Assembly of AUF1 Oligomers on U-rich RNA Targets by Sequential Dimer Association*

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Many labile mammalian mRNAs are targeted for rapid cytoplasmic turnover by the presence of A + U-rich elements (AREs) within their 3′-untranslated regions. These elements are selectively recognized by AUF1, a component of a multisubunit complex that may participate in the initiation of mRNA decay. In this study, we have investigated the recognition of AREs by AUF1 in vitro using oligoribonucleotide substrates. Gel mobility shift assays demonstrated that U-rich RNA targets were specifically bound by AUF1, generating two distinct RNA-protein complexes in a concentration-dependent manner. Chemical cross-linking revealed the interaction of AUF1 dimers to form tetrameric structures involving protein-protein interactions in the presence of high affinity RNA targets. From these data, a model of AUF1 association with AREs involving sequential dimer binding was developed. Using fluorescent RNA substrates, binding parameters of AUF1 dimer-ARE and tetramer-ARE equilibria were evaluated in solution by fluorescence anisotropy measurements. Using two AUF1 deletion mutants, sequences C-terminal to the RNA recognition motifs are shown to contribute to the formation of the AUF1 tetramer-ARE complex but are not obligatory for RNA binding activity. Kinetic studies demonstrated rapid turnover of AUF1-ARE complexes in solution, suggesting that these interactions are very dynamic in character. Taken together, these data support a model where ARE-dependent oligomerization of AUF1 may function to nucleate the formation of a trans-acting, RNA destabilizing complex in vivo.

The control of cytoplasmic mRNA turnover plays a major role in regulating both the level and timing of expression of many gene products in eukaryotes (reviewed in Refs. 1 and 2). In many cases, sequence elements within individual mRNAs function as cis-acting determinants of their stability, either constitutively or in response to external stimuli. Conceptually, modulation of mRNA turnover rates may be envisioned as altering the activity or accessibility of one or more ribonucleases toward mRNA. The activities of the AUF1 tetramer-ARE complex but are not obligatory for RNA binding activity. Kinetic studies demonstrated rapid turnover of AUF1-ARE complexes in solution, suggesting that these interactions are very dynamic in character. Taken together, these data support a model where ARE-dependent oligomerization of AUF1 may function to nucleate the formation of a trans-acting, RNA destabilizing complex in vivo.

AREs are potent cis-acting determinants of rapid cytoplasmic mRNA turnover in mammalian cells. They generally consist of one or more overlapping AUUUA pentamers contained within or near a U-rich tract (3–5). These elements are present in the 3′-untranslated regions (3′-UTRs) of many labile mRNAs, including several encoding inflammatory mediators, cytokines, oncoproteins, and G protein-coupled receptors (3, 6–10). mRNA turnover mediated by AREs is usually characterized by rapid 3′ to 5′ shortening of the poly(A) tract followed by decay of the mRNA body (11–15). In addition, ARE-directed mRNA decay is dependent upon active translation of the mRNA in many cellular systems (16–20), suggesting that some link also exists between protein synthesis and mRNA decay mechanisms.

AUF1 is an RNA-binding protein that exhibits many characteristics of a trans-acting factor participating in ARE-directed mRNA turnover (reviewed in Ref. 21). Current evidence indicates that AUF1 may function as a targeting system for AREs, either recruiting or promoting the assembly of multisubunit trans-acting complex(es) at these sites (22–24). Several cytoplasmic proteins co-immunoprecipitate with AUF1, indicating that AUF1 associates with additional factors in vivo (25). Some of these have been identified immunologically as the translation initiation factor eIF4G, poly(A)-binding protein, heat shock protein 70, and the 70-kDa heat shock cognate protein (26). The identification of these associated proteins is evidence of a physical link between AUF1 and factors involved in translation and mRNA turnover.

Elucidation of the mechanisms contributing to rapid mRNA turnover by AREs will require further understanding of both the molecular architecture of the trans-acting complex(es) as well as the molecular events involved in recognition of AREs by these factors. In particular, the direct interaction of AUF1 with target RNA sequences may serve to nucleate factor binding or transduce some signal to activate pre-assembled complexes. In this study, we have investigated the interaction of AUF1 in vitro with the following two U-rich oligoribonucleotides: the core ARE from tumor necrosis factor α (TNFα) mRNA, and a uridylate homopolymer. We present evidence that recognition of U-rich RNA sequences by AUF1 initiates the assembly of AUF1 multimers involving both RNA-protein and protein-protein interactions by sequential binding of AUF1 dimers. The application of fluorescence anisotropy to the study of RNA: AUF1 solution equilibria allowed estimation of the equilibrium constants for both initial and secondary binding events as well as an assessment of complex dynamics by off-rate analyses.

