NEMO Trimerizes through Its Coiled-coil C-terminal Domain

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NEMO/IκB kinase (IKK) γ is the regulatory component of the IKK complex comprising the two protein kinases, IKKα and IKKβ. To investigate the self-assembly properties of NEMO and to understand further the mechanism of activation of the IKK complex, we purified wild-type and mutant NEMO expressed in Escherichia coli. In the absence of its IKK partners, recombinant NEMO (rNEMO) is a metastable functional monomer correctly folded, according to its fluorescence and far-UV CD spectra, which is binding specifically to the IKK complex. A minor fraction of rNEMO was found tightly associated with DnaK (E. coli Hsp70). We also examined the interaction of NEMO with prokaryotic and eukaryotic Hsp70, and we showed that the Hsp70-NEMO complex forms a supramolecular structure probably corresponding to an assembly intermediate. In vivo cross-linking experiments indicate that native NEMO in association with IKK is in equilibrium between a dimeric and a trimeric form. Similarly to native NEMO, a NEMO mutant deleted from its IKK binding N-terminal domain (residues 242–388) forms a stable trimeric coiled-coil, suggesting that the association of NEMO with IKK or with Hsp70 prevents incorrect interdomain pairing reactions that could lead to aggregation or to a non-native oligomeric state of rNEMO. We propose a model in which the activation of the IKK complex occurs through the trimerization of NEMO upon binding to a not yet identified upstream activator.
tion (19, 20) and probably for recruitment by upstream activators. Mutagenesis experiments mapped the region of NEMO responsible for its self-association (21), but no correlation with its oligomeric state could be established.

To understand further the molecular role of NEMO in the activation of the IKK complex, we have studied the biochemical properties of purified murine NEMO recombinant protein (rNEMO) as well as of a truncated C-terminal domain produced in Escherichia coli. The homologous Hsp70 of E. coli, DnaK, was found tightly associated to rNEMO, and we characterized this association by gel filtration and analytical ultracentrifugation methods. As the Hsp70 protein family is relatively well conserved, the in vitro and in vivo association of NEMO with human Hsp70 was examined. We also investigated the oligomeric state of NEMO in vivo by protein cross-linking experiments. Consistent with our results a model was proposed in which the bipartite function of the C-terminal of NEMO modulates the activation of the IKK complex.

**EXPERIMENTAL PROCEDURES**

**Materials**—Octyl glucoside (OG) and dodecyl maltoside (DDM) were from Fluka (Buchs, Switzerland). Biochemicals, ethylene glycol mono-octyl ether (TGME) and Brij 35 were from Sigma, and the switzen detergent, zwittergent 3-16, was from Calbiochem. Bis[4-(maleimidomethyl)-alanine]ethane (BMOE) were from Pierce.

**Expression and Purification of rNEMO and of Its C-terminal Domain**—Murine NEMO was expressed in E. coli with the pRSETa/NEMO expression system (Invitrogen). The NEMO cDNA (14) was cloned in-frame with the vector into BamHI and PexII sites to give plasmid pRSETa/NEMO. The encoded polypeptide (rNEMO) contains a N-terminal extension of 34 residues (MRGS(H)6GMASMTGGQQMG—RDLYDDDDKDRW) inserted at position 2 in NEMO. This sequence contains a His tag and a site of proteolysis by enterokinase. A truncated mutant of NEMO corresponding to a part of its C-terminal domain (amino acids 242–388) was made by PCR mutagenesis using the plasmid pRSETa/NEMO as template. Briefly, NEMO cDNA was amplified between the 5’ primer oligonucleotide 5’-GACGCTAGCTACGACAGCCACATTAAGG and the 3’ primer oligonucleotide 5’-GACGACGTCCTAGTATGAGGTTCCTTCAGGAG. This introduced NheI and BamHI sites (underlined) and a stop codon (bold) at position 388 in the polypeptide sequence. After amplification, the fragment was digested with NheI and BamHI and cloned into a pET-28b expression vector (Novagen). The nucleotide sequences of NEMO and of the D242-F388Stop fragment were verified by DNA sequencing.

Purification of rNEMO was performed starting from a 2-liter culture of BL21(DE3) cells transformed with pRSETa/NEMO grown at 22 °C in 2× YT/ampliphen (50 μg/ml) medium to A600 = 1, and 1 μM isopropyl-1-thio-β-D-galactopyranoside was then added to the medium for 5 h. All subsequent steps were conducted at 4 °C. After ultracentrifugation, the cells were washed twice in a buffer, 100 mM Tris-HCl, pH 7.5, 80 mM MgCl2, 1 mM dithioerythritol, resuspended in extraction buffer (50 mM Tris-HCl, pH 7.5, 20 mM KCl, 5% glycerol, 1 mM dithioerythritol) containing a protease inhibitor mixture (Sigma), and broken in a French press at 1,500 pounds/square inch. The lysate was then diluted 2.5-fold with the equilibrium buffer of the Ni-NTA column (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 0.1 mM DDM) and centrifuged at 12,000 × g for 30 min. The supernatant was loaded on a 25-ml Ni-NTA-agarose column (Qiagen, 1.6 × 12.5 cm) charged with Ni2+. After washing with the equilibrium buffer containing 10 mM imidazole, the bound material was eluted at 110 mM imidazole by a 180-ml linear gradient of imidazole (250 mM) as compared with rNEMO. Fractions (5 ml) containing the C-terminal domain were pooled and dialyzed against buffer 50 mM Tris-HCl, pH 7.5, containing 50 mM KCl, 1 mM dithioerythritol, and 0.1 mM DDM (buffer B). The protein sample (~15 mg) was applied on a Resource Q column (6 ml, Amersham Biosciences) equilibrated in the buffer B. A majority of the protein sample (80%) passed through the column and corresponded to homogeneous D242-F388Stop fragment (see Fig. 5). 20% was bound to the matrix and eluted with a 120-mI gradient (50 mM to 1 M) of KCl. This fraction contained truncated rNEMO associated to DnaK with a stoichiometry of 1:1 as judged by SDS-PAGE. Both fractions were dialyzed twice against 50 mM Tris-HCl, pH 7.5, 100 mM KCl, 1 mM dithioerythritol, 50% glycerol, and 1 mM DDM and stored at −20 °C. Protein concentrations were determined either by the method of Bradford or by absorbance at 280 nm using an extinction coefficient of 0.352 unit-mg−1·cm−1 for rNEMO and of 0.181 unit-mg−1·cm−1 for the truncated rNEMO. Microsequencing of an internal peptide of the DnaK protein was performed as described previously (22), and amino acid sequence comparisons were carried out using the protein data bank (PDB).

