Single-molecule FRET supports the two-state model of Argonaute action

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Introduction

Prokaryotes and eukaryotes have developed distinct antiviral defense mechanisms using small RNAs for the recognition of invading nucleic acids. While the CRISPR/cas system is in place in bacteria and archaea,¹ the evolutionary unrelated RNA interference (RNAi) mechanism can be found in eukaryotes.² In addition to antiviral defense, RNAi is a critical mechanism for cell differentiation as it controls cellular transcript and protein levels. At the functional core of the eukaryotic RNA-silencing pathway, a member of the Argonaute (Ago) family can be found.³ This key protein binds small non-coding RNAs (20–30 nucleotides in length) that direct the silencing machinery to the target mRNA, which subsequently leads to Ago-catalyzed degradation of the mRNA or to translational inhibition.⁴ Four Ago proteins are present in human cells but Ago2 is the only catalytically active form. Initially, Ago2 is loaded with double-stranded RNA followed by the ejection of one strand (passenger strand) leaving the counter strand (guide strand) protein-bound.⁵⁻⁸ Owing to the sequence complementarity between the guide strand and a mRNA (target strand), Ago recognizes and cleaves the target mRNA.

Interestingly, homologs of Ago are also present in bacteria and archaea, but their physiological role is still unknown.⁹,¹⁰ Nevertheless, the bacterial and archaean homologs have been instrumental to unravel Ago structure and function. The first Ago structure published was that of an archaean homolog isolated from Pyrococcus furiosus.⁸ Since then, structures of Ago derived from bacterial,¹¹,¹² archaean,⁸,¹³ and recently from eukaryotic sources¹⁴⁻¹⁶ have been solved and show a high degree of similarity.¹⁷,¹⁸ The protein is composed of an N-terminal domain, a PAZ, middle (MID), and PIWI domain (Fig. 1A), which are divided into two lobes. The PAZ and N-terminal domain form one and the PIWI and MID domain form the other lobe. The PAZ domain is responsible for the binding of the 3’ terminus of the guide strand, and the guide strand in complex with Ago forms the binary complex. Accommodation of a target strand leads to the formation of the ternary complex. The 5’ end of the guide strand is buried in a deep pocket at the interface between the MID and PIWI domain. Furthermore, in the prokaryotic structure, the phosphate at the 5’ end is bound to a magnesium-ion that is coordinated by the protein’s C terminus. The protein contacts the guide strand mainly via its phosphate-sugar backbone. Biochemical and mutational studies confirmed that the PAZ domain, which adopts a typical RNaseH fold, harbors the catalytic site.¹⁹,²⁰ Like many other nucleases that cleave both RNA and DNA, a divalent cation-binding motif DDX (aspartate, aspartate, X being a glutamate, aspartate, or histidine) can be found in the PIWI domain. A recent crystallographic study of a ternary complex composed of Ago, a 21 nucleotide (nt) guide DNA, and a 20 nt target RNA, uncovered the dynamic nature of the protein.²¹

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conformation in which the N-terminal and PAZ domains are rotated away from the MID-PIWI domains to accommodate the target RNA. The rotation of the PAZ domain leads to the release of the 3′ end of the guide strand from the PAZ domain, which in turn allows the correct positioning of the cleavage site in the RNA toward the catalytic residues in the PIWI domain. Despite those structural insights, a description of the dynamic behavior of the Ago PAZ-domain and the loaded nucleic acid strands remains elusive.

In our single-molecule fluorescence resonance energy transfer (smFRET) study, the Ago protein from the hyperthermophilic archaeal organism *Methanocaldococcus jannaschii* (MjAgo) served as a model system to test the dynamic rearrangement of the nucleic acids bound by MjAgo. We probe the interplay between Ago and nucleic acids using fluorescent dyes as steric and conformational reporters. Applying this probing technique, we followed structural rearrangements of the protein scaffold and the bound nucleic acids when propagating from the binary to the ternary complex. We show that MjAgo is catalytically active at elevated temperatures and exclusively processes DNA target strands out of DNA hybrids. Our homology model of MjAgo served as guide for the rational selection of suitable labeling sites for an acceptor fluorophore in the protein. Using a donor-labeled guide strand, fluorescence resonance energy transfer between donor and acceptor dye was monitored upon binary and ternary complex formation. Proceeding from the binary to ternary complex, we monitored the conformational changes between nucleotide 18 and 13 of the guide strand relative to the N-terminal, PAZ, and MID domain. Our results show that the 3′ end of the guide DNA is released from the PAZ domain once the target strand of 85 °C. Therefore, the recombinant archaeal MjAgo could easily be isolated from the bacterial proteome by simple heat denaturation of the cellular extract. The protein was heat stable up to 85 °C leaving almost exclusively the archaeal protein in the soluble fraction. The heat stability of the protein indicates that the protein adopts its natural fold. An additional affinity purification step resulted in homogenous protein preparations (Fig. S1).

**Structural model of the Argonaute protein from *M. jannaschii***

The MjAgo protein is a prokaryotic homolog of the human Ago2 protein (23% sequence identity), which is an integral part of the human RISC machinery. However, MjAgo shares a higher degree of similarity with the archaeal Ago protein from *Pyrococcus furiosus* (PfAgo, 28% sequence identity). The structure of PfAgo is available, and therefore, we built a homology model of MjAgo using PfAgo as the template structure (Fig. 1B) based on a comprehensive sequence alignment (Fig. S2). A structural alignment of MjAgo with PfAgo (Fig. 1B) with a target-template Co-root mean square deviation of 1.45 Å as well as the modeling statistics (Table S2) show the similarity between the two structures and demonstrate that MjAgo adopts a domain organization and topology typical for the Ago protein family. In comparison to the human Ago2, structural differences can be found especially in the relative domain organization of the N-terminal and PAZ domain (Fig. S3). Furthermore, we used the homology model to choose suitable sites for fluorophore derivatization for the single-molecule experiments. We selected a position in the N-terminal domain (N76), PAZ domain (S221), and a position in the structural rigid MID domain (I410) for the attachment of fluorescent dyes. All three positions are surface exposed ensuring incorporation of the target strand.