Finally, we discuss potential functional consequences of RNA-
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RNA Substrates—All RNA oligonucleotides (2’-hydroxyl) were synthesized by Dharmacon Research (Boulder, CO). The sequence of each RNA probe is listed in Fig. 1A. Following 2’-O-deprotection according to the manufacturer’s instructions (27), RNA oligonucleotides were quantified by absorbance at 260 nm. Estimates of the extinction coefficients for each RNA probe at 260 nm were calculated as described (28). For fluorescein-tagged probes, absorbance at 260 nm was corrected by quantification of the fluorescein moiety at 495 nm as described (29).

The substrate “TFNA ARE” corresponds to the core ARE from the 3’-UTR of human TNFα mRNA. The RNA substrate “U32” contains a uridylic homopolymeric sequence, and “R” encodes a fragment of the rabbit β-globin coding region. Duplicate oligonucleotides containing 5’-fluorescein labels were also synthesized and are designated Fl-TFNA ARE, Fl-U32, and Fl-R8, respectively. For gel mobility shift assays, TFNA ARE, U32, and R8 substrates were radiolabeled using T4 polynucleotide kinase (Promega, Madison, WI) and [γ-32P]ATP (4500 Ci/mmol) (ICN Biomedicals, Costa Mesa, CA) to specific activities of 3–5 x 10^6 cpm/nmol. Unincorporated radiolabel was removed by spin column chromatography using G-25 Quick Spin columns (Roche Molecular Biochemicals). Probe-specific activity was estimated by liquid scintillation counting, and RNA integrity was verified by denaturing polyacrylamide gel electrophoresis and autoradiography. 32P-Labeled RNA probes were detected as single bands (data not shown) indicating that they were predominantly (>99%) full length.

Preparation of Recombinant Proteins—The construction of plasmids pTrcHisB-AUF1-(1–257) and pTrcHisB-AUF1-(1–229) was described previously (24). pTrcHisB-AUF1-(1–257) encodes a stable, N-terminal His6-tagged mutant of human p37AUF1 lacking 30 amino acid residues from the C terminus but showing comparable ARE binding activity to full-length p37AUF1 (24). pTrcHisB-AUF1-(1–229) encodes a truncation mutant of p37AUF1 lacking all sequences C-terminal of the RNA recognition motifs (RRMs). Recombinant His6-AUF1 mutant proteins were expressed and purified as described (30) and were judged to be 99% pure by SDS-PAGE. For protein cross-linking studies, His6 proteins were dialyzed against 10 mM HEPES-KOH (pH 7.5) prior to concentration. Where indicated, a 3500-Da N-terminal fragment containing the His6 tag was removed from His6-p37AUF1-(1–257) using the recombinant enterokinase kit (Novagen, Madison, WI) according to the manufacturer’s instructions. A mock-digested reaction was also assembled to control for changes in protein activity resulting from prolonged incubation at room temperature (2 h). All recombinant proteins were quantified by the method of Bradford (31) using bovine serum albumin as standard. Protein concentrations were also evaluated by comparison of Coomassie Blue-stained SDS-PAGE gels containing recombinant proteins and a titration of bovine serum albumin. Determination of protein concentration in these methods yielded estimates within 10% of previously determined values.

Gel Mobility Shift Assays—Binding reactions for gel mobility shift assays were performed with a range of His6-AUF1 fusion protein concentrations and 0.15 nM 32P-labeled RNA in a final volume of 10 μl containing 10 mM Tris-HCl (pH 7.5), 100 mM potassium acetate, 5 mM magnesium acetate, 2 mM dithiothreitol, 0.1 mM spermine, 1 μg/ml acetylated bovine serum albumin, 8 units of RNasin (Promega), 33% glycerol, and 1 μg/ml heparin. Reactions were incubated for 10 min at room temperature and immediately fractionated through 6% (40:1 acrylamide:bisacrylamide) non-denaturing gels as described (30). Reaction products were visualized by PhosphorImager scan (Molecular Dynamics, Sunnyvale, CA).