In Vitro Binding Assay with Probaratory and Eubaryotic Hsp70s and Co-immunoprecipitation—Ni-NTA magnetic agarose beads suspensions (25 μl, Qiagen) equilibrated with the buffer C (20 mM Tris-HCl, pH 8.0, 20 mM imidazole, 300 mM NaCl, 1 mM DDM, 5% glycerol, and 0.1 mM dithioerythritol) were incubated for 30 min at 4 °C with the purified His-tagged NEMO. After separation of beads with a magnet, the excess protein was removed, and beads captured with His-NEMO were washed twice with a 100-μl volume of buffer D (20 mM NaPO4, pH 7.0, 20 mM imidazole, 150 mM NaCl, 1 mM DDM, 5% glycerol, and 0.1 mM dithioerythritol). DnaK (StressGen) or human Hsp70 (Sigma) was then added and incubated for 1 h at 4 °C in buffer C containing 0.1 mM phenylmethylsulfonyl fluoride. Magnetic beads were separated and washed 3 times with buffer D (100 μl). Hsp70 proteins trapped by His-tagged NEMO were recovered by elution with buffer C containing 300 mM imidazole followed by SDS-PAGE with silver staining.

Circular Dichroism and Fluorescence Spectroscopy—Circular Dichroism Spectroscopy—The CD spectrum of rNEMO (15 μM) or of the truncated C-terminal domain (36 μM) was recorded from 182 to 280 nm at 20 °C and pH 7.0 in a buffer 20 mM potassium phosphate containing 1 mM DDM and 1 mM dithioerythritol (buffer E) using a Jobin-Yvon CD6 spectrodocichrograph (Longjumeau, France). Cells (20 μl) with a path length of 0.1 mm were used, and the product of the time constant and the rate of scanning were below 0.33 nm (23). Each spectrum was the result of the average of three scans taken from the same sample minus the average of three scans from the reference buffer. Deconvolution of CD spectra was performed according to the method of Chang et al. (24) using the CDFTT program as described previously (25). The fluorescence spectrum was recorded in buffer E at 20 °C on a PTI spectrofluorometer Quantamaster24. The excitation wavelength was 295 nm to minimize the contribution of tyrosyl residues to the total fluorescence. The excitation and emission bandwidths were both set to 2 nm. The fluorescence yield was determined as described previously (26).
**Analytical Gel Filtration**—The apparent Stokes radius of rNEMO was determined both at 20 and 4 °C by filtration of 400-μl samples on a Superdex 200 HR 10/30 column (Amersham Biosciences) equilibrated in 50 mM Tris- HCl, pH 7.5, containing 200 mM KCl, 0.2 mM DDM, and 1 mM diethyelthritol (buffer F), developed at a constant flow rate of 0.7 ml/min. The exclusion volume of the column was determined as described previously (33, 34). When the molecular mass of the protein or protein plus detergent was determined, the molecular mass of albumin (67 kDa, RS/H9263 and lin (669 kDa, with blue dextran 2000 and dithioerythritol, respectively. Thyroglobulin 50 mM Tris-HCl, pH 7.5, containing 200 mM KCl, 0.2 mM DDM, and 1 mM dithioerythritol (buffer F), developed at a constant flow rate of 0.7 ml/min. The exclusion volume of the column was determined as: (1/Sv) = Vx – Vg = (Sv – V0) (1/S0) where V0 is the volume of the elution peak calculated by centrifugation, using the equilibrium buffer of the column as reference. Sedimentation velocity experiments were performed at 10 °C to minimize protein aggregation on a Beckman Optima XL-A analytical ultracentrifuge equipped with an An-Ti60 ti-tanium four-hole rotor with two-channel 12-mm path length centerpieces. Samples of 400 μl were centrifuged at 50,000 rpm, and radial scans of absorbance at 280 nm were taken at 1-min intervals. Data were analyzed using the computer programs Sedweb (27), kindly provided by John Philo (Agen, Inc.). The first scans with incomplete clearing of the meniscus were not taken into account for the fitting function. The XLA-VELOC program supplied by Beckman was used for the calculation of the apparent sedimentation coefficient g(ε°) from the time derivative of the sedimentation velocity concentration profile as described previously (28). The sedimentation coefficient of species M2 determined using two species model with Sedweb corresponded to the peak position in the g(ε°) profiles. Sedimentation and diffusion coefficients were corrected to standard conditions, s20,w and D20,w. A partial specific volume of 0.720 cm3/g at 10 °C for rNEMO was calculated from its amino acid composition according to Ref. 29. Solvent density and viscosity at 10°C were 1.007 and 1.07 cP, respectively, determined from published tables (29). Hydrodynamic parameters such as frictional ratio f/f0 and Stokes radius were deduced from the Teller method (30) using the SEBDTNP program provided by John Philo.