**Results**

Cloning, expression, and purification of *M. jannaschii* Ago (MJ_1234)

According to phylogenetic studies, MjAgo belongs to the PAZ-containing prokaryoticagos and encodes the conserved DDD motif making it highly likely that this protein is catalytically active. The protein spans over 713 amino acids yielding a molecular weight of 84.5 kDa. The gene (GI: 1452130) corresponding to the hypothetical Ago protein (MJ_1234) from *M. jannaschii* could be successfully cloned from genomic DNA extracts into conventional expression vectors. Using this cloning strategy, the protein of interest is tagged with a 6xHis tag at its N or C terminus, which allows purification via Ni-NTA matrices. *M. jannaschii* is a hyperthermophilic organism with an optimal growth temperature

![Figure 1. Homology model of Argonaute from *Methanocaldococcus jannaschii*. (A) The protein is composed of an N-terminal domain (blue), a Piwi-Agoonaute-Wzille (PAZ) domain (salmon), a MID domain (orange), and a PIWI domain (green). (B) Homology model of *M. jannaschii* Argonaute using Argonaute from *P. furiosus* (PDB: 1Z25) as the template structure. Fluorophore modification sites at position Asparagine 76, Serine 221, and Isoleucine 410 are highlighted in red. (C) Structural alignment of the *M. jannaschii* model (colored in cyan) with the structure of *P. furiosus* Argonaute (domain coloring according to panel A). The target-template Co-root mean square deviation is 1.45 Å. The coordinated metal ion is highlighted as a pink sphere. The arrow indicates the outward movement of the PAZ and N-terminal domain of Argonaute upon loading of the target strand. See also Figures S2 and S3 and Table S2.](image-url)
sterical accessibility of the dye for the coupling reaction. In order to prevent any structural changes in the protein, we carefully selected amino acids that are located in disordered loop regions and are not highly conserved.

*M. jannaschii* Ago binds short nucleic acids in vitro

Prokaryotic Ago proteins have been shown to bind DNA guide strands with high affinity. We performed binding studies using fluorescently labeled DNA oligonucleotides employing gel mobility shift assays (EMSA) in order to test the binding capacity and specificity of MjAgo (Fig. 2). First, we studied whether MjAgo binds short DNA oligonucleotides. The sequence of the 21 nt guide strand used was derived from the human miRNA hsalet-7a-5p and carried a 5'-phosphate. The fluorescently labeled guide strand was bound in a concentration-dependent manner yielding a heparin-stable complex (Fig. S4). In order to rule out a strong sequence dependency of the binding efficiency, we tested alternative short DNA sequences that yielded comparable results with respect to binding efficiency (data not shown).

In order to identify suitable dye modification positions in the DNA for the smFRET experiments that still allow efficient binary and ternary complex formation, we tested different dye attachment points in the guide DNA. The fluorescent dye is a bulky moiety attached to the nucleobase, and hence, represents a sterical block. Consequently, structurally confined binding pockets and conserved interaction sites between the protein scaffold and the DNA should not tolerate an additional volume leading to sterical clashes between the dye and the protein. In contrast, flexible and less specifically bound stretches of the DNA should not be affected by the introduction of the dye. For example, the additional volume introduced by the organic dye Alexa488 amounts to 718 Å³ (considering the dye as a rigid molecule). We chose four different attachment points (nucleotide 13, 14, 18, and the 3’ end) for the organic dye Atto550 in the single-stranded guide DNA (for an overview of the oligonucleotides used in this study see Table S1), and determined the relative binding efficiency. Structural studies showed that the 3’ end of the guide strand

![Figure 2](image_url)

**Figure 2.** Interaction of *M. jannaschii* Argonaute with single-stranded and double-stranded DNA. (A) Complex formation between MjAgo (0, 1, 3, 5 µM) and guide strands (333 nM) that vary in the dye attachment position (nucleotide 13, 14, 18). (B) Quantification of the bound DNA fraction as a function of dye position and MjAgo concentration. The numbers reflect the DNA amount bound as compared with amount of DNA bound by 5 µM MjAgo using the labeled DNA guide at position 18 (set to 1). Data are mean ± SD of at least three independent experiments. See also Figures S4 and S5. (C) EMSA showing the complex formation between MjAgo (2.8 µM) and the DNAguide strand, DNAguide/RNAtarget, and DNAguide/DNAguide labeled with the fluorescent dye Atto550 at different positions of the guide strand (13 and 18). In addition a dsDNA carrying a mismatch at position 10–11 has been included in the experiment (denoted as m18). (D) EMSA showing the complex formation between MjAgo (2.8 µM) and the DNAguide/RNAtarget and DNAguide/DNAguide labeled with the fluorescent dye Atto550 at the 3’ end of the guide strand. All nucleic acids have been used at a concentration of 333 nM. Complex formation with dsDNA labeled at position 13 and single-stranded DNA is loaded for comparison. (E) EMSA showing the complex formation between MjAgo (2.8 µM) and ssDNAguide, ssDNAtarget, or dual labeled dsDNA (guide: Atto550 modification at position 18, target: Alexa647 modification at position 17). Atto550 fluorescence is shown in green, Alexa647 fluorescence in red and co-localization of Atto550 and Alexa647 fluorescence results in a yellow color. All nucleic acids have been used at a concentration of 333 nM in the EMSAs shown in this figure.
is buried in a binding pocket located in the PAZ domain where the nucleotide only loosely interacts with the protein scaffold.\textsuperscript{12} The electron density for positions 13 and 14 is not resolved in this structure. As the phosphate at the 5’ end is crucial for the recognition of the 5’ end by the MID and PIWI domains, coupling of the dye to the 5’ end was avoided. Our data clearly show that the positioning of the label at the 3’ end prevents an efficient binding of the DNA guide by MjAgo, indicating that the 3’ binding pocket in the PAZ domain is sterically restricted and an additional volume cannot be accommodated (Fig. 2B and D; Fig. S5C). Moving the label to position 13, 14, or 18 increases the total amount of bound DNA (Fig. 2B). If the dye is attached to nucleotide 18 the most efficient binding is observed, leading to a nearly 2-fold increased relative binding efficiency as compared with the attachment at nucleotide 13 or 14.

