RNA Sequence Elements Required for High Affinity Binding by the Zinc Finger Domain of Tristetraproline

CONFORMATIONAL CHANGES COUPLED TO THE BIPARTITE NATURE OF AU-RICH mRNA-DESTABILIZING MOTIFS

Brandy Y. Brewer‡, Joanna Malicka‡, Perry J. Blackshear§, and Gerald M. Wilson‡¶

From the ‡Department of Biochemistry and Molecular Biology, Center for Fluorescence Spectroscopy, and ¶Greenebaum Cancer Center, University of Maryland School of Medicine, Baltimore, Maryland 21201 and §Laboratory of Signal Transduction and the Office of Clinical Research, NIEHS, National Institutes of Health, Department of Health and Human Services, Research Triangle Park, North Carolina 27709

Tristetraproline (TTP) binds AU-rich elements (AREs) encoded within selected labile mRNAs and targets these transcripts for rapid cytoplasmic decay. RNA binding by TTP is mediated by an ∼70-amino acid domain containing two tandemly arrayed CCCH zinc fingers. Here we show that a 73-amino acid peptide spanning the TTP zinc finger domain, denoted TTP73, forms a dynamic, equimolar RNA-peptide complex with a 13-nucleotide fragment of the ARE from tumor necrosis factor α mRNA, which includes small but significant contributions from ionic interactions. Association of TTP73 with high affinity RNA substrates is accompanied by a large negative change in heat capacity without substantial modification of RNA structure, consistent with conformational changes in the peptide moiety during RNA binding. Analyses using mutant ARE substrates indicate that two adenylate residues located 3–6 bases apart within a uridylate-rich sequence are sufficient for high affinity recognition by TTP73 (Kd < 20 nM), with optimal affinity observed for RNA substrates containing AUUUA or AUUUUA. Linkage of conformational changes and binding affinity to the presence and spacing of these adenylate residues provides a thermodynamic basis for the RNA substrate specificity of TTP.

Cytoplasmic mRNA stability is an important mechanism in the regulation of gene expression. In mammalian cells, some mRNAs encoding regulatory proteins like cytokines, inflammatory mediators, and oncproteins decay very quickly, often exhibiting cytoplasmic half-lives of 30 min or less (reviewed in Refs. 1–3). Rapid turnover of these mRNAs is mediated by AU-rich elements (AREs), a loosely defined collection of uridylate-rich sequences localized to the 3′-untranslated regions of many labile transcripts. The increased turnover rates of ARE-containing transcripts are typically manifested through acceleration of deadenylation, where the poly(A) tail is progressively shortened in a 3′ → 5′ direction prior to extremely rapid degradation of the mRNA body (reviewed in Ref. 4).

The mRNA destabilizing activity of AREs is mediated by association of cytoplasmic trans-acting factors (reviewed in Ref. 5). Some factors, like members of the Hu family of RNA-binding proteins, prevent mRNA degradation (6, 7), whereas others, like AUF1 (8–10) and KSRP (11), promote rapid decay of ARE-containing transcripts. Association of tristetraproline (TTP) with ARE-containing mRNAs also promotes their rapid cytoplasmic catabolism, involving acceleration of deadenylation rates (12, 13). The significance of this mechanism is apparent from a TTP knock-out mouse model, where enhanced stability of tumor necrosis factor α (TNFα) mRNA in macrophages from TTP-deficient mice (14) produces constitutive enhancement of circulating TNFα, ultimately leading to development of a systemic inflammatory syndrome (15).

TTP is the prototype of the CCCH family of eukaryotic tandem zinc finger proteins (reviewed in Ref. 16). Interactions with selected AU-rich RNA substrates occur through the zinc finger domain, which is necessary and sufficient for high affinity RNA recognition (12). Preliminary characterization of TTP substrate selectivity suggests a preference for RNA sequences containing AUUUUA motifs, common among AREs from mRNAs encoding cytokines/lymphokines and inflammatory mediators (17–19). However, the apparent selectivity of TTP for a subset of ARE targets, coupled with the existence of several ARE-binding proteins promoting common functions (i.e. mRNA destabilization), underscores the need to understand the biophysical basis for RNA substrate preferences of these trans-acting factors.

