The 2'-5'-Oligoadenylate Synthetase 3 Enzyme Potently Synthesizes the 2'-5'-Oligoadenylates Required for RNase L Activation

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ABSTRACT

The members of the oligoadenylate synthetase (OAS) family of proteins are antiviral restriction factors that target a wide range of RNA and DNA viruses. They function as intracellular double-stranded RNA (dsRNA) sensors that, upon binding to dsRNA, undergo a conformational change and are activated to synthesize 2'-5'-linked oligoadenylates (2-5As). 2-5As of sufficient length act as second messengers to activate RNase L and thereby restrict viral replication. We expressed human OAS3 using the baculovirus system and purified it to homogeneity. We show that recombinant OAS3 is activated at a substantially lower concentration of dsRNA than OAS1, making it a potent *in vivo* sensor of dsRNA. Moreover, we find that OAS3 synthesizes considerably longer 2-5As than previously reported, and that OAS3 can activate RNase L intracellularly. The combined high affinity for dsRNA and the capability to produce 2-5As of sufficient length to activate RNase L suggests that OAS3 is a potent activator of RNase L. In addition, we provide experimental evidence to support one active site of OAS3 located in the C-terminal OAS domain and generate a low-resolution structure of OAS3 using SAXS.

IMPORTANCE

We are the first to purify the OAS3 enzyme to homogeneity, which allowed us to characterize the mechanism utilized by OAS3 and identify the active site. We provide compelling evidence that OAS3 can produce 2'-5'-oligoadenylates of sufficient length to activate RNase L. This is contrary to what is described in the current literature but agrees with recent *in vivo* data showing that OAS3 harbors an antiviral activity requiring RNase L. Thus, our work redefines our understanding of the biological role of OAS3. Furthermore, we used a combination of mutagenesis and small-angle X-ray scattering to describe the active site and low-resolution structure of OAS3.

The 2'-5'-oligoadenylate synthetases (OAS) are a family of interferon (IFN)- and virus-induced antiviral restriction factors (1–3) that offer protection against a wide spectrum of RNA and DNA viruses (for a review, see Silverman [4]). The OAS family belongs to a nucleotidyltransferase superfamily (5). Instead of the 3'-5'-phosphodiester linkage found in RNA and DNA, when activated by dsRNA the OAS enzymes synthesize 2'-5'-phosphodiester-linked oligoadenylates (2-5As) with the general formula $p_3A(2'p5'A)_n$, where $n \geq 1$ (6, 7). The OAS proteins share structural homology and the ability to make 2' specific phosphodiester bonds with the recently discovered pattern recognition receptor for cytosolic dsDNA, cGAS (8–11). In humans, the OAS family consists of four members: OAS1, OAS2, OAS3, and OASL (12, 13). OAS1, OAS2, and OAS3 have 2'-5'-oligoadenylate synthetase activity (14), whereas OASL is devoid of this activity despite sharing significant sequence similarity with the other OAS proteins (15, 16). The 42-kDa OAS1 consists of one OAS domain, while the 69-kDa OAS2 consists of two OAS domains and the 120-kDa OAS3 consists of three OAS domains (16–18). The active site of all polymerases (to which family the OAS enzymes belong) harbors a catalytic triad of three carboxyl acids (aspartic acid or glutamic acid), which coordinate the two magnesium ions required for catalysis (19). The crystal structures of porcine OAS1 (pOAS1) alone and of human OAS1 (hOAS1) bound to dsRNA reveal that three aspartic acids constitute the catalytic triad (20, 21). In pOAS1, these three aspartic acid residues are D74, D76, and D147, and in hOAS1 they are D75, D77, and

D148. Only in the third OAS domain of OAS3 are the three aspartic acid residues D816, D818, and D888, corresponding to the catalytic triad in pOAS1 and hOAS1, conserved (Fig. 1A), suggesting that the active site is located within this domain (22). The OAS proteins bind dsRNA in a groove of positively charged residues (20, 21). Upon binding to dsRNA, the OAS proteins undergo a conformational change and are activated to synthesize 2-5As (20, 23). The 2-5As are second messengers that bind to and activate the latent endoribonuclease RNase L (24, 25). The binding of 2-5A to RNase L causes dimerization and activation of RNase L (26, 27) and subsequent restriction of viral growth by degradation of cellular and viral RNA (28). It is a requirement for RNase L activation that the activating 2-5A consists of three or more linked ATP molecules with the 5' phosphates preserved (29). It was reported that OAS3 responds to dsRNA at a much lower concentration than OAS1 (30, 31). However, OAS3 seems to synthesize primarily dimeric 2-5As [$p_3A(2'p5'A)_2$], which has sparked a debate over whether or not OAS3 exerts its antiviral effect through an unknown RNase