Protein-Protein Cross-linking—Dithio-bis(succinimidyl propionate) (DSP)-mediated protein-protein cross-linking was performed in 10 μl reactions containing 10 mM HEPES-KOH (pH 7.5), 100 mM potassium acetate, and 5 mM magnesium acetate. In this buffer system, HEPES-dialyzed His6-p37AUF1-(1–257) or His6-p37AUF1-(1–229) was diluted to 5 μM in the presence or absence of 1.5 μM RNA. DSP (Pierce) was then added to a final concentration of 2.5 mM, and reactions were allowed to proceed for 10 min at room temperature. Cross-linking was then quenched by addition of a 100-fold molar excess of unlabeled RNA that was incubated for a further 15 min. Reaction products were fractionated by SDS-PAGE in the absence of reducing agents. Complexes containing AUF1 were identified by probing immunoblots with anti-AUF1 antiserum (25). Secondary antibody detection was performed using the SuperSignal Chemiluminescent Detection Kit (Pierce) and exposure to x-ray film.

Fluorescence Anisotropy—Fluorescence anisotropy measurements were made using the Beacon 2000 variable temperature fluorescence polarization system (Panvena, Madison, WI) equipped with fluorescein excitation (490 nm) and emission (535 nm) filters. Binding reactions were assembled as described for gel mobility shift assays (above) except that no glycerol was added, and the final volume was 100 μl. For equilibrium binding experiments, the polarimeter was operated in the static mode, with each sample of radiolabeled RNA competitor added to the fluorescein-labeled RNA probes. Following probe addition, samples were incubated for 1 min before anisotropy was measured. Preliminary on-rate analyses demonstrated that anisotropic equilibrium was reached within 10 s (data not shown). Data points represent the mean of 10 measurements for each binding reaction. Samples for off-rate analyses were similarly assembled, except that following an initial reading (t = 0), a 5000-fold molar excess of unlabeled RNA competitor was added to the binding mixture and rapidly mixed. Anisotropy measurements were taken in kinetic mode in intervals of 15 s, with five measurements taken at each time point. All non-linear regression fitting of anisotropic data and statistical evaluations were performed using PRISM software version 2.0 (GraphPad, San Diego, CA).

RESULTS

AUF1 Binding to U-rich Oligoribonucleotide Substrates Generates Two Complexes in Vitro—Human TNFsα mRNA contains an ARE in its 3’-UTR which contributes to its rapid turnover in vivo (32, 33). It is also sufficient to destabilize a heterologous mRNA in transfected cell systems (12), and cytoplasmic proteins have been identified binding this element in vitro (34). The core sequence of the TFNA ARE is similar to others identified as high affinity AUF1-binding sites (23, 25). Taken together, these features make the TFNA ARE a strong candidate for high affinity interaction with AUF1.

In gel mobility shift assays, binding of His6-p37AUF1-(1–257) to the TFNA ARE and U32 sequences generated two distinct complexes (Fig. 1, B and C). No detectable binding was observed to the rabbit β-globin substrate (Fig. 1D), consistent with the selectivity of AUF1 for a A + U-rich RNA sequences (23, 25). For both the TFNA ARE and U32 probes, the distribution of complexes with RNA was dependent on protein concentration, with the faster migrating complexes (complex I) appearing at lower concentrations of His6-p37AUF1-(1–257) (0.5–5 nM). Levels of complex I diminished as the abundance of complex II increased, consistent with the possibility of a precursor-product relationship. The diffuse smearing observed below binding complexes is likely due to RNA-protein dissociation in the gel. No additional binding events were observed in these assays, with protein concentrations tested up to 500 nM (data not shown).

AUF1 Forms Tetramers in the Presence of a High Affinity RNA-binding Site—Previous hydrodynamic studies demonstrated that AUF1 forms dimeric structures in solution involving an N-terminal alanine-rich region (24). Monomeric AUF1 was not detected in these experiments, indicating that dimers are generated with high affinity. To determine whether AUF1 oligomerization might contribute to the formation of complexes with the ARE, protein-protein binding reactions were treated with the chemical cross-linker DSP, permitting covariant linkage through primary amino groups. In the absence of cross-linker, His6-p37AUF1-(1–257) migrates at an apparent Mr of ~43,000 by SDS-PAGE, larger than its predicted Mr of 32,600 (data not shown). In cross-linking reactions lacking RNA, His6-p37AUF1-(1–257) was primarily detected as a dimer (Fig. 2, lane 1), consistent with the hydrodynamic studies of the full-length p37AUF1 (24). Cross-links were generated specifically through the DSP linker, since they were cleaved following treatment with reducing agents (data not shown). The presence of a non-binding RNA substrate (R8) did not alter the distribution of cross-linked protein products (Fig. 2, lane 2). However, larger protein complexes up to and including tetramers were observed in the presence of high affinity RNA targets (U32 and TFNA ARE; Fig. 2, lanes 3 and 4). The generation of these
larger AUF1 complexes in the presence of U32 indicated that these species were unlikely to be the result of RNA bridging, since there is a paucity of primary amino groups contained within U32 RNA. Furthermore, the lack of detectable AUF1 tetramers in the absence of a high affinity RNA target even at high concentrations of protein (5 μM) suggests that dimer-dimer association does not occur in the absence of U-rich RNA sequences.