In this study, we continue our examination of sequence-specific RNA recognition by trans-acting, ARE-binding factors by evaluating interactions between the zinc finger domain of TTP and model ARE substrates. A previous study employing a synthetic 73-amino acid peptide containing the tandem zinc fingers of TTP, termed TTP73, demonstrated that specific RNA-peptide complexes could be formed in solution using RNA substrates spanning regions of the ARE from TNFα mRNA (20). Here we have used the interaction between the TTP73 peptide and a series of short RNA oligonucleotide substrates to characterize the thermodynamics, dissociation kinetics, and substrate requirements of TTP73-RNA complexes. While providing information about the dynamics and physical forces contributing to ARE recognition by the TTP zinc finger domain, this work also presents thermodynamic evidence supporting preliminary NMR data that indicated a conformational change.
Lyophilized TTP73 peptide was resuspended in 10 mM Tris-Cl (pH 8.0), as described previously (20), and is termed TTP73. The TTP73 peptide was synthesized, purified, and refolded by Albachem Ltd. (Edinburgh, Scotland, UK), as described previously (20), and is termed TTP73. The TTP73 peptide was synthesized, purified, and refolded by Albachem Ltd. (Edinburgh, Scotland, UK), as described previously (20), and is termed TTP73. The TTP73 peptide was synthesized, purified, and refolded by Albachem Ltd. (Edinburgh, Scotland, UK), as described previously (20), and is termed TTP73. The TTP73 peptide was synthesized, purified, and refolded by Albachem Ltd. (Edinburgh, Scotland, UK), as described previously (20), and is termed TTP73.

The TTP73 peptide accompanying association with high affinity RNA substrates (20). Subsequent studies using mutant RNA substrates provide evidence that conformational changes in the peptide-RNA complex are coupled to high affinity binding and are linked to the presence of two adenylate residues within a U-rich RNA sequence, thus establishing a thermodynamic basis for discrimination of RNA substrates by TTP73. Furthermore, the moderately rapid dissociation kinetics of RNA complexes containing the TTP zinc finger domain support a model of regulated mRNA turnover involving dynamic competition between various ARE-binding proteins in the cytoplasm.

**EXPERIMENTAL PROCEDURES**

**RNA Substrates**—Sequences of RNA oligonucleotides used in this study are listed in Table I. Synthesis, deprotection of 2'-hydroxyl groups, and purification of RNA substrates were performed by Dharmaciaon Research (Lafayette, CO) or Integrated DNA Technologies (Corvali, OR). Dried RNA pellets were resuspended in 10 mM Tris-Cl (pH 8.0) and quantified by Coomassie Blue-stained SDS-PAGE following electrophoresis through 6% (40:1 acrylamide:bisacrylamide) native gels at 4 °C. Following electrophoresis, gels were dried, and the location of reaction products was identified by PhosphorImager scan (Amersham Biosciences).

**Fluorescence Anisotropy Assays**—For quantitative analyses of RNA-protein binding equilibria, a fluorescence anisotropy-based assay was employed to that described previously (21, 22). Briefly, fluorescein (Fl)-labeled RNA substrates were incubated with TTP73 in reactions similar to those described for GMSAs (above), except that glycerol was omitted; final volume was brought to 100 μl, and binding reactions were performed at 25 °C unless otherwise noted. Fluorescence anisotropy was measured with a Beckman 2000 Fluorescein Polarization System (Pavilion 2, Madison, WI) using fluorescein excitation (λex = 490 nm) and emission (λem = 535 nm) filters. Association of TTP73 with Fl-RNA substrates was detected by an increase in anisotropy of Fl-RNA emission, because of restricted segmental motion and retarded rotational correlation time in the RNA-protein complex relative to the free RNA substrate (23-25).

In all experiments reported in this work, protein binding did not induce a change in the fluorescence quantum yield of Fl-RNA substrates. As such, the measured fluorescence anisotropy (A0) of each binding reaction was interpreted as a function of the intrinsic anisotropy (A0) and fractional concentration (f) of each fluorescent species using Equation 1 (24, 26).