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FIG 1 Alignment of pOAS1, hOAS1, and OAS domains 1, 2, and 3 of OAS3 and purification of recombinant OAS3. (A) An alignment displaying residues conserved between hOAS1, pOAS1, and the three OAS domains of OAS3, denoted D1, D2, and D3, respectively. The numbers of amino acids spanned by each OAS domain in the full-length OAS3 are given in parentheses. Asterisks denote the three aspartic acid residues constituting the active sites of pOAS1 and hOAS1. Daggers denote residues crucial for dsRNA binding. A plus sign denotes the conserved lysine at position 212 in pOAS1. (B) A total of 1.5 mg OAS3 was loaded on a HiLoad 16/60 Superdex 200 column. OAS3 eluted in a single peak from the HiLoad 16/60 Superdex 200 column, and fractions D15 to E9 were collected. The chromatograms of the molecular mass markers used to estimate the mass of OAS3 is shown. (C) Coomassie-stained 8% SDS-PAGE. Load, sample loaded onto the HiLoad 16/60 Superdex 200 column; D15 to E9, fractions D15 to E9; Pool, pooled peak fractions; Concentrated, concentrated pool of the peak fractions; Marker, a low-molecular-mass marker (in kDa); pOAS1, the purified pOAS1.

L-independent pathway (30, 32, 33). This debate was fuelled by the first results showing that a single-nucleotide polymorphism (SNP) leading to an R844X truncated form of OAS3 lacking one of the amino acids of the putative active site only partially abrogated its antiviral activity against chikungunya virus, an enveloped RNA virus (34). Interestingly, it was also reported that OAS3 could exert antiviral activity against dengue virus in an RNase L-dependent manner, showing that OAS3 does synthesize 2-5As of a sufficient length for RNase L activation (35).

MATERIALS AND METHODS

Protein expression and purification. Wild-type (WT) OAS3 and the mutant forms D816A/D818A and R844X, without an affinity tag, were expressed in *Drosophila melanogaster* S2 cells using the DES expression system (Invitrogen). The established stable S2/OAS3 cell lines were induced for 10 days with cadmium. At the end of induction, cells were

pelleted. The cells were lysed in 5 ml per gram of cell pellet with 150 mM NaCl, 50 mM HEPES, pH 6.8, 10% (vol/vol) glycerol, 0.2% (vol/vol) NP-40, 1 mM β -mercaptoethanol (BME), and Complete protease inhibitor cocktail (Roche) on ice for 5 min. Cell debris was pelleted at $18,000 \times g$ for 30 min at 4°C. The supernatant was diluted 5 $\times$ with buffer A (25 mM HEPES, pH 6.8, 5% [vol/vol] glycerol) and loaded onto a 50-ml HiTrap SP Sepharose fast flow (FF) column (GE Life Sciences). The column was washed in a 2 $\times$ column volume (CV) of 90% buffer A and 10% buffer B (0.6 M NaCl, 25 mM HEPES, pH 6.8, and 5% [vol/vol] glycerol). Bound OAS3 was eluted in a linear gradient of 6 $\times$ CV up to 100% buffer B.

N-terminally 6 $\times$ His-tagged OAS3 was codon optimized for baculovirus expression and cloned into the pOET1 vector (Oxford Expression Technologies). The baculovirus was generated using the flashBAC system on Sf9 cells according to the manufacturer's protocols (Oxford Expression Technologies). A volume of 2.8 liters of High Five cells was grown to 1.5×10^6 cells/ml and infected at a multiplicity of infection of 5 for 48 h