**Equilibrium Sedimentation**—Sedimentation equilibrium experiments with rNEMO were carried out at 10 °C at 8,000 or 12,000 rpm in a Beckman Optima XL-A analytical ultracentrifuge. Initial loading concentrations (120 μl) were either 0.54 mg/ml in a buffer 50 mM potassium phosphate, pH 7.0, containing 100 mM KC1, 10 mM OG, and 1 mM diethyelthritol or 0.25 mg/ml in a buffer 20 mM potassium phosphate, pH 7.0, containing 150 mM KC1, 10 mM TMGE, and 1 mM diethyelthritol. In the study of the C-terminal fragment, experiments were performed using two loading concentrations (1 and 1.2 mg/ml in 120 μl) and two rotor speeds (12,000 and 18,000 rpm) in a single run as described in Table II. Protein samples were allowed to equilibrate for 30 h, and duplicate scans (2 h apart) were overlaid to determine that there were no further changes in the sample cell. After collecting data at equilibrium, the samples were centrifuged at 50,000 rpm for 12 h to sediment the protein, and radial scans were again collected to obtain a base-line correction for each cell. To determine the buoyant molecular mass, we followed the formalism of Reynolds and Tanford (31) in which the contribution of the bound detergent δD is expressed in g/g by the relation:

\[ M^* = M + (M - M_0) \delta D = M_0 (1 + \phi) + \delta D \phi \delta (1 - \phi) \]

(Eq. 1)

where \( M^* \) is the molecular mass of the anhydrous protein-detergent complex, \( M \) its partial specific volume, \( M_0 \) is the molecular mass of the anhydrous protein, \( \phi \) its partial specific volume, \( \rho \) is the buffer density, and \( \rho_0 \) the partial specific volume of detergent. Because the density of the detergent TMGE (C1,2E4) was close to that of buffer, the second term in the second member of Equation 1 was negligible. In contrast, the \( \delta D \) term of detergent micelle OG was low (0.92 ± 0.004 ml/g at 20 °C (32)) and a densifier such as the sucrese is often added to the buffer to enhance the change of detergent-OG (\( \phi = \rho_0 - \rho_0 \)) However, the addition of sucrose can change dramatically the elution volume of the protein or the protein–detergent association. We used an OG concentration of 10 mM below its critical micelle concentration (30 mM) to prevent detergent micelle formation such that the second term becomes negligible due to a low \( \delta D \). At 10 °C the densities were 1.009 and 1.016 g/ml in the buffers containing 10 mM TMGE and 10 mM OG, respectively. The partial specific volumes of DnaK and of DnaK-rNEMO complex calculated from their amino acid composition were 0.731 and 0.726 ml/g, respectively, at 10 °C. All data were fitted with one, two, or three species models as described previously (33, 34).

**In Vivo Chemical Cross-linking**—HeLa cells were purchased from American Type Culture Collection and were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Chemical cross-linkings in vivo were performed with a homobifunctional cross-linker as described previously (35, 36). We used BMH or BMOE which is very similar to BMH except that the two carbodiimide groups are linked by a shorter spacer arm (8 A). BMH and BMOE were purchased from Pierce and were stored in Me2SO at −20 °C at 20 mM. The in vivo cross-linking with BMH gave the same results as BMOE except that the efficiency of cross-linking was lower. Briefly, cells (8 × 107) were concentrated into 1 ml, resuspended twice with fresh medium by successive centrifugations (100 × g for 10 min), and incubated on ice for 30 min. 25 μl of stock BMOE (20 mM) was then added to half of cells (4 × 107 cells in 500 μl) at 37 °C to give a final concentration of BMOE of 1 mM. The other half were mock-treated by 25 μl of Me2SO added as control. 100 μl of cells were withdrawn either immediately after adding BMOE or after 10 min or 2 h, incubated for 10 min at 37 °C, and mixed with 20 μl of 180 mM diethyelthritol (final concentration 30 mM) to quench the cross-linking reaction. Cells were then pelleted at 4 °C and washed twice with cold phosphate-buffered saline. The Laemml loading buffer supplemented with 6 x urea (100 μl) was added to cell pellets (40 μl), and the mixtures were boiled for 10 min. The precipitates were removed by centrifugation at 20,000 × g for 4 °C for 30 min. Supernatants (40 mg/ml) were diluted 50-fold with Laemml buffer so that 16 μg of protein were loaded in each well. Western blots were performed as described previously (37) using a final antibody concentration of 0.5 μg/ml with polyclonal anti-NEMO (14) or with monoclonal anti-IKKβ (PharMingen).

