tensor for the whole complex) are the same as those obtained by singular value decomposition fitting to the coordinates of the two proteins individually. A table of structural statistics for the BAF₂-Em¹⁰⁰ complex is provided in Table 2. A superposition of the backbone (N, Cα, C') atoms of 180 simulated annealing structures (Em¹⁰⁰, green, and the two subunits of the BAF₂ dimer shown in red and blue). Reweighted atomic probability density maps (drawn at a value of 25% maximum and calculated from the final 180 simulated annealing structures) for the interfacial side chains, illustrating the interactions between Em¹⁰⁰ (gray mesh) and the red (B) and blue (C) subunits of the BAF₂ dimer (red meshes). The backbone (with the same color scheme as in A) is represented by flat ribbons. The side chains of the restrained regularized mean coordinates are color coded according to atom type (carbon, gray; oxygen, red; and nitrogen, blue).

The BAF-Emerin Interface—The interaction surface between the BAF₂ dimer and Em¹⁰⁰ is formed by a convex protrusion on Em¹⁰⁰ comprising helix α1, the subsequent loop, and the N-terminal end of α2; and a deep concave cleft on the BAF₂ dimer comprising the C-terminal end of α2, the subsequent hairpin turn and α3 of the red subunit of BAF₂, and the hairpin turn between α2 and α3, the C-terminal end of α3, and the central portion of α4 of the blue subunit of BAF (Fig. 8A). (For clarity we distinguish the two subunits of BAF by color coding.) There is no overlap between the single Em¹⁰⁰ binding site and the two symmetry related DNA binding sites on the BAF₂ dimer. The latter comprises the N terminus of α1, the 3–10 helix/turn/α2 motif, and the N-terminal portion of α5. 969 Å² of accessible surface area are buried at the interface of which 462 Å² originates from BAF₂ and 507 Å² from Em¹⁰⁰. The loop connecting helices α1 and α2 of Em¹⁰⁰ interact with the red subunit of BAF, whereas α1 and the following loop interact with the blue subunit. The binding surfaces on both BAF₂ and Em¹⁰⁰ consist of a central hydrophobic portion surrounded by a rim of polar and charged residues (Fig. 8B), typical of many protein-protein complexes (47). The key hydrophobic interactions involve Val⁵¹, Leu⁵², Leu⁵⁸, Val⁵¹, Phe³⁹, and Gly³⁸ of BAF (where lowercase letters indicate residues from the blue subunit) and Leu⁴¹, Gly⁴₂, Phe⁴³, and Val⁴⁰ of Em¹⁰⁰ (italics denote residues of Em¹⁰⁰). The preponderance of hydrophobic interactions at the interface and the displacement of ordered water from these hydrophobic surfaces upon binding accounts for the positive entropic change upon complex formation observed by ITC. Key electrostatic interactions occur between Arg⁴⁷, Gln⁵⁸, and Asp⁶⁵ of the red subunit of BAF and Asp⁹, Lys⁵⁰, and Lys⁵⁶, respectively, of Em¹⁰⁰ (Fig. 7B) and between glu⁴⁰ of the blue subunit of BAF and Arg₁⁰⁰ of Em¹⁰⁰, and possibly the hydroxyl groups of Thr³⁰ and Thr³³ via water-mediated interactions as well (Fig. 6C). Additional electrostatic interactions include possible water bridged contacts between Gln⁴⁸ of the red subunit of BAF and the hydroxyl groups of Ser²⁹ and Thr³⁰ of Em¹⁰⁰ (Fig. 7B). Trp⁶² of the red subunit of BAF is principally involved in hydrophobic contacts with Thr³⁰ and Leu³³ (Fig. 7B), and trp⁶² of the blue subunit with Val²⁷ (Fig. 7C). The observed interactions between BAF and Em¹⁰⁰ are fully consistent with mutagenesis data that showed that the G24A/P25A/V26A/V27A, T30A/