before harvesting. The cells were lysed in 5 ml per gram of cell pellet with 150 mM NaCl, 50 mM HEPES, pH 6.8, 10% (vol/vol) glycerol, 0.2% (vol/vol) NP-40, 20 mM imidazole, 1 mM BME, and Complete protease inhibitor cocktail (Roche) on ice for 5 min. The lysate was cleared at $18,000 \times g$ for 45 min before incubating with 1 ml Ni-Sepharose excel beads (GE Healthcare) for 1.5 h. The beads were washed in $15 \times$ CV of 500 mM NaCl, 50 mM HEPES, pH 8, 10% (vol/vol) glycerol, 20 mM imidazole, and 1 mM BME and in $15 \times$ CV of 500 mM NaCl, 50 mM HEPES, pH 6, 10% (vol/vol) glycerol, 20 mM imidazole, and 1 mM BME. Nickel-bound protein was eluted in $20 \times$ CV of 40 mM NaCl, 50 mM HEPES, pH 6.8, 10% (vol/vol) glycerol, 500 mM imidazole, and 1 mM BME. The eluted OAS3 was diluted $2 \times$ in 25 mM HEPES, pH 6.8, 10% (vol/vol) glycerol, and 1 mM BME before loading onto a 5-ml HiTrap heparin column (GE Healthcare). Bound OAS3 was eluted in a linear gradient from 25 mM to 600 mM NaCl, 50 mM HEPES, pH 6.8, 10% (vol/vol) glycerol, and 1 mM BME. The fractions containing OAS3 were pooled, concentrated to 1.5 mg/ml in 1 ml, and further purified on a HiLoad 16/60 Superdex 200 (GE Healthcare) in 200 mM NaCl, 10 mM HEPES, pH 6.8, 5% (vol/vol) glycerol, and 1 mM BME. The fractions containing OAS3 were pooled and concentrated to 0.5 mg/ml before flash freezing in liquid nitrogen and storage at -80°C .

pOAS1 was expressed in *E. coli* BL21(DE3) (Novagen) and purified as described previously (36).

For the transient expression of WT OAS3 and mutants, 7.5×10^5 HEK293T cells per well were seeded in a 6-well plate in 1 ml medium. The constructs were transfected into the HEK293T cells using 2 μg DNA and 6 μg polyethylenimine (PEI). After 24 h of expression, the cells were lysed (in 500 μl per well) with 50 mM HEPES, pH 7.5, 10 mM MgCl_2 , 150 mM NaCl, 0.5% (vol/vol) NP-40, 10% (vol/vol) glycerol, 25 mM NaF, 1 mM NaVO_4 , and Complete protease inhibitor cocktail (Roche). The cleared lysate was gently stirred with 20 μl M2 magnetic anti-FLAG slurry equilibrated in lysis buffer (Sigma) overnight at 4°C . After incubation, the beads were washed in 2×1 ml 50 mM HEPES, pH 7.5, 10 mM MgCl_2 , 150 mM NaCl, and 10% (vol/vol) glycerol using a magnetic rack before being resuspended in 20 μl of the same buffer. Four- μl beads resuspended in wash buffer were used for an activity assay with a total volume of 20 μl .

OAS activity assay. The OAS proteins were diluted in OAS dilution buffer (50 mM NaCl, 25 mM HEPES, pH 6.8, 5% glycerol). The activity was measured by mixing 4 μl $5 \times$ OAS buffer (20 mM Tris-HCl, pH 7.5, 75 mM magnesium acetate, 1 mM dithiothreitol [DTT], 0.2 mM EDTA, pH 7.5, 0.5 mg/ml bovine serum albumin [BSA], and 10% [vol/vol] glycerol), 4 μl OAS protein in OAS dilution buffer, 4 μl poly(I-C) (synthetic dsRNA analog), 4 μl 4 mM ATP, pH 7.5, including 2.5 μCi α - ^{32}P -labeled ATP (PerkinElmer), and 4 μl water. Care was taken to use only the same batch of poly(I-C), as there can be batch-to-batch variation in the ability to activate OAS. The reaction mixture was incubated for 1 h at 37°C . Heating the mixture for 5 min at 95°C terminated the reaction. The ATP and the 2-5As were separated by thin-layer chromatography (TLC) on a PEI cellulose plate (Merck) run in 30 mM MgCl_2 and 0.4 M Tris-HCl, pH 8.6. The radioactive spots were visualized using a Phosphor screen (Molecular Dynamics) exposed to the TLC plate and quantified using a Typhoon PhosphorImager with Quantity One software (Bio-Rad). The background is estimated and subtracted using a control sample with OAS but without poly(I-C). Linear regression was used to estimate the linear interval of enzymatic rates. To estimate the dsRNA 50% effective concentration (EC_{50}) for the activation of OAS3 and pOAS1, the poly(I-C) concentration was transformed to logarithmic scale, and a nonlinear regression with the equation $V_{\text{max}}/[1 + 10^{(\log \text{EC}_{50} - [\text{dsRNA}])}] \times \text{exponential coefficient}$ was fitted to the data (37). The equation is used for log(dose) response relationships to determine the EC_{50} of the agonist. The exponential coefficient is used as a fitting parameter to account for the nonstandard slope. It is equivalent to the Hill coefficient used to describe allosteric activation, but we avoid this term, as the activation here is not strictly allosteric.