Furthermore, we tested whether the fluorescent label leads to an unspecific interaction between protein and oligonucleotide. By swapping Atto550 (a rhodamine dye) with Alexa647 (a cyanine dye), a complete change in dye chemistry was achieved but the affinity of the labeled oligonucleotides was unaltered (Fig. S5). This result indicates that the fluorophore does not exert any influence on the protein–oligonucleotide interaction. Also, the hexahistidine-tag did not influence the guide DNA binding efficiency (Fig. S5).

Taken together, we present a fluorescence-based tool to probe the sensitive interaction sites between the Ago protein scaffold and the DNA guide. Our studies are in very good agreement with structural and functional evidence of prokaryotic Ago proteins suggesting that MjAgo is a functional member of the Ago family.

Next, we investigated whether MjAgo efficiently binds double-stranded nucleic acids. We formed ternary complexes using double-stranded DNA or a DNA\text/_RNA hybrid as binding partner. We found that MjAgo readily binds DNA/RNA hybrids and dsDNA (Fig. 2C). As shown in Figure 2C, the ternary complex can be clearly distinguished from the binary complex in a native gel with the ternary complex migrating slower than the binary complex. Interestingly, the binary complex is detectable even though the hybridization was performed with a 2-fold excess of target strand to ensure saturation of the guide strand. Providing a 10-fold excess of the target strand did reduce the amount of binary complex formed but no complete shift toward the ternary complex could be observed (data not shown). In order to find out whether the two detected bands are two different isoforms of the ternary complex or whether the lower band represents residual binary
complex, we incubated MjAgo with the guide strand carrying the Atto550 label and a target strand modified with Alexa647. The single-stranded target DNA did not associate with MjAgo but efficient complex formation was observed for the dual-labeled dsDNA (Fig. 2E). The fluorescence signal of both guide and target strand was found in the upper band while the lower band emitted the donor fluorescence only, and thus, represents a genuine binary complex. Binding of ssRNA, dsRNA, and RNAguide/DNAtarget hybrids to MjAgo was observed to be much less efficient than the DNA counterparts (data not shown).

Furthermore, the fluorescent probing allowed us to follow the arrangement of the complex in the ternary complex. The dsDNA carrying a label at the 3′ end of the guide strand is found in complex with MjAgo, which is not detectable for the ssDNA even though binding appears to be less efficient as compared with the dsDNA labeled at position 13 (Fig. 2D). This result suggests that in case of the dsDNA, the fluorophore at the 3′ end of the guide is not interfering with the protein–DNAguide interaction anymore.

Characterization of the catalytic activity of M. jannaschii Ago

Bioinformatic analysis classified MjAgo as catalytic competent Ago variant. In order to assess the catalytic activity of MjAgo, we performed cleavage assays. Here, a fluorescently labeled target strand was used (Fig. 3A) and the resulting cleavage products were separated on a denaturing sequencing gel. We first tested which nucleic acid type is utilized as substrate by MjAgo (Fig. 3B). Our data showed that MjAgo exclusively cleaves DNA strands out of a DNA/DNA hybrid. RNA target strands are not cleaved irrespective of the guide strand chemistry. Similarly, the DNA target strand is not cleaved if a RNA guide strand was used. The cleavage reaction occurred in a concentration-dependent, time-dependent, and temperature-dependent manner (Fig. 3C; Fig. S6). Efficient cleavage is found at temperatures above 75 °C. Target DNA cleavage did not occur if bivalent cations were removed by the addition of EDTA, demonstrating that MjAgo, like all other Agos characterized so far, requires bivalent cations for its catalytic reaction (Fig. 3D). The target strand was not cleaved in the absence of the DNA guide strand (Fig. 3D). Furthermore, using a 10 nt DNA guide did not mediate cleavage of the target strand, which might also be a result of the extreme instability of short duplexes at elevated temperatures required for MjAgo activity (Fig. S6). Interestingly, cleavage of the target strand by MjAgo yielded multiple products. Two major and two minor products were found corresponding to cleavage sites between nucleotide 9/10, 10/11, 13/14, and 14/15 (as counted from the 5′ end of the guide strand). In order to verify the specificity of the cleavage reaction, we additionally used a substrate that carried a tandem mismatch at nucleotide positions 10 and 11 (“mismatch target”) and a DNA target 41 nt in length (Fig. 3B; Fig. S7). The “mismatch substrate” was not utilized by MjAgo and no cleavage occurred at any of the cleavage sites (Fig. 3B). We tested the mismatch duplex in binding
Figure 5. Conformational changes between position 13 in the guide strand and the MjAgo domains on proceeding from the binary to the ternary complex. FRET efficiency histograms for binary (first row) and ternary complexes (second row) containing a guide strand labeled at nucleotide 13 with Atto550 and MjAgo modified with the acceptor dye DyLight650 either in the PAZ domain (S221AzF, right column) or the MID domain (N76AzF, left column). The histograms were fitted to a single or double-Gaussian model to determine the mean FRET efficiency (red line: sum of Gaussians). Data analysis as in Figure 5.