A0 = A0 + A0pK(TTP73) + K(TTP73)  

This equation represents the intrinsic anisotropy constant, and A0 and A0p represent the intrinsic anisotropy values of the free and protein-associated Fl-RNA substrates, respectively. A0 was measured as the anisotropy of each RNA substrate in the absence of TTP73 (n ≥ 3), whereas all other constants were solved by nonlinear least squares regression of A0 versus [TTP73] using PRISM version 3.0 (GraphPad, San Diego). To measure RNA-peptide complex dissociation rates, binding reactions containing TTP73 and Fl-RNA substrates were assembled as described above with anisotropy measured at equilibrium (τ = 0). Following addition of a 5000-fold excess of unlabeled RNA, anisotropy was measured in intervals of 15 s, with five measurements taken for each time point.

**Thermodynamic Analyses of Peptide-RNA Binding Equilibria**—Enthalpic (ΔH°) and entropic (ΔS°) contributions to the free energy of TTP73 binding to RNA substrates were estimated from the temperature dependence of equilibrium binding constants by van’t Hoff plots of log K versus 1/T. This plot is linear when ΔH° is independent of temperature (27, 28) and may be resolved by Equation 5, where R is the gas constant (1.987 × 10-3 kcal mol⁻¹ K⁻¹).

ln(K) = (ΔH°/RT) + (ΔS°/R)  

When significantly nonlinear, ln(K) versus 1/T plots were resolved using Equation 4, where ΔC°p,obs represents the change in molar heat capacity, and T0 and Tp represent characteristic temperatures at which enthalpy and entropy, respectively, contribute no net energy to formation of the peptide-RNA complex (28).

ln(K) = (ΔC°p,obs/T0)(T/T0 - ln(T/T0))  

Solution of ΔC°p,obs, T0, and Tp then allows the contributions of enthalpy and entropy to the energy of peptide-RNA complex formation to be calculated as a function of temperature using Equations 5 and 6, respectively.

ΔH° = ΔC°p,obs(T - T0)  

ΔS° = TΔC°p,obs ln(T/T0)  

**Analysis of RNA Folding by Fluorescence Resonance Energy Transfer (FRET)**—Potential conformational changes in ARE-containing RNA substrates induced by peptide binding were evaluated by FRET, based on the intramolecular distance between the 5'-Fl (donor) and 3'-Cy5 (acceptor) moieties of a model RNA substrate (Fl-ARE-AU-A-Cy5).

### Table I

RNA substrates used in this study

| Name | Sequence (5'→3') |
|------|-----------------|
| Fl-ARE15 | F1-AUUUUAAUAAUA |
| ARE15 | AUUUUAAUAAUA |
| Fl-ARE9 | F1-AUUUUAA |
| Fl-AREU | F1-AUUUUAAU |
| Fl-REV | F1-AUUUUUUA |
| Fl-ARU | F1-AUUUUUUA |
| Fl-AU-A | F1-AUUUUUUA |
| Fl-AU-U | F1-AUUUUUUA |
| Fl-AU-A | F1-AUUUUUUA |
| Fl-AU-A | F1-AUUUUUUA |
| Fl-AU-A-Cy5 | F1-AUUUUUUAUUUUA-UUUU |
| Fl-AU-A-Cy5 | F1-AUUUUUUAUUUUA-UUUU |
| Fl-ARE-AU-A-Cy5 | F1-AUUUUUUAUUUUA-UUUU-Cy5 |

*“Fl” and “Cy5” indicate the positions of the fluorescein and cyanine-5 moieties, respectively, conjugated to applicable RNA substrates.*
RNA Recognition by Tristetraprolin Zinc Finger Domain

FRET efficiency ($E_{FRET}$) is dependent on the scalar distance ($r$) between the donor and acceptor by Equation 7.

$$E_{FRET} = R_0^2/(R_0^2 + r^2)$$  \hspace{1cm} (Eq. 7)