PAGE separation of 2-5As. Following an OAS activity assay, synthesized 2-5As were separated by PAGE on a 20% polyacrylamide and 7 M urea gel (20% acrylamide-bisacrylamide [19:1], 7 M urea, $1 \times$ Tris-borate-EDTA [TBE], 0.05% ammonium persulfate [APS], and 0.1% tetramethylethylenediamine [TEMED]) performed at 10 W for 165 min. The radioactive spots were visualized using a Phosphor Screen (Molecular Dynamics) exposed to the gel and quantified using a Typhoon PhosphorImager.

Assay of RNase L activity. Cell lines stably transfected with C-terminally FLAG-tagged hOAS1 and C-terminally FLAG-tagged OAS3 were generated using the Flp-In T-REx 293 system (Life Technologies) according to the manufacturer's protocol. A total of 8×10^5 cells were induced to express the stable transfected protein using 1 $\mu\text{g}/\text{ml}$ tetracycline for 24 h. The cells subsequently were either mock transfected or transfected with 8 μg poly(I-C) using Lipofectamine 2000 (Life Technologies) according to the manufacturer's protocol and incubated for 7.5 h. Total cellular RNA was extracted using the E.Z.N.A. total RNA kit 1 (Omega Biotek) according to the manufacturer's protocol. Five ng cellular RNA was separated using the Experion RNA HighSens analysis kit (Bio-Rad) according to the manufacturer's protocol.

Western blotting. Protein samples were separated by 8% SDS-PAGE. The proteins then were transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) and blocked with 5% milk in Tris-buffered saline (TBS) with 0.05% Tween 20 (TTBS) at room temperature for 1 h. The membrane was washed $3 \times$ for 5 min each in TTBS and blotted with rabbit polyclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; diluted 1:2,500) (sc-25778; Santa Cruz), mouse monoclonal anti-FLAG (diluted 1:5,000) (F3165; Sigma), mouse polyclonal anti-OAS3 antiserum (diluted 1:1,000) (kindly provided as a gift from Ara Hovanessian), or 0.5 $\mu\text{g}/\text{ml}$ rabbit polyclonal anti-OAS3, C terminus (AP6228a; Abgent), at 4°C overnight. The membrane was washed $3 \times$ for 5 min each in 1% milk in TTBS. The bound primary antibodies were detected using either a goat monoclonal anti-mouse horseradish peroxidase (HRP)-conjugated antibody (diluted 1:4,000) (Pierce) or a goat monoclonal anti-rabbit HRP-conjugated antibody (diluted 1:4,000) (Pierce) in 1% milk in TTBS at room temperature for 1 h. After secondary blotting, the membrane was washed $4 \times$ for 5 min each in TTBS before visualizing the antibody-bound proteins on Kodak film by the enhanced chemiluminescence system (Pierce).

Small-angle X-ray scattering. Synchrotron radiation small-angle X-ray scattering data were collected at the I911-4 beamline at MAX Lab (38), Lund, Sweden, at a wavelength of 0.91 $\text{\AA}$ using a Pilatus detector and 90-s exposure time. Solutions of OAS3 were measured at 25°C in the buffer used for size-exclusion chromatography at protein concentrations of 5 mg/ml, 2.5 mg/ml, and 1.25 mg/ml. The sample-to-detector distance was 2.058 m, covering a range of $0.08 < s < 5.0 \text{ nm}^{-1}$ [$s = 4\pi \sin(\theta)/\lambda$]. To avoid radiation damage, the sample was exposed in the oscillation mode. The data were processed with the ATSAS software package (39). Scattering from the buffer solution was subtracted using PRIMUS (40). Linear Guinier plots in the Guinier region ($s \times R_g < 1.3$, where R_g is radius of gyration) were determined. Analysis of $\log(I)/\log(s)$ plots (where I is scattering intensity) determined that no significant aggregation or repulsion was observed for the 5 mg/ml data set compared to the two lower-concentration data sets. Moreover, R_g and the maximum particle dimensions (D_{max}) were similar between the data sets. Subsequent analysis and modeling was performed using the 5 mg/ml data set. R_g and D_{max} were determined by GNOM (41) and AUTOPOROD (42). An ensemble of 12 low-resolution *ab initio* models were made using the *ab initio* bead modeling program DAMMIF (43). The representative DAMMIF model was obtained through DAMAVER (44), with an average normal spatial discrepancy (NSD) of 0.735, while only one model was discarded. The pdb2vol function of SITUS (45) was used to create a volumetric map of the representative model. A rigid-body model of OAS3 was generated from the experimental scattering of three molecules of pOAS1 (PDB entry 1PX5) to model the three OAS domains of OAS3 using SASREF (46). The crystal structure of pOAS1 was chosen for rigid-body modeling, as this structure