Two binding complexes of AUF1 and RNA were detected by gel mobility shift assay (Fig. 1, B and C). The slowest mobility complex (complex II) likely represents RNA associated with the AUF1 tetramer, since it represents the largest change in mobility relative to the free probe, and its abundance increases with protein concentration. Complex I is observed at lower concentrations of protein and could thus represent an AUF1 monomer, dimer, or trimer associated with RNA. However, because unbound AUF1 is dimeric (24), interpretation of complex I as an AUF1 dimer rather than a monomer or trimer bound to RNA is the sole case in which monomeric AUF1 species are not required. Given both the absence of additional intermediate binding species observed by gel mobility shift analysis (Fig. 1) and the absence of detectable monomeric AUF1 by gel filtration and sedimentation velocity experiments (24), the contribution of monomeric AUF1 to the binding equilibrium is likely to be negligible.

**A Model for ARE-dependent Assembly of AUF1 Multimers Based on Sequential Association of Protein Dimers**—Given the following findings, (i) His6-p37AUF1-(1–257) association with TNFα ARE and U32 RNAs generates two RNA-protein complexes, (ii) formation of these complexes is dependent on protein concentration, (iii) AUF1 complexes as large as tetramers are associated with these RNA targets, and (iv) AUF1 dimers do not interact in the absence of U-rich RNA targets, we propose that AUF1 associates with these RNA probes by sequential dimer binding. In this model, complex I (Fig. 1) represents the AUF1 dimer-bound RNA (P2R), and complex II represents an RNA-associated AUF1 tetramer (P4R). Whereas the re-iterative nature of AREs suggests that AUF1 oligomers may be the result of multiple binding sites on the RNA target, RNA-dependent tetramer cross-linking indicates that adjacent dimers are held in close proximity in the P4R complex, making interaction between these subunits likely. Furthermore, AUF1:ARE binding equilibria evaluated by remaining free probe concentration (Fig. 1, see also Refs. 23, 24, 35) are not resolved by Scatchard analysis, suggesting that binding events involving multiple AUF1 dimers are not independent (data not shown). However, the model does not exclude the possibility that the initial dimer binding event may occur at one of several sites on a given RNA target. For this reason, the AUF1 dimer-RNA equilibrium may reflect an average of multiple simultaneous P2R variants.

**An AUF1 Mutant Protein Lacking Sequences C-terminal of the RRMs Is Defective in RNA-dependent Tetramer Formation**—The generation of tetrameric His6-p37AUF1-(1–257) structures on U-rich RNA targets indicates that protein-protein interactions may be generated between AUF1 dimers in an RNA-dependent manner. Whereas previous studies demonstrated that formation of AUF1 dimers in the absence of RNA required sequences near the N terminus, sequences C-terminal to the RRMs were dispensable for dimer assembly (24). In order to evaluate the contribution of C-terminal sequences to tetramer formation in the presence of an RNA target, gel mobility
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A

Fig. 3. Association of His<sub>6</sub>-p37<sup>AUF1</sup>-(1–229) with RNA substrates. Interaction between His<sub>6</sub>-p37<sup>AUF1</sup>-(1–229) and TNF<sub>a</sub> ARE was monitored by gel mobility shift assay as described under “Experimental Procedures” and Fig. 1, with the positions of RNA-protein complexes and free TNF<sub>a</sub> ARE RNA indicated (A). Protein-protein contacts between His<sub>6</sub>-p37<sup>AUF1</sup>-(1–229) subunits were monitored by cross-linking with DSP as described in Fig. 2 in the presence or absence of RNA oligonucleotides (B).

shift assays were also performed using the truncation mutant His<sub>6</sub>-p37<sup>AUF1</sup>-(1–229), which lacks all sequences C-terminal of the RRs. Association of His<sub>6</sub>-p37<sup>AUF1</sup>-(1–229) with the TNF<sub>a</sub> ARE generated primarily a single complex consistent with P<sub>2</sub>R (Fig. 3A, complex I), whereas a complex consistent with P<sub>2</sub>R (complex II) was detected only weakly at higher concentrations of His<sub>6</sub>-p37<sup>AUF1</sup>-(1–229) (0.5 nM), complete association was not detected (Fig. 3A), even at protein concentrations up to 2 μM (data not shown). This suggests that maintenance of His<sub>6</sub>-p37<sup>AUF1</sup>-(1–229)-RNA complexes may be hindered by gel fractionation, possibly involving rapid dissociation in the sample wells or during electrophoresis.