$R_0$ is the Förster distance for the donor-acceptor pair or the scalar distance yielding FRET efficiency of 50% (29, 30). A value of $R_0$ for the Fl-Cy5 dye pair linked to single-stranded RNA was calculated using Equation 8.

$$R_0 = 0.211 \times (k^2 n^4 Q_d J(\lambda))^1/3$$  \hspace{1cm} (Eq. 8)

Here $k^2$ is the orientation factor for dipole-dipole coupling; $n$ is the refractive index of the medium; $Q_d$ is the quantum yield of the donor in the absence of the acceptor; and $J(\lambda)$ is the overlap integral between donor emission and acceptor absorbance, because Fl and Cy5 were conjugated to the RNA moiety using 6- and 3-carbon linkers, respectively. Further support for this approximation is given by the very low anisotropy of fluorescein emission ($r_0 = 0.022 ± 0.002$) of the Fl-ARE-AU substrate at 25 °C. Because all fluorescence measurements were performed in dilute aqueous solution, $n$ was set to 1.333. $Q_d$ was measured as 0.40 using the Fl-ARE-AU substrate as described (31), with 3-aminofluoranthene in Me2SO ($Q = 0.32$ at 25 °C) as a reference standard (32). The overlap integral $J(\lambda)$ was calculated from the fluorescence emission spectrum of Fl ($F_d(\lambda)$) and the absorbance spectrum of Cy5 ($c_\lambda(\lambda)$) using Equation 9 (data not shown).

$$J(\lambda) = \int F_d(\lambda)c_\lambda(\lambda)\lambda^4 d\lambda$$ \hspace{1cm} (Eq. 9)

From these data, $J(\lambda)$ resolved to $1.49 \times 10^{-20}$ cm$^2$mol$^{-1}$s$^{-1}$nm$^{-4}$, yielding $R_0$ of 47 Å.

Fluorescence emission spectra were measured using a Cary Eclipse fluorescence spectrophotometer (Varian Instruments, Walnut Creek, CA) with a Peltier temperature control cell ($\lambda_{exc} = 485$ nm, 10 nm bandwidth) at 25 °C. $E_{FRET}$ values were calculated from paired measurements of blank-corrected donor emission (518 nm) using RNA substrates containing either the FRET donor alone (Fl-ARE-AU; $F_d$) or the donor-acceptor pair (Fl-ARE-AU-A-Cy5; $F_{DA}$) by Equation 10 (30, 39).

$$E_{FRET} = 1 - (F_{DA}/F_d)$$ \hspace{1cm} (Eq. 10)

RESULTS

The Zinc Finger Domain of TTP Forms a Dynamic, Equimolar Complex with the ARE$_{13}$ Substrate—Previously we demonstrated that TTP73 could bind a 20-nucleotide fragment of the ARE from TNFα mRNA with high affinity (20). Whereas this equilibrium was largely consistent with 1:1 stoichiometry of peptide:RNA, a minor but detectable complex consistent with two protein binding events was detected at peptide concentrations above 5 nM. However, we and others (17) have detected interactions between the TTP zinc finger domain and RNA substrates as small as 9–13 nucleotides. Accordingly, this study focused on the recognition of small (≤13 nucleotides) RNA ligands by TTP73, to minimize the potential for multiple peptide binding events.

The RNA substrate ARE$_{13}$ encodes a sequence contained within the TNFα ARE. By GMSA, TTP73 formed a single RNA-peptide complex with the radiolabeled ARE$_{13}$ ligand in a concentration-dependent manner (Fig. 1A). Binding was absolutely dependent on the presence of Zn$^{2+}$, verifying that zinc finger-independent mechanisms did not make significant contributions to RNA binding activity in this system. Also, RNA binding activity was dependent on the ARE sequence, because TTP73 did not form detectable complexes with the unrelated Rb RNA substrate. Using the fluorescence anisotropy assay, TTP73 peptide binding to a fluorescein-labeled ARE$_{13}$ sub-

FIG. 1. Detection of TTP73-ARE$_{13}$ complexes by gel mobility shift assay. A, schematic of the TTP73 peptide. Solid circles indicate Cys and His residues contributing to co-ordination of Zn$^{2+}$ in each zinc finger. B, binding reactions containing $^{32}$P-labeled RNA substrates (0.2 nm) and varying concentrations of TTP73 peptide were fractionated by native gel electrophoresis. The position of the complex formed by TTP73 binding to the ARE$_{13}$ substrate is indicated. Samples lacking Zn$^{2+}$-included EDTA (0.5 mm) to scavenge residual Zn$^{2+}$ from other reaction components.