represents the inactive OAS form. Theoretical scattering data from pOAS1 was generated using CRY SOL (47). A second rigid-body model of OAS3 was generated using Bunch (46). As Bunch relies on high-resolution structures of domains of OAS3 and requires identical amino acid sequences between the high-resolution domains and the sequence of OAS3, homology models of the individual OAS domains of OAS3 were generated using Modeler (48). The models of the individual OAS domains of OAS3 (OAS3_D1, OAS3_D2, and OAS3_D3) were based on the crystal structure of pOAS1 (PDB entry 1PX5). Furthermore, Bunch requires linkers between the high-resolution domains; therefore, the Modeler-derived model of OAS3_D2 was truncated by 6 amino acids to allow for flexibility between the second and third domains. These 6 amino acids instead served as a linker. Docking of the rigid-body models obtained from SASREF and Bunch into the representative DAMMIF-derived *ab initio* shape envelope was performed by SUPCOMB (49), yielding NSD values of 1.00 and 1.01, respectively. Figures of the docked rigid-body models in the SITUS-generated volumetric map were made using UCSF Chimera (50). An analysis of the interdomain flexibility was conducted using the ensemble optimization method (EOM) (51). EOM was fed the Modeler-derived homology models of OAS domains 1, 2, and 3 of OAS3. OAS3_D2 was truncated by six amino acids that instead served as a linker between domains 2 and 3. The EOM algorithm creates an initial ensemble of OAS3 with random conformations of the domains relative to each other from the homology-modeled OAS domains of OAS3. An ensemble is selected from the initial pool if the computed theoretical scattering of that ensemble fits the experimental scattering. If the radius of gyration distribution in the selected ensembles are as broad as the R_g in the initial ensemble pool, the protein likely is flexible. In contrast, a narrow R_g distribution indicates that the protein is rigid.

RESULTS

OAS3 expression and purification. Codon-optimized OAS3 with an N-terminal 6× His tag and a total mass of 124 kDa was expressed using the baculovirus system in High Five insect cells. Insects do not have OAS proteins; therefore, contamination with endogenous OAS is not an issue. Following immobilized metal ion affinity chromatography and heparin affinity chromatography, OAS3 was purified to homogeneity by size exclusion chromatography (Fig. 1B and C). OAS3 eluted at ~70 ml, corresponding in mass to a globular protein of ~160 kDa (Fig. 1B). The increase in apparent size suggests that OAS3 is elongated. pOAS1 was purified to homogeneity by established protocols (Fig. 1C) (36). In this study, we compared the enzymatic properties of human OAS3 to those of pOAS1, which shares 75% sequence identity to hOAS1.

OAS3 is activated at a substantially lower concentration of poly(I·C) than pOAS1. To establish the linear range of the 2-5A synthetase activity in the assay used, 2-5A production was measured at a saturating concentration of poly(I·C) and plotted against protein concentration for pOAS1 and OAS3 (Fig. 2A). The measuring points included in the linear regression analysis in order to obtain the best fit were determined manually. The linear fit was used to determine k_{cat} values of 9 s^{-1} and 13 s^{-1} for pOAS1 and OAS3, respectively. As 2 nM OAS3 and 6 nM pOAS1 are at the center of the linear intervals, those were selected as the enzyme concentrations used to examine the sensitivity of OAS3 to dsRNA. Enzymatic activities of OAS3 and pOAS1 were plotted against the poly(I·C) concentration, and a nonlinear dose-response curve was fitted to the measuring points (Fig. 2B). Remarkably, the calculated EC_{50} s indicate that OAS3 is on the order of 1,000 to 10,000 times more sensitive to poly(I·C) than pOAS1 (Table 1).

OAS3 synthesizes longer 2-5As than pOAS1 that can activate RNase L. To investigate the length of the 2-5As synthesized by OAS3 and pOAS1, we performed activity assays at increasing OAS concentrations but within the linear range. The synthesized 2-5As subsequently were separated by 20% PAGE. Surprisingly, OAS3 synthesizes longer 2-5As than pOAS1 at comparable or even lower levels of activity than pOAS1 (Fig. 3A). Furthermore, OAS3 seems to have the ability to produce long 2-5As having seven or more linked ATP molecules. pOAS1 does not readily synthesize these long 2-5As even when using a large amount of pOAS1, leading to activity outside the linear range (Fig. 3A, leftmost lane). Quantifying the relative amounts of the individual 2-5A species at the similar enzymatic rate of ~0.015 nmol 2-5A per min shows that OAS3 synthesized 71% $p_3A(2'p5'A)_2$, 19% $p_3A(2'p5'A)_3$, and 10% $p_3A(2'p5'A)_{4-12}$, while pOAS1 synthesized 96% $p_3A(2'p5'A)_2$ and 4% $p_3A(2'p5'A)_3$ but negligible $p_3A(2'p5'A)_{4-12}$ oligomer species (Fig. 3B). To verify that OAS3 can synthesize the 2-5As required to activate RNase L intracellularly, we generated Flp-In T-REx 293 cell lines that were mock transfected or stably transfected with C-terminally FLAG-tagged hOAS1 or C-terminally FLAG-tagged OAS3. The expression of the proteins was induced, and the cells were mock transfected or transfected with poly(I·C). Assaying the integrity of 28S rRNA and 18S rRNA clearly demonstrates that OAS3 activates RNase L at levels at least comparable to those of hOAS1 (Fig. 3C).