Structure of the BAF-Emerin Complex

**FIGURE 8.** The BAF$_2$-Em$_{LEM}$ interface. A, ribbon diagram of the BAF$_2$-Em$_{LEM}$ complex (color coded as in Fig. 7A) also illustrating the position of the two DNA duplexes observed in the crystal structure of the BAF$_2$-DNA$_2$ complex (5). B, surface representations illustrating the binding surfaces involved in the BAF$_2$-Em$_{LEM}$ complex. The binding surface on BAF$_2$ is shown on the left panel and on Em$_{LEM}$ on the right panel. The binding surfaces are color coded with hydrophobic residues in green, polar residues in light blue, positively charged residues in dark blue, and negatively charged residues in red. The relevant portions of the interacting partner are shown as gold tubes. The surface of the non-interacting residues of the BAF$_2$ dimer is shown in dark gray for the red subunit and light gray for the blue subunit (as depicted in A). Residues of the blue subunit of BAF$_2$ are labeled in lowercase, and residues of Em$_{LEM}$ in italics. The view in the right-hand panel is related to that in the left-hand panel by a 180° rotation about an axis parallel to the printed lines on the page.

R31A, and Y34A/E35A/K36A/K37A mutations significantly reduce binding of emerin to BAF (52). (Note that a fourth emerin mutation that disrupts BAF binding, E11A/L12A (52), does not involve the interaction surface but is predicted to destabilize the Em$_{LEM}$ core through the introduction of a cavity as a consequence of the replacement of a leucine by the much smaller alanine side chain.)

**Modulation of the Interaction of BAF with LEM Domain Proteins**—The structure of the BAF$_2$-Em$_{LEM}$ complex reported here, together with the structure of BAF$_2$ in complex with DNA (5), places constraints on how the interaction of BAF with LEM domain proteins is regulated. BAF and LEM domain proteins function as part of large nucleoprotein networks; attempts to fish out interacting partners of BAF and LEM domain proteins by biochemical techniques yields numerous proteins, most of which presumably interact indirectly.\(^3\) LAP2 was first identified as a BAF-interacting protein in a yeast two-hybrid screen, and deletion analysis mapped a region encompassing the LEM domain to be sufficient for this interaction (53). The structure of the BAF$_2$-Em$_{LEM}$ complex establishes the basis of this interaction. In **in vitro** binding studies, the LAP2 constant domain has a higher affinity for BAF bound to DNA than for BAF alone and this was taken as evidence for a conformational change in BAF upon DNA binding (54). It is now clear that no conformational changes in BAF occur upon binding either DNA or the LEM domain. Alternative explanations for the higher affinity of the LAP2 constant domain for BAF bound to DNA include the possible interaction of regions outside of the LEM domain with DNA or stabilization of the complex through binding of multiple units of BAF to DNA. Modulation of the BAF-LEM interaction by regions outside the LEM domain is also suggested by the different affinities of various LAP2 isoforms for BAF (54). In addition, studies of the behavior of fluorescently labeled BAF and emerin in cells also suggest modulation of the BAF-LEM interaction (55). A direct interaction between BAF and emerin at the nuclear envelope was demonstrated by FRET analysis. However, fluorescence recovery after photobleaching experiments showed that whereas BAF was highly mobile at the nuclear envelope, emerin was much less mobile. On the basis of these results a “touch and go” model was proposed in which BAF binds emerin frequently but transiently during interphase. This association of BAF and emerin agrees nicely with the transient interaction (\(k_{off} \approx 78 \text{ s}^{-1}\)) we observe by NMR between BAF and the LEM domain of emerin. In contrast, BAF associates much more stably with LEM domain proteins at the “core” region of telophase chromosomes (55). This stable interaction cannot be accounted for by the interaction of BAF with the LEM domain alone, which is transient, and additional protein factors are likely involved.

**Concluding Remarks**—The structures of the BAF$_2$-Em$_{LEM}$ and BAF$_2$-DNA$_2$ (5) complexes provides a structural basis for how BAF both bridges DNA and binds nuclear membrane proteins that contain the LEM domain. The BAF dimer is required for DNA bridging, but binding of the BAF dimer to a single LEM domain ensures that each BAF dimer
interacts with only a single LEM-domain protein and prevents assembly of mixed complexes with multiple nuclear envelope proteins.

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