We also tested the Atto550-modified guide strands in the cleavage reactions. Here, the Atto550 label was used as a Lester probe again and the cleavage products were analyzed via the Alexa647 label attached to the target strand as before (Fig. S7). Incorporation of Atto550 into the guide strand at position 13, 14, and 18 did not lead to a decrease in cleavage efficiency as compared with a non-modified guide strand, while the attachment of Atto550 to the 3′ end led to a clearly reduced product formation. This is in agreement with the binding data that showed that the 3′ end-labeled DNA guide strand is not efficiently bound by MjAgo. It is noteworthy that the usage of the guide strand labeled at position 13 caused a slightly different cleavage pattern with the majority of the DNA cleaved at position 14/15.

Conformational changes on progressing from the binary to the ternary complex

Being able to probe the interaction of the DNA guide strand with MjAgo in the binary and ternary complex using fluorescent reporters, we next aimed to monitor the structural changes in the complex when proceeding from the binary to the ternary complex. Here, we performed smFRET experiments, a powerful tool to study conformational changes within heterogeneous populations as it can report on changes of intra- and intermolecular distance changes.26 In our dual-color smFRET experiments, the acceptor fluorophore DyLight650 is site-specifically introduced in the protein chain at chosen positions (Fig. 1) and the donor fluorophore Atto550 resides within the DNA guide. As the MjAgo protein contains six potentially functional important cysteine residues, we developed an alternative labeling strategy to site-specifically introduce a fluorescent probe into the protein. We engineered the unnatural amino acid p-Azidophenylalanine (AzF) into the N-terminal domain, the flexible PAZ domain, or the MID domain (amino acid position 76, 221, and 410, respectively; Fig. 1B) of MjAgo using an amber suppression strategy.27 This allowed the site-directed modification of the protein via the unique azide group of the unnatural amino acid with fluorescent dyes via the Staudinger ligation,28 irrespective of the number and position of the multiple intrinsic cysteines encoded in Ago with labeling efficiencies of 30–40% (Fig. S8). MjAgo that does not contain an unnatural amino acid was not labeled, indicating the specificity of the labeling reaction (Fig. S8A and B). The introduction of the mutations did not abrogate the functionality of the protein as demonstrated by efficient binary and ternary complex formation (Fig. S8C and D). Subsequent to protein labeling, either the binary or ternary complex was formed and the excess of fluorophore as well as the excess of DNA was removed from the protein–nucleic acid complex by size-exclusion chromatography. We employed confocal microscopy with alternating laser excitation to detect the MjAgo–nucleic acid complexes as this method allows for the sorting of freely diffusing molecules according to two parameters, their dye stoichiometry (expressed in the S value), and FRET efficiency (expressed in the E value)29,30 (Fig. S9). This enables the extraction of information about the FRET efficiency for the dual-labeled complexes even in the presence of a large background of fluorescing molecules that do not show FRET. Furthermore, this method provides the opportunity to analyze heterogeneous samples containing species with different conformations, information that is lost in standard ensemble assays.

Forming the binary complex with the guide strand labeled at position 18 resulted in well-defined FRET populations for all MjAgo-guide strand complexes, indicating that a stable complex between MjAgo and the guide strand is formed. In order to follow conformational changes of the domains relative to each other and relative to the DNA, we specifically incorporated the acceptor dye in three different domains of MjAgo and nucleotide 18 of the guide DNA. The PAZ and N-terminal domain have been shown to undergo extensive structural rearrangements when progressing from the binary to the ternary complex.12,21,22,31 In contrast, the MID-PIWI lobe of Ago is conformational stable and the 5′ end of the guide strand and the guide/target hybrid bound via the MID-PIWI domains can be clearly assigned in the crystal structure. Figure 4 shows the FRET efficiency distribution derived from the FRET populations for the binary complex of MjAgo with the acceptor position in the N-terminal, PAZ and MID-domain with mean FRET efficiencies of EN-18 = 0.82, EPAZ-18 = 0.82, and EMID-18 = 0.83. Formation of the ternary complex using all three labeled MjAgo variants gave rise to two clearly separate populations. The high FRET populations exhibit comparable mean FRET efficiency values as found for the binary complexes (EN-18 = 0.80, EPAZ-18 = 0.78 and EMID-18 = 0.84), while the second population was clearly shifted toward lower FRET efficiencies (EN* = 0.63, EPAZ* = 0.35, and EMID* = 0.42). Here, the complexes that carried the acceptor in the PAZ domain showed
the most pronounced FRET shift indicating that the distance between nucleotide 18 and this position changed dramatically. Possibly, the low FRET population could represent a structurally rearranged complex after cleavage of the target strand. Notably, using a model protein derived from a hyperthermophilic organism, we were able to control and trigger nucleic acid binding and cleavage independently by choosing the appropriate temperature. Efficient complex formation occurs already at 65 °C, but we did not observe cleavage at temperatures below 75 °C. Therefore, we chose conditions for the single-molecule measurements that prevent target cleavage (Fig. S6). Consequently, the conformational changes observed can be ascribed to target loading and are not correlated with target cleavage. As a control, we included the mismatched dsDNA in our measurements, which did not result in efficient ternary complex formation. No low FRET population was observed in this case. The mean FRET efficiencies (E^N-18 = 0.84, E^PAZ-18 = 0.79, and E^MID-18 = 0.75) correspond to values determined for the binary complex, indicating that the 3′ end of the guide is still anchored in the PAZ domain as seen for the binary complex.