OAS3 has one active site located in the C-terminal OAS domain. To verify the putative location of the active site in OAS3, several mutants were generated and expressed. The activity of each OAS3 mutant was assayed and quantified, and if the displayed activity was less than 1% compared to the wild type, the mutant was considered inactive. The double mutant D816A/D818A and the truncated R844X were expressed without a tag for affinity purification in S2 cells and purified by a single step of cation exchange chromatography. Even at a high concentration of approximately 20 nM, D816A/D818A showed no activity, confirming that OAS3 harbors one active site located in the C-terminal OAS domain (Fig. 4A). The expression and purification of R844X proved difficult, presumably due to the improper folding of the protein (Fig. 4B). Using the maximum amount of protein possible, corresponding to approximately 5 nM R844X, no activity was observed for R844X (Fig. 4A).

To identify amino acids crucial for catalytic activity, WT OAS3 and several mutants carrying a FLAG tag were transiently expressed in HEK293 cells and purified by immunoprecipitation. The double mutants D74A/E76A and D473A/E475A carry mutations in the residues corresponding to two of the three aspartic acids of the putative catalytic triads in the first (OAS3_D1) and second (OAS3_D2) OAS domain (Fig. 1A). Both D74A/E76A and D473A/475A displayed activity, confirming that 2-5A synthesis is not severely affected by these mutations (Fig. 4C). To map the third aspartic acid residue of the catalytic triad, the activity of D886A, D888A, and D886A/D888A was investigated. Only D886A was active (Fig. 4C), confirming that D888 is the third aspartic acid residue in the catalytic triad, as predicted by our alignment (Fig. 1A). K207, K606, and K950 correspond to K212 in pOAS1 and to K213 in hOAS1 (Fig. 1A), which are suggested to interact with the phosphate of the incoming ATP (21). K207A, K606A, and K950A were assayed for activity; however, only K950 lost the ability to synthesize 2-5As (Fig. 4C), again agreeing with one active site in OAS3_D3. Homogenous WT OAS3 and transiently ex-

FIG 2 Determination of the linear enzymatic rate and sensitivity toward dsRNA for pOAS1 and OAS3. (A) Enzymatic activities were investigated for pOAS1 and OAS3 and plotted against the protein concentration. The enzymatic rate is displayed as the synthesis of 2-5A in nmol per min. The cutouts show an enlarged section of the linear interval of enzymatic rates with the best linear regression. (B) Sensitivity toward dsRNA was investigated using fixed concentrations of 2 nM OAS3 (circles) and 6 nM pOAS1 (squares) plotted against increasing amounts of poly(I:C). The experimental data were analyzed by nonlinear regression using the model indicated in Materials and Methods.

pressed immunoprecipitated WT OAS3 were used as positive controls. The activity without OAS and with empty vector served as negative controls as well as for background estimation. Western blotting verified that all mutants had been expressed and immunoprecipitated (Fig. 4D).

Small-angle X-ray scattering reveals an elongated conformation of OAS3. To determine the overall domain structure of OAS3, a low-resolution model was created using small-angle X-

ray scattering (SAXS). The experimental scattering is presented in Fig. 5A. The radius of gyration and $D_{\max}$ were determined to be $47 \pm 0.26 \text{ \AA}$ and $155 \text{ \AA}$, respectively. This suggests an elongated structure of OAS3. The pair-distance distribution function, $P(r)$, displays a small shoulder peak at greater distances relative to the major peak and is tailing at long distances, indicating that OAS3 has a nonspherical, elongated shape (Fig. 5B). Both an *ab initio* dummy atom model and two rigid-body models were generated from the SAXS data to independently determine the low-resolution structure of OAS3. The DAMMIF-generated *ab initio* model provides a fit to the experimental data with $\chi = 1.04$. The SASREF-derived rigid-body model provides a fit to the experimental data with $\chi = 1.66$. The rigid-body model obtained through Bunch provides a very good fit to the experimental data with $\chi = 1.06$. The computed scattering curves are compared to the experimental data in Fig. 5A. The rigid-body models were docked into the envelope of the representative DAMMIF-gener-