**RESULTS**

**Expression and Purification of Recombinant NEMO**—Recombinant His-tagged NEMO (rNEMO) was expressed in E. coli at 22 °C to prevent the formation of inclusion bodies and purified following the protocol under “Experimental Procedures.” The analysis by SDS-PAGE of each chromatographic step is presented in Fig. 1A. The specific binding on chromatographic matrix bearing a nitrilotriacetic group charged with nickel (Ni-NTA) was strictly dependent on the presence of a neutral detergent such as dodecyl maltoside (DDM), which prevented the formation of aggregates. Despite the addition of protease inhibitors, a partial proteolysis of rNEMO protein was detected by Western blot. This partial degradation occurred even if the bacteria were directly boiled in SDS/urea lysis buffer (data not shown) and was likely due to in vivo endogenous proteases, possibly fostered by the lack of interaction with IKKa or - kinases. The proteolyzed fragments were easily removed using ion exchangers (compare lanes Ni and HA in Fig. 1A). The analysis by Ni-NTA pool by SDS-PAGE also revealed the presence of 40- (p40) and 70-kDa (p70) proteins (lanes Ni and Q). These proteins were not found in the eluate when extracts without tagged NEMO were loaded on Ni-NTA column (data not shown). The high imidazole concentration used suggests that both proteins port the view that p70 forms a protein complex with rNEMO. Whereas p40 could be separated by chromatography on ceramic hydroxyapatite column (lane HA), p70 remained associated with rNEMO throughout all purification steps (compare lanes Ni and HA in Fig. 1A) as well as in additional gel filtration and hydrophobic chromatographies (not shown). These results support the view that p70 forms a protein complex with rNEMO. Two additional minor bands with molecular masses of 110 and 160 kDa, respectively, were observed in SDS-PAGE (lane HA, asterisks) and recognized by anti-NEMO antibodies in Western blotting (data not shown). Incomplete dissociation of oligomeric proteins upon SDS-PAGE will be present in the loading buffer, and these two polypeptides could correspond to dimeric and trimeric forms of rNEMO Oligomerization Domain.
NEMO Oligomerization Domain

also called DnaK, in the E. coli protein data bank. Lowering the
DDM concentration from 1 to 0.2 mM induced immediate pro-
tein precipitation. Fig. 1B shows a change in the DnaK/rNEMO
ratio at this lower detergent concentration. Very little DnaK
was found in the pellet fraction (lane P, DnaK/rNEMO ratio of
1:20), whereas the two proteins were in a ratio of 1:3 in the
supernatant (lane S). Because DnaK co-elutes with rNEMO in
all chromatographic columns used, reflecting the formation of
a protein complex, these data indicate that DnaK can act as a
molecular chaperone protecting rNEMO from aggregation.
DnaK-rNEMO complex was further characterized by develop-
ing an in vitro assay using the purified rNEMO as bait and the
commercially available pure DnaK. His-tagged rNEMO was
captured on magnetic beads (Ni-NTA). After incubation with a
variable amount of DnaK, the protein complex was detected by
silver staining of the SDS-PAGE analysis after elution of His-
tagged rNEMO (Fig. 2). Ni-NTA beads not saturated with
His-NEMO were used as control. As shown in Fig. 2a
the addition of DnaK induces an increase of a specific DnaK-
rNEMO complex with 1:1 stoichiometry. Note that although
the interaction was weaker in the presence of ATP/Mg2+, it was
not abolished (compare 4th and 5th lanes).

As Hsp70 protein family has been conserved in evolution, we
next examined whether the human counterpart of DnaK could
also interact specifically with rNEMO, using a similar in vitro
assay. As shown in Fig. 2b, the His-tagged NEMO interacts
specifically with Hsp70 forming a protein complex with 1:1
stoichiometry. The in vivo association of Hsp70 was also inves-
tigated using co-immunoprecipitation experiments. In these
experiments NEMO was transiently expressed in human 293
cells, and crude extracts were used for immunoprecipitation
with anti-NEMO antibodies. The immunoprecipitates were
then analyzed by Western blotting using anti-Hsp70 antibody.
The preimmune serum was used as negative control. As shown
in Fig. 2c both constitutive (73 kDa) and inducible (72 kDa)
forms of Hsp70 were detected in the immunoprecipitate, indi-
cating that human Hsp70-like DnaK interacts specifically with
NEMO in vivo.

Intrinsic Fluorescence and Secondary Structure of rNEMO—
Because NEMO has no enzymatic activity, we checked whether
the recombinant protein was correctly folded by recording its
CD spectra (Fig. 3A) and by measuring its fluorescence yield
(Fig. 3B). Far-UV CD and fluorescence spectra of rNEMO were
recorded in the presence of 1 mM DDM. Under these conditions,
the signal contribution of DnaK was negligible. The CD profile
exhibited two negative dichroic bands with minima at 208 and
222 nm and a positive dichroic band with a maximum at 192
nm characteristic of a protein with a high α-helix content.
Deconvolution of CD spectra using the method of Chang et al.
(24) estimated the fractions of the α-helix, β-form, and unor-
dered form to 44, 0, and 56%, respectively. This result is in
agreement with the secondary structure prediction derived
from the amino acid sequence using the DSC software (38).

Recombinant NEMO contains two Trp residues (Trp-34 and
Trp-39), located in the N-terminal part of the protein. To min-
imize the contribution of the 6 Tyr, the fluorescence spectrum
was recorded with an excitation at 295 nm (Fig. 2b). The
emission spectrum displayed a maximum at 345 nm indicating
that at least one of the two Trp was accessible to the solvent. In
addition, the fluorescence quantum yield of 0.3 was high as
compared with that of N-acetyl-l-tryptophanamide (φ_F = 0.14)
indicating that rNEMO is correctly folded.

rNEMO Binds Specifically to the IKK Complex—The struc-
tural integrity of rNEMO was also checked by determining
whether the pure His-tagged recombinant protein could bind
specifically to the IKK complex through the interaction with