Next, we moved the position of the donor to nucleotide 13 in the guide DNA. In most structures available, this position can be resolved indicating that this position is less flexible than the 3′ end. Here, we formed binary and ternary complexes with MjAgo with the acceptor dye attached either to the PAZ domain or to the MID domain as these showed the most pronounced FRET shift (Fig. 5). The binary complexes exhibited a single FRET population (E^PAZ-13 = 0.81 and E^MID-13 = 0.82), while the ternary complexes gave rise to two populations (E^PAZ-13 = 0.88 and E^MID-13 = 0.90; E^PAZ-13 = 0.63 and E^MID-13 = 0.65) comparable to the situation found for the label at position 18. Notably, the FRET shift is strongly reduced (ΔFRET^PAZ-18 = 0.43 vs. ΔFRET^PAZ-13 = 0.25; ΔFRET^MID-18 = 0.42 vs. ΔFRET^MID-13 = 0.25) congruent with a situation where the 3′ end undergoes large structural re-orientation while position 13 is less affected since the 5′ end is fixed in the MID domain binding pocket and the bases of the seed sequence are fixed by extensive interactions with the MID-PIWI domains.

Discussion

The last 10 years have seen a leap forward in the understanding of Ago function and structure, revealing that the protein is composed of four domains (N-terminal, PAZ, PIWI, and MID) arranged in a bilobal manner. The overall organization of the binary Ago-guide strand complex and ternary Ago-guide/target strand complex has been captured employing X-ray crystallography. In this work we present complementary approaches to probe the interaction of the guide and target strand with the protein scaffold and present single-molecule FRET measurements using an archaeal Ago model system. These techniques allow a structural investigation of Ago in its apo-form and the binary and ternary complexes and extend to the regions that are not accessible by conventional structural methods.

We examined the arrangement of the guide strand employing fluorescent dyes as reporter molecules. Attached to the nucleic acids, the dye fulfils three functions, (1) as a label that traces the DNA in gels and indicates the complexation status of Ago, (2) as voluminous moiety that probes the protein scaffold for structurally confined interaction sites, and (3) as donor fluorophore for single-molecule FRET experiments. Using this probing approach, we found that the binding of the guide is hampered if the 3′ end of the guide DNA is blocked by the fluorophore. In contrast, placing the dye at position 18 poses the least influence...
on guide strand binding, indicating that nucleotide 18 is not part of the protein sidechain–DNA interaction network. This is in very good agreement with the structural data collected using the bacterial Ago protein.\textsuperscript{12,22} The 5′ end and the 3′ end of the guide strand are fixed in binding pockets in the MID/PIWI and PAZ domain, respectively, whereas nucleotide 18 is not buried in the protein (Fig. S7). The position of nucleotides 13 and 14 are not determined in the crystal structure but our data show that nucleotides 13 and 14 are contacted by MjAgo as the relative binding efficiency of the labeled guide DNA at these positions is reduced compared with position 18. The apparent micromolar affinity of MjAgo for DNA is rather weak compared to other prokaryotic Agos (affinity in the nanomolar range)\textsuperscript{13} and eukaryotic Ago that binds guide RNA with picomolar affinity.\textsuperscript{32} We cannot exclude that the dye label interferes with binding even though we observe the strongest signal for the binary complex with the label at position 18, and that therefore the actual affinity for the guide DNA is higher than observed in our experiments. Another explanation is that recombinant MjAgo co-purifies with endogenous nucleic acid of the expression host and the labeled nucleic acids added for the binding experiments undergo a competition reaction with the co-purified nucleic acid species.

Accommodation of the target strand and propagation toward the ternary complex requires the straightening of the guide strand, which is accompanied by a large conformational rearrangement of the N-terminal and PAZ domain to form a more open conformation (Fig. S10).\textsuperscript{11,33} We were able to follow ternary complex formation in gel shift experiments, which showed that MjAgo efficiently binds DNA/DNA and DNA/RNA hybrids. Target strand incorporation was not observed if a DNA/DNA hybrid was used that carried a tandem mismatch as common in miRNAs that are recognized by human Ago2. Most likely, this is due to the reduced melting temperature of the mismatch dsDNA. As complex formation and cleavage requires 65 °C and temperatures above 75 °C, respectively, the mismatched target cannot be utilized by MjAgo. Interestingly, the melting temperature of the standard DNA hybrid used is 58 °C, indicating that MjAgo is able to stabilize the duplex in a stable configuration that allows cleavage of the target strand even at elevated temperatures. Unlike bacterial Ago that utilizes both RNA and DNA as target\textsuperscript{22} in vitro and appears to use a RNA-guided DNA-target cleavage mechanism in vivo,\textsuperscript{34} MjAgo shows a clear selectivity for DNA for its nucleolytic reaction, even though a target RNA strand promotes ternary complex formation equally well. Whereas Ago2 and the bacterial Agos show cleavage products that correspond to a single cleavage site opposite nucleotide 10–11 of the guide strand, we observe multiple cleavage products for MjAgo. Even though we cannot explain this finding based on our current knowledge, we were able to show that activity is dependent on bivalent cations as observed for Ago2 and bacterial Ago,\textsuperscript{5,7,22} and that MjAgo also accepts long DNAs as target.