TABLE 1 OAS3 and pOAS1 sensitivity toward dsRNA ^a			
OAS	EC ₅₀ (μg/ml) (95% CI)	Exponential coefficient ^b	R ²
OAS3	0.0026 (0.0021–0.0033)	2 (±0.4)	0.94
pOAS1	23 (14–38)	1.4 (±0.3)	0.99

^a The EC₅₀ and 95% confidence interval (CI), exponential coefficient, and R² values were estimated from the fit shown in Fig. 2B.
^b Standard errors are given in parentheses.

FIG 3 Lengths of 2-5As synthesized by OAS3 and pOAS1 and their capability in activating RNase L. (A) Radiogram of a 20% PAGE showing the lengths of 2-5As synthesized by OAS3 and pOAS1 at various levels of enzymatic activity. Enzymatic activity levels are displayed as synthesized 2-5A in nmol per min for each lane. ATP and 2-5As of various lengths are depicted. The concentration of pOAS1 in the outermost left lane is 48 nM. The concentration of pOAS1, from left to right, is 2.4 nM, 3.6 nM, 4.8 nM, and 6 nM. The concentration of OAS3, from left to right, is 0.8 nM, 1.2 nM, 1.6 nM, and 2 nM. The OAS proteins were activated with 100 μ g/ml poly(I:C) for 1 h. (B) Quantification of the 2-5A species synthesized at similar enzymatic activity for OAS3 (0.013 nmol/min) and pOAS1 (0.015 nmol/min). (C) RNase L activity was determined by assaying the integrity of 28S and 18S rRNA. Flp-In T-REx 293 cells stably transfected with hOAS1-FLAG or OAS3-FLAG were either mock transfected or transfected with poly(I:C), and cellular RNA extracts were separated using the Experion HighSens RNA analysis kit. Comparable expression levels of hOAS1-FLAG and OAS3-FLAG were confirmed by Western blot analysis on cell lysates using antibodies against FLAG and GAPDH.

ated *ab initio* model, with NSD values of 1.01 and 1.00 for the Bunch- and SASREF-derived rigid-body models, respectively (Fig. 5D and E). The *ab initio* shape envelope does not formally indicate orientation. As a result, the docking of the SASREF- and Bunch-generated rigid-body models in the *ab initio* shape envelope yielded two opposite facing models in the *ab initio* shape envelope model (Fig. 5D and E). The flexibility allowed by Bunch generates a rigid-body model that adopts a more open conformation that fits the *ab initio* shape envelope well compared to the inflexible SASREF-generated rigid-body model. The inflexible SASREF-generated rigid-body model does not account for all *ab initio* shape density, which suggests some flexibility in OAS3 (Fig. 5E). To analyze the interdomain flexibility, we utilized EOM. Interestingly, the EOM analysis yields a bimodal R_g distribution with the highest peak in the lower R_g ranges, indicating that OAS3 primarily adopts a rigid conformation (Fig. 5C). However, there is a second, smaller peak in the upper R_g distribution indicating a flexible protein (Fig. 5C). This suggests that OAS3 adopts two conformations in solution.

DISCUSSION

We purified OAS3 to homogeneity for biochemical analysis. The measured k_{cat} values of 13 s^{-1} and 9 s^{-1} for OAS3 and pOAS1, respectively, are comparable within the sensitivity of the OAS activity assay (Table 1) and are comparable to those of previously published studies (21). We demonstrate that OAS3 is between 1,000 and 10,000 times more sensitive to dsRNA than pOAS1 (Fig. 2B and Table 1). This is in agreement with previous findings (30, 31). However, in contrast to previous reports, we show that OAS3 readily synthesizes 2-5As of three or more linked ATPs even within the linear range of activity (Fig. 3A and B) (30, 32, 33). There are several noteworthy differences between earlier studies and ours. We purified OAS3 to homogeneity, while previous studies used immunoprecipitated OAS3 immobilized on beads. Using immobilized OAS3 makes it difficult to control the amount and purity of OAS3 used during the assays.