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**Fig. 1. Purification of recombinant NEMO and effect of DnaK on rNEMO.** A, analysis by SDS-PAGE of the purification steps of
NEMO. The crude extracts from transformed E. coli cells expressing
(lane E) or not expressing NEMO (null plasmid) (lane C) were analyzed
by SDS-PAGE. An arrowhead indicates the polypeptide corresponding
to recombinant NEMO. The analysis of the pooled fractions from each
purification step is shown as follows: lanes Ni, Q, and HA are the pools
of Ni-NTA, POROS HQ, and ceramic hydroxyapatite columns, respec-
tively (see “Experimental Procedures”). Lane M corresponds to protein
markers. Arrows indicate rNEMO and co-eluting p40 and p70 proteins.
Asterisks indicate 110- and 160-kDa protein bands that are specifically
recognized by rNEMO antibodies. B, chaperone role of DnaK on the
recombinant NEMO. Purified rNEMO containing 1 mM DDM was di-
luted in a buffer containing 0.2 mM DDM. After centrifugation at 13,000
rpm, the supernatant (lane S) and the pellet (lane P) were analyzed by
SDS-PAGE, and the ratio DnaK/rNEMO was determined by densitom-
etry after Coomassie staining.
the IKKα kinase (Fig. 4). S100 extracts were prepared either from a parental pre-B cell line (70Z/3) which contains the native endogenous NEMO associated to IKK complex or from a NEMO-deficient mutant pre-B cell line (1.3E2) (14). The His-tagged NEMO captured on Ni-NTA beads was then incubated in extracts from both cell types, and the interaction with IKK complex was detected by Western blotting after elution of the His-tagged NEMO. The purified His-tagged C terminus mutant of rNEMO lacking the N-terminal IKK binding domain was used as control (see below). As shown in Fig. 4A, a specific interaction of rNEMO lacking the N-terminal IKK binding domain was used as control (see below). As shown in Fig. 4a, a specific interaction of rNEMO with the IKK complex was detected in 1.3E2 cells (lane 2), whereas no association with IKK complex was observed with His-tagged rNEMO in 70Z/3 cells nor with His-tagged C terminus mutant in 1.3E2 cells (1st and 3rd lanes). To determine the recovery of IKK complex bound, we analyzed by Western blotting unbound materials in different extracts (Fig. 4b). About 50% of IKK complex in the 1.3E2 extract were captured by Ni-NTA beads saturated with His-NEMO, indicating that the interaction is highly specific. Taken together, CD and fluorescence spectra as well as the interaction assay showed that rNEMO is a functional recombinant protein that is correctly folded with a high α-helical content.

Quaternary Structure of rNEMO and of the DnaK-rNEMO Complex—The states of association of the recombinant rNEMO and the DnaK-rNEMO complex were analyzed by a combination of gel filtration and ultracentrifugation experiments. To increase the fraction of DnaK-rNEMO complex in solution, a part of free rNEMO was removed by precipitation using a lower concentration of DDM (0.2 mM) (see Fig. 1B, lane S). Fig. 5A shows the analysis by gel filtration of the DnaK/rNEMO mixture at 20°C on a Superdex 200 HR10/30 column in a buffer containing 0.2 mM DDM. All of rNEMO eluted in a single symmetrical peak both at 20 and at 4°C. SDS-PAGE analysis showed that each fraction contained both rNEMO and DnaK proteins in a ratio 3:1 (data not shown). The elution volume, between that of ferritin and thyroglobulin, corresponds to a very high Stokes radius ($R_s = 73 \text{ Å}$ (inset of Fig. 5A)), corresponding to an apparent mass of 500 kDa for a globular protein that could indicate the presence of multimeric species.

The fraction corresponding to the median peak of the column shown in Fig. 5A (0.8 mg/ml) was analyzed by sedimentation velocity in the same buffer (Fig. 5B). Using the Svedberg software (27), the data were poorly fitted with a single species, and the best fit was obtained with a two species model, $M_1$ and $M_2$. 

FIG. 2. Association of NEMO with Hsp70. a, in vitro association with E. coli DnaK. Ni-NTA beads (25 μl) saturated with (Ni-NTA/His-NEMO) or without (Ni-NTA) His-tagged rNEMO were incubated with variable amounts of DnaK in the absence or in the presence of 5 mM ATP as indicated. After thorough washing, His-tagged rNEMO was eluted, and the protein complex was analyzed by SDS-PAGE and visualized by silver staining as described under “Experimental Procedures.” b, in vitro association with human Hsp70. Similar experiment as a except that Ni-NTA beads with or without bound His-NEMO were incubated with a saturating concentration of Hsp70 (10 μg, 0.2 mg/ml). In the right lane (Hsp70) 1 μg was loaded alone to show purity. c, in vivo association with human Hsp70. Extracts from 293 cells transiently expressing NEMO were immunoprecipitated with anti-NEMO antibodies or a preimmune serum (control). Immunoprecipitates (IP) were then analyzed by Western blot (WB) with anti-Hsp70 as described under “Experimental Procedures.”
representing 75 and 25% of the material, respectively. The sedimentation and diffusion coefficients of species M1 ($s_{20,w} = 4.1 \text{ S}$ and $D_{20,w} = 7.0 \times 10^{-7} \text{ cm}^2/\text{s}$, Table I) and the corresponding molecular mass calculated from the Svedberg relation (mass = 53,000 Da) indicated that it corresponded to monomeric rNEMO (calculated mass = 51,796 Da). Moreover, its Stokes radius ($R_S = 29.7 \text{ Å}$) and frictional ratio ($f/f_0 = 1.20$) indicated that it behaved as a globular molecule. These results contrast strongly with the data from size-exclusion chromatography experiments where all of rNEMO was eluted as a single peak with a $R_S$ of 73 Å (see “Discussion”). The proportion of species M2 relative to M1 (25 and 75%, respectively) strongly suggests that M2 may correspond to the DnaK-rNEMO complex. Both the values of the average sedimentation coefficient ($s_{20,w} = 5.5 \text{ S}$) and the very large diffusion coefficient ($D_{20,w} = 10.9 \times 10^{-7} \text{ cm}^2/\text{s}$) reflect an equilibrium between rNEMO and DnaK-rNEMO complex. The apparent sedimentation coefficient distribution function, $g(s^*)$ versus $s^*$, supports this analysis because the distribution profile exhibited a large asymmetric peak toward the high $s^*$ with a maximum at 4.1 S (inset of Fig. 5B).