Only in the double-stranded configuration the binding of the Atto550-labeled 3′ end was monitored. No binary complex was found when the ternary complex was formed, including the 3′ end-labeled guide indicating that MjAgo is able to bind the dsDNA without an intermediate step via the binary complex. A possible explanation for this observation is that dsDNA is typically found as a more stretched and rigid B-form helix that cannot be accommodated by MjAgo and leads to the release of the 3′ end. It is also conceivable that the additional or altered interactions of MjAgo with the nucleic acids in the ternary complex compensate for the presence of a bulky dye moiety at the 3′ end. In the bacterial system, a DNA/RNA hybrid composed of a 21 nt DNA and 15 nt RNA triggered the release of the 3′ end of the DNAguide\textsubscript{15,17} suggesting a comparable molecular mechanisms for archaeal Agos. This assumption is furthermore supported by our single-molecule FRET data in solution. Here, FRET between a donor dye positioned in the guide DNA and an acceptor fluorophore site-specifically introduced into MjAgo in either the N-terminal, PAZ, or MID domain revealed structural rearrangements upon the transition from the binary to the ternary complex. The formation of the ternary complex resulted in a second FRET population with significantly increased distances between position 18 in the guide DNA and each of the labeled MjAgo domains. Taking into account the structural data, this result can be best explained if this population is ascribed to a MjAgo conformation in which the 3′ end of the guide DNA is released from the PAZ domain and the N-terminal-PAZ domain lobe adopts an opened position\textsuperscript{14,16,21,22} (see Fig. 6 for a model summarizing our data). Most likely, this conformational change is a consequence of the rigidity of double-stranded DNA resulting in a 3′-unlocked conformation where pairing of the target strand provides the energy necessary to disrupt the interactions in the PAZ domain. The hybridization of the target strand accumulates tension on the protein–guide DNA interaction that ultimately releases the 3′ end from the PAZ domain. We did not observe a full transition of the high FRET population toward the low FRET state. This can be explained by the incomplete incorporation of the target strand into the labeled MjAgo protein, as shown in binding experiments, which result in MjAgo harboring the guide strand only or the guide–target strand duplex. The latter complex is characterized by a low FRET efficiency. A dynamic equilibrium between binary and ternary complex with concomitant conformational rearrangements of the PAZ domain faster than the diffusion time can be ruled out due the clearly separated populations in the E-S-histogram (a dynamic equilibrium would result in a smeared population spanning from the low FRET to the high FRET position, see also Fig. S11). As the low FRET population was not observed when a mismatched DNA hybrid was used that only leads to binary complex formation, we can clearly assign the conformational change to the loading of a fully complementary dsDNA substrate forming a catalytic competent complex. In accordance with our model, the mismatch does not allow the build-up of sufficient tension to break the DNA-PAZ domain interaction. Notably, the archaeal model system employed in this study allowed us to monitor the conformational changes associated with target strand binding only, unrelated to cleavage as cleavage is temperature-inducible. We demonstrate that PAZ domain opening is connected to target strand association and does not require cleavage supporting the “two-state model” proposed by Tomari and Zamore\textsuperscript{8} (see Fig. 6). This is in agreement with a recent study that employed ensemble
pre-steady-state kinetic techniques to delineate the mechanistic aspects of siRNA-dependent target RNA slicing by Ago2, which are in support of the “two-state model.” A detailed structural analysis of the eukaryotic Ago protein led to the hypothesis that the release of the DNA guide from the PAZ domain and the insertion of a glutamate finger into the active site (plugged-in conformation) enables cleavage of the target strand.

Furthermore, surveying multiple FRET-based distances between the individual Ago domains and two positions in the guide DNA, we gained insights into the path of the 3′ end of the guide strand in the ternary complex that is not resolved in the crystal structures available. However, we are not able to speculate on the pathway of the target strand at its 5′ end. The FRET changes that accompany ternary complex formation are most pronounced for the distance between the MID and PAZ domain and position 18. In contrast, the FRET change between MID and PAZ domain and position 13 as well as the N-terminal domain and position 18 are less drastic. These results fit a model of the ternary complex best where the 3′ end of the guide DNA passes the N-terminal domain facing toward the PIWI domain. Thus, the distance between position 18 and the MID/PAZ domain is increased but the distance to the labeling position in the N-terminal domain is just slightly changed. However, a tightly protein-associated seed segment (up to position 8) restricts the conformational flexibility of position 13 resulting in smaller FRET changes.

Taken together, we present a single-molecule FRET study of an Ago protein in solution yielding data that support the “two-state model” of Ago function and propose a model where the altered pathway of the 3′ end after PAZ domain release leads along the PIWI-facing side of the N-terminal domain.