Covalent cross-linking of His<sub>6</sub>-p37<sup>AUF1</sup>-(1–229) with DSP confirmed that this protein forms dimers in the absence of RNA (Fig. 3B, lane 1). A small amount of trimeric AUF1 was also detected but is likely the product of partial oxidation or aggregation in the protein preparation. Inclusion of the R<sub>6</sub> RNA oligonucleotide in the cross-linking reaction did not alter the distribution of covalently linked products (Fig. 3B, lane 2).

Similarly, only minimal changes in the recovery of trimeric AUF1 were observed by addition of the TNF<sub>a</sub> ARE (Fig. 3B, lane 4), and tetrameric species were not detected. However, binding reactions containing U<sub>32</sub> displayed both trimeric and tetrameric cross-linked species (Fig. 3B, lane 3). Taken together, these data indicate that the ability of AUF1 to form RNA-dependent tetramers is compromised but not completely abrogated by removal of sequences between amino acid residues 229 and 257. In particular, tetramer formation with His<sub>6</sub>-p37<sup>AUF1</sup>-(1–229) was observed only with the uridylate homopolymer, where the possibility of multiple identical binding sites exists on the RNA. This suggests that a bona fide ARE, like that in TNF<sub>a</sub> mRNA, presents a hierarchy of AUF1-binding sites consistent with a sequential binding model, in which AUF1 sequences between 229 and 257 contribute to secondary binding events.

Evaluation of AUF1 ARE Equilibrium Constants by Fluorescence Anisotropy—In order to evaluate the validity of the sequential dimer-binding model for AUF1 oligomerization on an ARE, it was necessary to first express the equilibrium relationships between each component mathematically. Subsequently, fluorescence-based solution binding experiments performed under equilibrium conditions were employed to test the accuracy of these equations in describing the sequential association of AUF1 dimers with a high affinity RNA target.

The steady state concentrations of P<sub>2</sub>R and P<sub>4</sub>R may be described in terms of the concentrations of RNA [R] and dimeric protein [P<sub>2</sub>] by Equations 1 and 2.

\[
[P_2R] = K_2[R][P_2] \quad (\text{Eq. 1})
\]

\[
[P_4R] = K_4[R][P_2]^2 \quad (\text{Eq. 2})
\]

By titrating excess protein [P<sub>2</sub>]<sub>free</sub> against a constant concentration of RNA substrate where [R]<sub>tot</sub> ≪ 1/K<sub>1</sub>, [P<sub>2</sub>]<sub>free</sub> remains in vast excess over [P<sub>2</sub>R] and [P<sub>4</sub>R]. Accordingly, [P<sub>2</sub>]<sub>free</sub> is well approximated by [P<sub>2</sub>]<sub>tot</sub> and is henceforth referred to simply as [P<sub>2</sub>].

By using fluorescein-labeled RNAs, RNA-protein complexes are distinguishable in solution based on differences in the intrinsic fluorescence anisotropy exhibited by each species resulting from changes in molecular volume under conditions of constant temperature and viscosity (36, 37). An initial experiment was performed to determine whether the quantum yield of the fluorescein-labeled RNA changed as a result of AUF1 binding. By using Fl-TNF ARE and a titration of His<sub>6</sub>-p37<sup>AUF1</sup>-(1–229), no significant change in fluorescence intensity was observed with protein concentrations up to 250 nM His<sub>6</sub>-p37<sup>AUF1</sup>-(1–229) dimer (Fig. 4A), demonstrating that AUF1 binding did not alter the quantum yield of the fluorescein-labeled RNA. Accordingly, the measured anisotropy A<sub>i</sub> of the fluorescent RNA probe may be interpreted using Equation 3.