To obtain additional information on species M2 and to confirm the monomeric state of rNEMO, we next analyzed the mixture DnaK/rNEMO by equilibrium sedimentation (Fig. 6 and Table I). For this experiment, the detergent used could not be DDM because its high density would require the presence of a densifier such as sucrose to make the detergent transparent (31). To minimize the possible contribution of the detergent to the calculated mass of the protein, we chose two different approaches. First, we used the detergent TGME (cmc of 7 mM in 0.1 M NaCl) with a density close to that of the buffer in order to achieve gravitational transparency (31). Second, we used the detergent OG with a high cmc (cmc of 25 mM in 0.1 mM NaCl) so that its working concentration was below its cmc to minimize micelle formation. Fig. 6 shows a sedimentation equilibrium experiment of the mixture DnaK/rNEMO with a ratio 1:2 in a buffer containing 10 mM OG. Again, the radial distribution was poorly fitted with a single species model, and the best fitting, represented by the curved line in Fig. 6, was obtained with a two species model. As shown in Table I the values found (49,000 ± 3,000 Da for M1 (55%) and 340,000 ± 20,000 Da for M2 (45%)) corresponded to monomeric rNEMO and to a heavy protein complex between DnaK and rNEMO. No significant improvement was obtained when fitting was performed using a three-component model either in fixing the masses of protein partners or in allowing them to float. This indicated that no
free DnaK was detectable during the centrifugation, implying again a tight binding between DnaK and rNEMO. Similar results were obtained when the experiments were performed with a buffer containing 10 mM TGME. In this case, the molecular masses of $M_1$ and $M_2$ were $55,000 \pm 3,000$ Da (66%) and $360,000 \pm 10,000$ Da (34%), respectively. Given a complex
FIG. 6. Sedimentation equilibrium of rNEMO. Equilibrium distribution of rNEMO measured by its absorbance at 280 nm is plotted as a function of radial distance at 12,000 rpm and 10 °C. Initial protein concentration was 0.35 mg/ml with DnaK/rNEMO ratio of 1:2 in a 50 mM Tris-HCl, pH 7.5, containing 100 mM potassium chloride, 1 mM dithioerythritol, and 10 mM β-OG. Data (symbols) were fitted (curves) as described under “Experimental Procedures.” The line shows the best-fitting curve for an ideal two-species model with a molecular mass of 49,000 ± 3,000 Da for species M₁ and 340,000 ± 20,000 Da for species M₂ (see also Table I). The random distribution of residuals as function of radial distance is shown above.

stoichiometry of 1:1 (see Fig. 2), this mass matches with the mass of a DnaK-rNEMO complex containing 3 molecules of DnaK bound to 3 rNEMO molecules (theoretical mass of 360,540 Da).

Altogether, our ultracentrifugation experiments show that a fraction of the recombinant rNEMO is present as a monomer while the remainder is tightly associated to the chaperone DnaK. The DnaK-rNEMO complex forms a supramolecular structure (3:3) that may correspond to an assembly intermediate of rNEMO trapped in its trimeric state.

The C-terminal Domain of rNEMO Forms a Trimeric Coiled-coil—In order to understand which region of NEMO mediates its oligomerization, we compared the sequences of NEMO and of the related proteins NRP/FIP-2 (39, 40). The best conserved C-terminal half (amino acids 240–412 in NEMO) includes both the coiled-coil CC2 and the LZ domains, as well as the ZF motif (Fig. 7). The analysis of the 7-residue repeat composing each coiled-coil domain using the MultiCoil program (41) predicts that the CC2 shows a propensity to form trimeric coiled-coils, whereas LZ, similar to the wild-type GCN4 LZ (42), is likely to self-associate into dimer. To determine which type of oligomer can be formed with the C-terminal part of NEMO, we tried to express the C-terminal domain in E. coli. Unfortunately the complete fragment was poorly produced in E. coli, making its purification difficult. We next decided to express a fragment corresponding to the C-terminal part of NEMO devoid of the 24 C-terminal amino acids composing the zinc finger motif (residues 242–388). This fragment was well expressed in E. coli, and the use of the purification procedure, described under “Experimental Procedures,” resulted in 6 mg of homogeneous protein from 1 liter of culture. As the mutant protein also showed a propensity to form aggregates, all buffers were also supplemented with DDM (0.1 mM). The apparent molecular mass observed by SDS-PAGE (inset of Fig. 8a) was in agreement with the calculated molecular mass of purified truncated mutant of 19,625 Da. During the last chromatographic step, the fraction of the truncated mutant protein which passed through the Q-column (80%) was homogeneous (inset of Fig. 8a), whereas the part (20%) eluted with a salt linear gradient was associated to DnaK in a stoichiometry of 1:1 (data not shown).

The CD spectrum of the purified truncated mutant is shown in Fig. 8a. It is similar to that of WT rNEMO with a maximum at 192 nm and two minima at 208 and 222 nm, but the amplitude of each dichroic band is significantly higher, yielding an α-helix content of 52% instead of 45% in the case of WT rNEMO. In contrast to the WT rNEMO where the same α-helical content was detected over a large protein concentration...
range (2–40 μM) (data not shown), molar dichroic absorption of the C-terminal fragment showed a strong concentration dependence. As shown in Fig. 8b, the increase of protein concentration induced a significant increase in helicity with a plateau at 10–11 μM, consistent with the formation of intermolecular coiled-coils induced by the oligomerization of the C-terminal fragment.