Materials and Methods

Comparative modeling

Template structures suitable for homology modeling of Methanocaldococcus jannaschii Ago (RefSeq. NP_248321.1) were identified from the PDB database using the Basic Local Alignment Search Tool. Top ranking with 28% sequence identity were the apo state and Mn 2+-bound crystal structures of PfAgo (PDB: 1Z25 and 1U04), of which the Mn 2+-bound state was used as a template. Based on an initial sequence alignment generated in Modeller version 9.9 (Build 7963), 20 models were built, all of which with a GA-341 score of 1.0. To test for further improvement, the initial alignment was iterated by adjusting the positions of insertions and deletions in 11 independent steps, each comprising 20 models as described in reference 40. All sequence iterations preserved the GA-341 score of 1.0. Further evaluation of all models was based on the global and per-residue DOPE score in comparison to the template scores as well as on the violation of stereochemical restraints. Since clusters of such restraint violations indicate putative alignment errors, alignment iteration was performed to minimize such clusters. The 11 independent alignment variations were combined pairwise to test for the most beneficial combination in terms of DOPE score and stereochemistry. The final target–template sequence alignment is shown in Figure S2. Please see the Supplementary Material for additional information on model quality (scores and stereochemistry).

Protein expression and purification

The full-length Ago gene (GI: DSM2661) from Methanocaldococcus jannaschii (MjAgo) was cloned into pET151/D-TOPO or pET101/D-TOPO (Invitrogen) vectors allowing the expression of the recombinant protein with an N-terminal or C-terminal hexa-histidine tag, respectively. The Ago gene was expressed in Rosetta(DE3)pLyS5 (Novagen) cells. After harvesting of the cells by centrifugation (8000 g, 20 min) the cells were resuspended in resuspension buffer (50 mM Tris/HCl pH 7.4, 100 mM NaCl, 10% glycerol). Cells were lysed by sonification and the soluble protein fraction was extracted. As the protein is derived from a hyperthermophilic organism, a first purification of the recombinant MjAgo could be achieved by a heat denaturation step (30 min at 85 °C). Here, approximately 90% of the endogenous E.coli protein could be removed. The heat stable recombinant protein was further purified by affinity chromatography using a HisTrap column (GE Healthcare). The eluted protein was concentrated using Amicon Ultra filter (Millipore) and stored in a buffer containing 50 mM Tris/HCl pH 7.1, 100 mM NaCl, 10% glycerol. The His-tag was removed by TEV cleavage (TEV protease purchased from Roboklon, Germany) for 1 h at 37 °C to create a tag-free MjAgo preparation. The cleaved His-tag and the His-tagged TEV protease were removed by Ni-NTA affinity chromatography while the tag-free MjAgo remains in the flow through. Protein concentrations were determined using an extinction coefficient at 280 nm of 122 510 M⁻¹ cm⁻¹ (calculated using the ProtParam tool from www.expasy.org).

For the production of fluorescently labeled variants, a nonsense-suppressor strategy was chosen, which allows the specific labeling of the cysteine-rich MjAgo via the unique unnatural amino acid p-azido-1-phenylalanine. Amber mutations (TAG) have been introduced at position Asparagine 76, Serine 221, or Isoleucine 410 into the MjAgo gene using either the QuikChange II site-directed mutagenesis kit (Agilent) or the SOE (splice by overlap extension) PCR strategy. The mutated gene was cloned into the pET101/D-TOPO vector to ensure purification of the full-length protein via a C-terminal His-tag. The recombinant protein was produced in BL21/DE3 that additionally carried the arabinose-inducible pEvolv-pAzF plasmid encoding multiple copies of an amber-suppressor tRNA (tRNA Cly) and an engineered tyrosyl-tRNA synthetase. Expression was performed in M9 minimal media with supplements as described earlier. Extraction of the mutated MjAgo protein was performed as described for the wild-type protein with the exception that the heat treatment step was done at 75 °C for 30 min.

Protein labeling of unnatural amino acids incorporated into MjAgo

MjAgo variants carrying p-azido-1-phenylalanine at position 76, 221, or 410 were labeled using the Staudinger-Bertozzi ligation between an azide and a triarylphosphine moiety (attached to the DyLight fluorophores). A final concentration of 50 µM DyLight650 (Pierce) was added to the purified protein solution and incubated for 30 min at 25 °C. The reaction was stopped by the addition of 10 mM DTT.
Synthetic oligonucleotides

HPLC-purified oligodeoxynucleotides were purchased from Eurofins-MWG (Ebersberg, Germany). Concentrations were determined by UV absorbance at 260 nm. The sequence of the 21-nucleotide guide strand was derived from the human miRNA hsa-let-7a-5p (5′-UGAGGUAGUG GGUUGUAAG U) and carried a 5′-phosphate. A full list of oligonucleotide sequences can be found in the supplementary material. The fluorescent dye Atto550 and Alexa647 were attached to either position 13, 14, 18, or to the 3′ end of the oligonucleotide. For cleavage assays, a target strand was used that carried an Alexa647 modification at position 17 (counted from the 5′ end of the target strand). Guide and target strands were annealed by heating them up to 95 °C and then immediately cooled down to 20 °C in 0.1 °C steps every 6 s. To ensure complete annealing of the labeled guide strand, the unlabeled target strand was added in 2-fold excess.

Electrophoretic mobility shift assay

Fluorescently labeled oligonucleotides (333 nM) were incubated with MjAgo at different molar ratios (see the corresponding figure legends) for 20 min at 65 °C in 1x NaGTT buffer (50 mM Tris/HCl, pH 7.4, 100 mM NaCl, 5 mM MgCl₂, 2% glycerol, 10 mM Dithiothreitol, 67 µg/ml BSA). Heparin was added to a final concentration of 67 µg/ml to suppress unspecific interactions of MjAgo with nucleic acids. The reaction was incubated for another 10 min. Next gel loading buffer (final concentration of 10 µl formamide-loading buffer and the resulting fragments were separated by non-denaturing Tris-Glycine gel electrophoresis (10 or 12% PAA gels). Subsequently, fluorescently labeled oligonucleotides and protein–nucleic acid complexes were visualized on a Fuji FLA7000 scanner and signals were quantified using ImageQuant TL software (GE Healthcare).