\[
A_i = \sum_j A_{if} \quad (\text{Eq. 3})
\]

where A<sub>i</sub> represents the intrinsic anisotropy of each fluorescing species (in this case R, P<sub>2</sub>R, or P<sub>4</sub>R), and f<sub>i</sub> its fractional concentration (38–40). Applying this to our binding model where the concentration of total fluorescent riboprobe [R]<sub>tot</sub> is limiting, the fractional concentration of each species is given by Equations 4–6.
resulting from the sequential dimer binding algorithm (residual plot was also prepared to detect any bias for data subsets with association of His6-p37AUF1-(1–229) with the TNF a fluorescence intensity was measured as a function of His6-p37AUF1-(1–229) concentration to verify that all fluorescent complexes exhibited similar effective quantum yields (0.049 as measured above) (Fig. 4B, dotted line). Comparison of the sum-of-squares deviations for each regression function using the F test (GraphPad PRISM version 2.0) indicates that interpretation of these data is significantly improved using the sequential dimer binding model ($p < 0.0001$).

**Calculation of AUF1 Equilibrium Binding Constants for Fluorescent RNA Substrates**—In general, calculation of $K_1$ and $K_2$ values within reasonable confidence intervals from fluorescence anisotropy isotherms using Equation 8 requires knowledge of the intrinsic anisotropy constants $k_R$ and $k_{P2R}$. $k_R$ can be measured directly as described above (0.049 for Fl-TNFα ARE, 0.039 for Fl-U32). However, an approximation of $k_{P2R}$ is difficult to obtain unless $K_1$ and $K_2$ are significantly different. Accordingly, the most confident estimate of $k_{P2R}$ was given by association of His$_6$-p37AUF1-(1–229) with the Fl-TNFα ARE RNA (Fig. 4B). In this case, regression of Equation 8 with $k_R$ held constant (0.049) and $k_{P2R}$, $k_{P4R}$, $K_1$, and $K_2$ left unfixed yielded a solution for $k_{P2R}$ of $0.080 \pm 0.005$. Triplicate assays yielded values of $k_{P2R}$ consistent with this estimate. Subsequently, solution of anisotropy plots using Equation 8 with $k_R$ and $k_{P2R}$ fixed in the regression allowed estimates of $K_1$ and $K_2$ to be calculated for the interactions of His$_6$-p37AUF1-(1–257) and His$_6$-p37AUF1-(1–229) with Fl-TNFα ARE and Fl-U32. A representative plot utilizing this technique for the solution of binding constants describing the His$_6$-p37AUF1-(1–257): Fl-TNFα ARE equilibrium is shown in Fig. 5 (solid circles). Substitution of the HEPES-based buffer system used in crosslinking assays (Figs. 2 and 3B) did not significantly alter the anisotropy profile, but heparin was required to minimize non-specific interaction of protein with RNA (data not shown). In an additional experiment, the N-terminal His$_6$ tag of His$_6$-p37AUF1-(1–257) was removed using enterokinase. Changes in anisotropy generated by association of the digested protein with Fl-TNFα ARE were not significantly different than those observed using mock-digested protein (data not shown), indi-
This indicates that, unlike Fl-U32, the Fl-TNF
analyzed by non-linear regression using Equation 8 with
A
in solution by off-rate analysis. First, binding reactions were
products. This is supported by the measured anisotropy value
cating that the His6 motif does not contribute to RNA binding
values (open circles).
Fig. 5. Determination of equilibrium constants for association of
His6-p37AUF1-(1–257) with the TNFα ARE. Anisotropy values for
Fl-TNFα ARE-His6-p37AUF1-(1–257) equilibria (solid circles) were ana-
yzed by non-linear regression using Equation 8 with
A
and
A
were observed with increasing dimer concentration of His6-p37AUF1-(1–257), consistent with the gel mobility shift results (Fig. 1D).
Calculated values of
K
1 and
K
2 for His6-p37AUF1-(1–257) and
His6-p37AUF1-(1–229) binding to the Fl-TNFα ARE and Fl-U32 RNAs are listed in Table I. Based on these data, we conclude that specific recognition of both the TNFα ARE and the uridy-
late homopolymer by AUF1 does not require sequences C-
terminal of the RRMs. In addition, binding affinity of the sec-
don dimer (K
2) is enhanced relative to
K
1 when multiple identical binding sites are present (Fl-U32: K
2/K
1 >1). How-
ever, for the TNFα ARE, binding of the second His6-p37AUF1-
(1–229) dimer occurred with much lower affinity than the first. This indicates that, unlike Fl-U32, the Fl-TNFα ARE probe does not present multiple high affinity sites for AUF1 binding. However, inclusion of the 29 amino acid residues C-terminal of the RRMs in His6-p37AUF1-(1–257) increases the affinity of AUF1 dimers for the AUF1 dimer-RNA complex, thus enhanc-
ing AUF1 tetramer formation on a physiologically relevant RNA target sequence.