The OAS enzymes work by a nonprocessive elongation mech-

FIG 4 Mapping crucial amino acids for enzymatic activity in OAS3. (A) TLC showing the activity of 2 nM OAS3 WT, 2 nM OAS3 WT without (w/o) poly(I-C), ~20 nM D816A/D818A, and ~5 nM R844X. The OAS3 WT was purified to homogeneity, while D816A/D818A and R844X were purified by one-step cation chromatography. ATP and 2-5A species are indicated. The activity is indicated under each lane by a plus sign for active or a minus sign for inactive (less than 1% activity compared to the wild type). (B) A Western blot confirming the presence of OAS3 WT, D816A/D818A, and R844X assessed for activity as described for panel A. (C) TLC showing the activity of homogenous OAS3 and several immunoprecipitated mutants. ATP and 2-5A species are indicated. The activity is indicated under each lane by a plus sign for active or a minus sign for inactive (less than 1% activity compared to the wild type). (D) A Western blot confirming the presence of the immunoprecipitated OAS3 mutants.

anism (52, 53), which means that the length of the oligomers synthesized strongly depends on the total enzyme activity and the ATP concentration used in the assay. The latter is due to the fact that ATP will compete with the previously formed 2-5As for elongation. This means that high ATP concentration and low enzymatic activity will favor the formation of dimeric 2-5As, and the opposite will favor the formation of longer 2-5A oligomers. For the reasons just mentioned, we took care to compare the length of 2-5As synthesized by OAS3 and pOAS1 at similar levels of activity and within the linear range of activity. In doing so, we conclude that OAS3 is capable of synthesizing the trimeric 2-5A required for RNase L activation. Furthermore, we demonstrate that OAS3 synthesizes the 2-5As required for RNase L activation intracellularly (Fig. 3C).

The production of longer 2-5A species has been observed previously for OAS2 (53). We observed that OAS3 produces minor amounts of 2-5As of 7 to 11 linked ATPs (Fig. 3A and B). It is difficult to explain the production of these long 2-5As by a strictly nonprocessive elongation mechanism. It remains to be clarified if OAS2 and OAS3 can change to a processive mode when the growing 2-5A reaches a certain length, as is observed for other poly-

merases (54). Nevertheless, it is quite possible that these longer oligomers (7 or more in length) are formed *in vitro* only at high enzyme concentrations. Taken together with the low concentration of dsRNA needed for OAS3 activation compared to that needed for activation of pOAS1 and OAS2 (53), it seems likely that OAS3 is a potent activator of RNase L during viral infections *in vivo*.

Through mutational studies we have mapped the active site of OAS3 to the C-terminal OAS domain. The R844X truncated form of OAS3 displaying an allele frequency of 0.5% in the Caucasian population (34) is incapable of producing 2-5As (Fig. 4A and B). Our data clearly demonstrate that the two first OAS domains in OAS3 are not playing a crucial role in 2-5A synthesis. However, the alignment suggests that these two domains have retained affinity toward dsRNA through the conservation of some of the RNA binding residues (Fig. 1A). It is likely that the high affinity of OAS3 toward long dsRNA, like poly(I-C), is caused by avidity where multiple OAS domains contribute simultaneously to the binding. This might explain the higher sensitivity toward dsRNA displayed by OAS3 compared to pOAS1 when using poly(I-C) as an activator.

FIG 5 Overview, analysis, and modeling of SAXS data of OAS3. (A) Experimental scattering from OAS3 (circles) and theoretical scattering curves of *ab initio* modeling by DAMMIF (continuous orange line) and rigid-body modeling by SASREF (continuous red line) and Bunch (continuous blue line). The inset shows the Guinier region. (B) $P(r)$ function for OAS3 computed from scattering patterns using GNOM. (C) Radius of gyration distribution of initial ensemble pool (purple line) and selected structures (black line) using EOM. (D) Docking of the rigid-body model obtained from Bunch to the representative DAMMIF-derived *ab initio* model shape envelope. (E) Docking of the rigid-body model obtained by SASREF to the representative DAMMIF-derived *ab initio* model shape envelope.

Our SAXS analysis of OAS3 and the *ab initio* and rigid-body models show that OAS3 adopts an elongated conformation consistent with the elution volume observed with size exclusion chromatography (Fig. 5 and 1B). The DAMMIF-generated *ab initio*

dummy atom model and the Bunch-derived rigid-body model provide the best prediction of the experimental scattering (Fig. 5A and D). The EOM analysis indicates that OAS3 primarily adopts a rigid conformation in solution; however, some degree of flexibil-

ity in OAS3 also is indicated, which most likely is due to the linker between the first and second OAS domains (Fig. 5C and 1A). This flexibility between the first two OAS domains might explain the unaccounted-for shape density in the N terminus of the DAMMIF-generated *ab initio* model when docking in the SASREF-derived rigid-body model (Fig. 5E).

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