Cleavage activity of M. jannaschii Ago

Cleavage assays were performed by combining 0.6 µM recombinant MjAgo with 1.7 µM guide DNA and 0.72 µM target DNA in a buffer containing 50 mM Tris/HCl pH 7.4, 100 mM NaCl, 5 mM MgCl₂, 2% Glycerol, 10 mM DTT, and 67 µg/ml BSA in a total volume of 15 µl. The target DNA is labeled with Alexa647 at position 17, which allows the detection of the cleavage products via the fluorescent signal (see Supplementary Materials for a list of oligonucleotides used). All components were combined on ice and the enzymatic reaction was initiated by incubating the reaction at 85 °C. 10 µl of the reactions were stopped by the addition of 10 µl formamide-loading buffer and the resulting fragments were separated on a 12% denaturing polyacrylamide gel for 80 min at 80W. The fluorescent signal was visualized using a FLA7000 scanner (GE Healthcare).

Binary and ternary complex formation in preparation for single-molecule analysis

DL650-labeled Ago was incubated either with the guide strand only or the guide–target duplex (final concentration 4 µM). In both cases, the Atto550-modification of the guide DNA strand at position 18 or 13 was used. The incubation was performed in a buffer consisting of 50 mM Tris/HCl pH 7.4, 100 mM NaCl, 10 mM MgSO₄, 2% Glycerol, 10 mM DTT, and 67 µg/ml BSA in a total volume of 250 µl for 20 min at 72 °C. Excess of DNA and the fluorophore were removed by subsequent size exclusion chromatography using a Superose 6 column (GE Healthcare). The eluate fractions containing the Ago-DNA complex were used for single-molecule measurements directly after the chromatography run. Cleavage assays showed that the target is not cleaved under these conditions using equivalent incubation steps (20 min at 72 °C and subsequently 22 °C for up to 3 h) (Fig. S6).

Single molecule fluorescence measurements

In order to detect fluorescence from individual diffusing proteins, the concentration of fluorescently labeled molecules was adjusted to the picomolar range. Measurements were performed in LabTek chamber slides (Nunc) in a total volume of 400 µl.

We employed a custom built confocal microscope to study fluorescence and FRET on the level of single molecules. An acousto-optical tunable filter (AOTFnc-V15, AA optoelectronic) allowed for alternation of the excitation lasers with a 100 µs period so that each molecule diffusing through the laser focus experienced several cycles of separate donor and acceptor excitation. The donor fluorophore Atto550 was excited with a continuous wave diode laser at 532 nm (Sapphire LP 532 nm 100 mW; 115 µW) and the acceptor fluorophore DyLight650 with an 80 MHz pulsed laser at 640 nm (LDH-D-C-640, Picoquant, 55 µW). The laser beam was coupled into an oil-immersion objective (UPLSAPO100XO, NA 1.40, Olympus) of an inverted microscope by a dual-band dichroic beam splitter (Dualband z532/633, AHF). The emitted fluorescence was collected by the same objective, focused onto a 50 µm pinhole, and split spectrally at 640 nm by a dichroic beam splitter (640DCXR, AHF). Two avalanche photodiodes (τ-SPAD-100, Picoquant) detected the donor and acceptor fluorescence with appropriate spectral filtering (Brightline HC582/75, Bandpass ET 700/75 min, both AHF, RazorEdge LP 532, RazorEdge LP 647, both Semrock). The detector signal was registered using a PC card for single-photon counting (SPC-830, Becker&Hickl) and evaluated using custom-made LabVIEW (National Instruments) software.

Data evaluation for ALEX measurements

Fluorescence bursts from individual molecules diffusing through the laser focus are detected with a burst search algorithm (parameters used: t = 500 µs, M = 30, L = 100).43 Molecules are alternating excited and the fluorescence of donor and acceptor is separately detected.26 This defines three different photon counts: donor emission due to donor excitation

\[ F_D^o \]

acceptor emission due to acceptor excitation

\[ F_D^a \]

and donor emission due to donor excitation

\[ F_D^d \]

which corresponds to the FRET signal. These measured photon counts were corrected for background signals (1.3 kHz, 1.3 kHz, and 1 kHz, respectively) and we determined the FRET efficiency and the stoichiometry value for each molecule. We therefore extracted the leakage value.

54 RNA Biology Volume 11 Issue 1
\[ I_k = \frac{F_D^A}{F_D} - \frac{L}{F_D} - \frac{D}{F_D} - \frac{A}{F_A} \]

from the donor only population of the E-S-histogram and the direct excitation factor

\[ d_x = \frac{F_A^D}{F_A} \]

from the acceptor-only population according to reference 30. These values account for bleed through of donor fluorescence in the acceptor channel and for the direct excitation of the acceptor fluorophore during donor excitation. The values for the individual measurements can be found in Table S3. This yields the corrected FRET counts

\[ F_{\text{FRET}} = \frac{F_D^A}{F_A} - \frac{L}{F_D} - \frac{D}{F_D} - \frac{A}{F_A} \]

and subsequently,

\[ E = \frac{F_{\text{FRET}}}{F_{\text{FRET}} + F_D^A} \]

The resulting E-S-histogram is further refined by the recently introduced ALEX-2CDE and FRET-2CDE filters. All E-S histograms are shown in Figure S10 and the respective filter settings are listed in Table S3.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Supplemental Materials
Supplemental materials may be found here: www.landesbioscience.com/journals/rnabiology/article/27446/
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