Dynamic Nature of AUF1ARE Equilibria—To evaluate the dynamics of AUF1ARE interactions, the stability of His6-
p37AUF1-(1–257) complexes with Fl-TNFα ARE was monitored in solution by off-rate analysis. First, binding reactions were generated with 10 nm His6-p37AUF1-(1–257) dimer and 0.2 nM Fl-TNFα ARE. Based on the estimates of
K
1 and
K
2 (Table I), this binding reaction produces a mixed population of binding products. This is supported by the measured anisotropy value (A
1 = 0.091 ± 0.002, n = 3; Fig. 6, t = 0), which falls between the estimated intrinsic anisotropy for P
R (0.089) and the calculated value of A
FL for this binding reaction (0.097 ± 0.001), as well as the corresponding distribution of binding products identified by gel mobility shift assay (Fig. 1B, 20 nm His6-p37AUF1-(1–257) monomer). Addition of a 5000-fold molar excess of unlabeled TNFα ARE to the binding reaction resulted in a rapid decrease in measured anisotropy with an apparent half-life of less than 10 s (Fig. 6, solid circles). The decrease in anisotropy resolved to values for protein-free samples (open circles), indicating complete dissociation of fluorescent com-
plexes. Similar dissociation kinetics were observed using the U32 probe as a competitor (data not shown). However, His6-
p37AUF1-(1–257)-Fl-TNFα ARE complexes were stable in the presence of excess unlabeled R8 RNA, as minimal changes in fluorescence anisotropy were observed following its addition to the binding reaction (Fig. 6, triangles). These data demonstrate that the interaction of AUF1 with high affinity RNA targets is dynamic in solution and that U-rich RNA sequences are suffi-
cient for protein recognition.

DISCUSSION

In this study, we have employed oligoribonucleotide target sequences to investigate mechanisms involved in the RNA-de-
dependent oligomerization of AUF1. We propose that AUF1 asso-
ciates with an ARE as a dimer in solution and that this initial binding event permits subsequent interaction with additional AUF1 dimers to form the oligomeric complex. In a previous work, AUF1 multimers as large as hexamers were identified complexed with the ARE from c-fos mRNA, a 75-nucleotide sequence contributing to the rapid decay of this transcript (24). In this study, shortened RNA targets (<40 nucleotides) served to limit the complexity of the AUF1 multimers, allowing math-
ematical models of their assembly to be derived and tested. Furthermore, the use of 5'-fluorescein-labeled RNA substrates allowed RNA-protein binding events to be evaluated in solution under true equilibrium conditions by fluorescence anisotropy.

Association of His6-p37AUF1-(1–257) and His6-p37AUF1-(1–
229) with both the core ARE from TNFα mRNA as well as a uridylate homopolymer were well described by a sequential dimer binding model, allowing equilibrium constants for both stages of tetramer assembly at 25 °C to be estimated. In each case, $K_d$ values resolved to the low nanomolar range indicating that C-terminal sequences are not requisite for efficient and specific recognition of RNA target sequences.

The sequential nature of the AUF1 binding mechanism is consistent with an induced fit model (41) for ARE recognition by this protein, in which structural rearrangements in one or more binding partners are requisite for high affinity interaction between them. In particular, binding of an AUF1 dimer to an “optimal” site on a target RNA may facilitate additional binding events at adjacent suboptimal sites due to free energy contributions from protein-protein contacts. This may partially explain the sequence heterogeneity observed among different AREs (3, 9, 21, 42), since stringent sequence conservation may only be required at the initial AUF1 contact site. Although protein-protein cross-linking suggests interaction between adjacent AUF1 dimers on an ARE, it has not been proven whether binding of subsequent dimers necessarily includes the generation of additional RNA-protein contacts. However, the relative binding affinities ($K_d/K_1$) for AUF1 binding to Fl-U$_{42}$ and Fl-TNFα ARE suggest that this is the case. With Fl-U$_{32}$, the potential exists for multiple identical AUF1-binding sites on a single RNA target. The observation that $K_d > K_1$ for both AUF1 mutants binding this probe, together with the potential for tetrameric cross-linked proteins on U$_{32}$, indicates that the binding affinity of the second dimer is likely the result of both RNA-protein and protein-protein contacts. By contrast, the free energy contributing to $K_1$ is largely, if not solely, attributable to the RNA-protein interaction. By this model, the absence of cross-linked His$_{6}$-p37AUF1-(1–229) tetraters on the TNFα ARE probe, combined with resolution of $K_d < K_1$ for this interaction, indicates that multiple identical AUF1-binding sites do not exist on this RNA. However, cross-linking of His$_{6}$-p37AUF1-(1–257) tetraters on the TNFα ARE suggests that interaction between dimers of this AUF1 mutant may improve the energetics of P$_R$ complex formation. The increased ratio of $K_d/K_1$ for this binding event relative to His$_{6}$-p37AUF1-(1–229) binding supports this model and furthermore implicates AUF1 sequences between residues 229 and 257 in RNA-dependent tetramer formation on the TNFα ARE.