The quaternary structure of the C-terminal fragment was determined by equilibrium sedimentation. Table II summarizes the results of two fits using either a monospecies model or a monomer-dimer-trimer model. All radial distributions obtained at 12,000 or 18,000 rpm with a loading concentration of either 1 or 1.2 mg/ml were poorly fitted with the one-component model (average molecular mass of about 35,000 Da) well above the 19,625 mass of monomer indicating oligomerization of the C-terminal fragment. The best fit at 18,000 rpm was obtained with a monomer-dimer-trimer model which gave a significant improvement of χ² and a random distribution of residuals as compared with monospecies or bispecies models (monomer-trimer or dimer-trimer). Therefore, in a protein concentration range of 0.5 to 2.1 mg/ml, the average distribution of the C-terminal fragment present in solution was monomer (54%), dimer (16%), and trimer (30%) with dissociation constants $K_{M\rightarrow D}$, $K_{D\rightarrow T}$, and $K_{M\rightarrow T}$ equal to 117, 17.6, and 8.8 μM, respectively, indicating that the affinity of the homotrimer is 13-fold higher than that for the homodimer.

**Quaternary Structure of Native NEMO**—Previous experiments (14) using gel filtration analysis of S100 extracts in different cell lines showed that native NEMO is always present in association with IKK kinases and that no free form of the protein could be detected. In order to determine the oligomeric state of the NEMO in these complexes, in vivo chemical cross-linking experiments in HeLa cells were performed using the permeable homobifunctional cross-linker, BMOE, which reacts specifically with the cysteine residues (see “Experimental Procedures”). The extent of total protein cross-linking was probed by the SDS-PAGE analysis of crude extracts, either treated or mock-treated with 1 mM BMOE. As shown in Fig. 9a, the pattern of treated cells only slightly differed from that of the control, indicating that only a small number of cellular proteins were cross-linked. When the cross-linked cells were compared with the mock-treated cells by immunoblotting with either NEMO antibodies or with anti-IKKβ antibodies, specific cross-links were detected for both proteins (Fig. 9b). Note that no band corresponding to either NEMO (48,200 Da) or IKKβ (86,564 Da) was observed in cells treated with BMOE, indicating that the cross-linking reaction was complete. Three species specifically reacted with anti-NEMO antibodies, with masses of 110, 160, and about 350 kDa, respectively. The 110- and 160-kDa species matched the masses of the cross-linked dimer and trimer of NEMO. The slightly slower migration as compared with the theoretical masses of NEMO dimer (96 kDa) and trimer (144 kDa) was probably due to the cross-linker molecules that may affect the electrophoretic migration. The detection of the cross-linked dimer of NEMO could reflect a partial cross-linking of the NEMO trimer. This was not the case because extended incubation of the cross-linker with HeLa (2 h) did not change the relative proportions of cross-linked dimer and cross-linked trimer (data not shown). When using anti-IKKβ antibodies, only one species of about 350 kDa was detected in treated cells. This 350-kDa species, which co-migrated with the third cross-linked species generated with anti-NEMO.
all experiments were performed in the presence of 10 mM OG at 10 °C as described under “Experimental Procedures.”

| Initial protein concentration | Speed | Single species | χ² | Monomer-dimer-trimer equilibrium | Distribution of molecular species | Deduced mass of monomer | χ² |
|------------------------------|-------|----------------|----|---------------------------------|---------------------------------|------------------------|----|
| mg/ml                        | rpm   | Da             |     |                                 |                                 |                        |     |
| 1.2                          | 12,000| 34,500 ± 1,000| 31  | 65% a; 35% (α₂ + α₃)            | 22,000 ± 3,000                  | 22                     |     |
|                              | 18,000| 37,500 ± 1,000| 47  | 53% a; 16% α₁; 31% α₃           | 21,000 ± 2,000                  | 20                     |     |
| 1.0                          | 12,000| 33,500 ± 1,500| 30  | 66% a; 34% (α₂ + α₃)            | 23,000 ± 4,000                  | 24                     |     |
|                              | 18,000| 35,300 ± 2,000| 49  | 54% a; 16% α₁; 30% α₃           | 22,000 ± 3,000                  | 33                     |     |

* No improvement of the fit was observed by a trispecies model (monomer-dimer-trimer). See text and “Experimental Procedures” for further details.

† Distribution of molecular species was calculated for a protein concentration range from 0.3 to 2.1 mg/ml.

antibodies (Fig. 9B), is likely to correspond to the cross-linked IKK complex.

DISCUSSION

In the present study, the oligomeric state of native and recombinant NEMO purified from *E. coli* was investigated leading to the identification of a domain responsible for its self-association as a trimer. A variety of biochemical methods showed that most of rNEMO is in a monomeric state. This demonstration mainly relies on ultracentrifugation experiments. The data deduced from the sedimentation velocity were best interpreted using a two-species model which identified unambiguously the presence of monomer (species M₁) in the rNEMO protein preparation. The fits of sedimentation profiles for species M₂ yielded an average sedimentation coefficient of 5.5 S and a diffusion coefficient of 10.9 × 10⁻⁷ cm²/s. This very large diffusion coefficient reflected an equilibrium with a heavier species whose presence was confirmed by the equilibrium sedimentation experiments. Monomeric rNEMO displays an aberrant retarded elution in gel permeation. This suggests that the molecular mass of the IKK complex previously determined by gel filtration (~700–900 kDa) was overestimated (14, 20, 43). This very high aberrant Stokes radius of the rNEMO monomer (73 Å) along with the poor resolution of the gel filtration made it impossible to separate the free form of rNEMO from that bound to DnaK. It should be noted that, in general, the early elution of a protein from a gel permeation column is either due to a denatured state, to a very elongated shape, or to a protein/detergent micellar structure. However, our data from velocity and equilibrium sedimentation experiments demonstrate that rNEMO monomer behaves as a globular protein. In addition the far-UV CD spectrum of rNEMO as well as the interaction assay with IKK show that this monomer is in a native state. It thus appears that interference with the matrix combined with the presence of some detergent molecules bound to rNEMO is the cause for the aberrant elution of the monomer from the gel permeation.

The presence of a DnaK-rNEMO complex was best seen in equilibrium sedimentation (Fig. 6) showing that rNEMO can form a high molecular weight complex (360 kDa) with DnaK comprising 3 molecules of each protein. Neither free DnaK, which exists in a monomer-dimer-trimer equilibrium (44), nor a DnaK-rNEMO complex with a stoichiometry of 1:1 could be detected. This was probably due to the presence of a molar excess of rNEMO in all experiments and to the high propensity of the DnaK-rNEMO complex to self-assemble at the concentration used (0.1–1.25 mg/ml). Because we showed that DnaK binds to NEMO in a stoichiometric ratio of 1:1 (see Fig. 2a), the DnaK-rNEMO complex is likely formed via the trimerization of rNEMO. Thus, the fraction of rNEMO bound to DnaK may represent an assembly intermediate of rNEMO in *E. coli*. It is usually thought that DnaK recognizes with high affinity proteins exposing locally short hydrophobic segments either in an extended conformation or as elements with no secondary structure such as loops (45, 46). We showed that the C-terminal fragment of rNEMO also binds to DnaK, although it was correctly folded forming a stable trimeric coiled-coil (data not shown). We hypothesize that the association with DnaK could occur with the monomer of the C-terminal fragment which
contains at least two coiled-coil motifs with a suitable hydrophobic α-helical interface. Indeed, the motif EEALVAKQE (positions 263–271) composing the CC2 coiled-coil was predicted to be a DnaK-binding site with a very high score (47). The question then arises why the rNEMO behaves mainly as a monomer, whereas the endogenous form in association with its IKK partners is in equilibrium between a dimer and a trimer. Our hypothesis is that the interactions of IKK partners through the N-terminal domain of rNEMO have a coupling effect in its self-assembly which is impaired in the absence of this interaction. Consistent with this hypothesis, the C-terminal fragment of NEMO deleted of the IKK binding domain forms a stable trimeric coiled-coil structure that was significantly stabilized upon oligomerization as shown by CD (see Fig. 8b).

We demonstrate an association of NEMO with the human protein Hsp70 homologous to DnaK. Previous work (48, 49) showed a role for eukaryotic Hsp70 and Hsp90 in the conformational maturation of signal transduction molecules, and recently, the requirements of Hsp70 and Hsp90 proteins in the NF-κB activation of lipopolysaccharide-induced cells were reported (50, 51). It is likely that Hsp70 alone or in association with Hsp90 is involved in maintaining the monomeric metastable NEMO in a state competent to bind to IKKα or IKKβ kinases. This association may facilitate the correct oligomeric assembly of NEMO through stabilization of its N-terminal domain. Our data also suggest that NEMO is more sensitive to proteolytic degradation in the absence of this interaction. We propose that Hsp70 and Hsp90 proteins may play a key role in controlling the biological activity of NEMO and thereby in the activation of NF-κB.

Even though there is strong genetic evidence that NEMO is essential for the activation of the IKK complex, the molecular mechanism by which it activates IKK kinases is poorly understood. It has been proposed that NEMO activates the IKK complex by recruiting it to a receptor, but this mechanism was recently questioned by results showing that the IKK complex was still recruited to tumor necrosis factor R1 in response to TNF in NEMO-deficient cells (18). In contrast the results by others (19–21) indicate that the oligomerization of NEMO plays a key role in the activation of IKK kinases. The biochemical characterization of the purified C-terminal fragment of NEMO shown in this paper suggests that it is based on a coiled-coil trimer rather than on coiled-coil dimers. The C-terminal domain contains both an LZ motif, well known to form stable homo- or heterodimers, and a CC2 coiled-coil motif, which is predicted to form a coiled-coil trimer. Thus, the trimeric assembly of NEMO is likely governed by the CC2 coiled-coil motif and not by the LZ motif, the latter being probably rather involved in a specific hetero-association. The expression of NEMO lacking only the CC2 domain does not restore the NF-κB activation in NEMO-deficient 1.3E2 cells after lipopolysaccharide stimulation,2 indicating that the trimerization is crucial for activation of IKK complex. Furthermore, the CC2 domain is a key element for NEMO biological function because a point mutation Ala→Gly within this domain leads to EDA-ID syndrome (17).

Fig. 10 shows a model for the regulation of NEMO function upon its oligomerization. In this model, the NEMO LZ forms heterodimers with upstream activators corresponding either to viral proteins (52) or to signaling proteins belonging to the interleukin-1/lipopolysaccharide or TNF pathways, for example the receptor-interacting protein involved in the response to TNF-α. We propose that the association of NEMO with these upstream regulatory components triggers the activation of the IKK complex by a conformational change via its trimerization. The formation of a homodimer through the leucine zipper would then prevent this association. Thus, different oligomeric states of NEMO (α2 or α3) shown in this study may correspond to inactive or active states of the IKK complex, respectively. Experiments attempting to correlate the dimer or trimer oligomerization of NEMO with the inactive or active state of IKK complex are in progress.

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Addendum—While this paper was being revised, the role of the Hsp90 in the assembly of NEMO to the IKK complex was reported (53).

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