In this study, interaction between AUF1 and the U$_{32}$ RNA was observed using several experimental techniques. These results demonstrate that AUUUA motifs are not requisite for AUF1 binding and further suggest that uridylate residues may be the primary determinants of RNA recognition by AUF1. However, in previous studies, gel mobility shift assays failed to detect a high affinity interaction between AUF1 and a U$_{32}$ homopolymer contained within a β-globin chimeric RNA fragment (23). This β-globin/U$_{32}$ cassette was also ineffective in initiating mRNA turnover in a transfected cell system (4). One explanation for this apparent discrepancy is that AUF1 may be sterically or conformationally hindered from binding U$_{32}$ in the context of the β-globin chimera as a result of flanking RNA sequences. Interaction between AUF1 and U-rich RNA sequences is also supported by the observation that cellular AUF1 can be purified by affinity chromatography over poly(U)-agarose (25) and that uridylate residues are poorly represented in some U-rich sequences that act as mRNA destabilizers (42). Taken together, these data indicate that the role of interspersed adenylate residues in AREs may be secondary to AUF1 recognition. However, the conservation of these A residues in AREs across species (6, 7) suggests that they function at some other level, possibly involving the association of ancillary factors or by contributing to the positioning of AUF1 dimeric units in the oligomeric state. This may also account for the His$_{6}$-p37AUF1-(1–229) tetraters generated by DSP-mediated cross-linking in the presence of U$_{32}$ but not TNFα ARE (Fig. 3B), since the lack of interspersed A residues in U$_{32}$ RNA may permit conformational variants of a P$_R$ complex that are unfavorable on a bona fide ARE.

Another interesting feature of the AUF1-TNFα ARE binding equilibria were their rapid off-rates ($t_{1/2} < 10$ s), which may contribute to the efficient recognition of AREs within complex RNA populations. Whereas some complex dissociation was observed by gel mobility shift assays, the clear resolution of AUF1-RNA binding events despite rapid dissociation kinetics (Fig. 1) suggests that gel “caging” effects (43) may be involved in maintaining these complexes during electrophoresis. Alternatively, the presence of multiple RNA-binding domains within the AUF1-ARE complex may allow for an “exchange” mechanism, where interaction of an ARE with accessible RNA recognition motifs in the AUF1 complex results in the release of an RNA molecule bound at another site. In this case, AUF1-ARE interactions may be stable under limiting RNA concentrations, whereas excess target RNA would induce rapid complex dissociation.

Current data indicate that the binding of AUF1 to ARE-containing mRNAs may function as a targeting system, perhaps directing the association of other factors necessary for the initiation of mRNA turnover (21). To this end, AUF1 oligomerization may represent the ARE-dependent generation of a binding surface for some other factor(s). This notion is also supported by the hydrodynamic properties of the c-fos ARE AUF1$_6$ complex, where changes in calculated frictional ratios were consistent with maximization of protein surface area (24). Given the dynamic nature of the AUF1-ARE interaction, it is likely that binding of ancillary factors also serves to stabilize the complex in solution. Recently, several cytoplasmic proteins associated with an AUF1-containing complex have been identified, including factors participating in the regulation of translation (eIF4G) and mRNA turnover (poly(A)-binding protein) (26). Heat shock protein 70, which also co-immunoprecipitates with cytoplasmic AUF1 (26), can independently associate with A + U-rich RNA sequences (44). Once assembled, this multimeric complex may somehow target mRNAs for decay, possibly by directing specific ribonucleases to mRNAs in an ARE-dependent fashion.

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