- **Fig. 1A**: A schematic of the TTP73 peptide shows the zinc finger domain of TTP. (i) The TTP73 peptide has a predicted isoelectric point of 9.34 and is expected to exhibit a net charge of +5 at pH 8. (ii) Coordination of two Zn$^{2+}$ ions contributes additional positive charge to the peptide. (iii) Several amino acid residues with basic side chains are located within each zinc finger motif. (iv) NMR structural analysis of an ARE-peptide complex containing the zinc finger domain of a TTP-related peptide, TIS11d, shows four basic amino acid side chains in close proximity to the RNA phosphodiester backbone (36). These basic residues are conserved in the zinc finger domain of TTP.
RNA Recognition by Tristetraprolin Zinc Finger Domain

To evaluate the net influence of ion pairs on the stability of TTP73-Fl-ARE13 ribonucleoprotein (RNP) complexes, equilibrium binding constants were measured under conditions of increasing ionic strength by varying the concentration of KCl from 50 to 500 mM, and plotted as log([K]) versus log([K]) (Fig. 4). The slope of this plot is influenced by the number ion pairs formed during RNA-protein complex assembly (37). The salt dependence of TTP73-Fl-ARE13 RNP formation yielded −dlog(K)/dlog([K]) = 1.1 ± 0.2 (regression solution ±95% confidence interval). This value is similar to RNP complexes containing trp RNA-binding attenuation protein (38), RNase A (35), and Hsp70 (39) but significantly lower than that expected for RNPs with large ionic components, including U1A (40) and ribosomal protein S8 (37) bound to cognate RNA substrates (−dlog(K)/dlog([K]) > 3). The modest value of this parameter indicates that ionic interactions make significant but relatively minor contributions to the stability of the TTP73-Fl-ARE13 RNP, confirming that nonelectrostatic mechanisms, possibly including base-specific contacts or stacking interactions, are critical for assembly of this RNA-protein complex.

RNA Sequence Elements Contributing to High Affinity Binding of the TTP73 Peptide—AREs constitute a diverse collection of mRNA-distabilizing sequences and may be recognized by many different cytoplasmic RNA-binding proteins (4, 5). Some ARE-binding factors, including AUFI (23, 41), Hsp70 (39), HnA (18), and HuD (17), appear to show little selectivity among ARE templates, other than a general preference for U-rich sequences. However, some recent studies (17–19) indicate that TTP binding may be selective for RNA substrates containing AUUUA motifs, whose appearance is restricted to a subset of known AU-rich mRNA-distabilizing sequences. To identify specific RNA sequence elements contributing to TTP association with U-rich RNA sequences, the binding affinity of the TTP73 peptide for a series of mutant ARE substrates was measured using the fluorescence anisotropy assay. Data generated through these experiments (Table II) revealed three principal RNA sequence features contributing to optimal TTP73 binding. First, optimal TTP73 binding requires an RNA substrate greater than 7 nucleotides in length (cf. Fl-ARE13 versus Fl-ARE9) and may be effectively attained with substrates as small as nine nucleotides (cf. Fl-ARE9 versus Fl-ARE13 and Fl-ARE-AU3A). Second, optimal binding of TTP73 requires two adenylyl residues within the U-rich motif (cf. Fl-ARE-AU3A and Fl-ARE-UU3A versus Fl-ARE-AU3A; Fl-ARE-AU3A and Fl-ARE-UU3A versus Fl-ARE9), with each adenylyl residue contributing to total binding affinity (i.e. K

**Fig. 2.** Measurement of TTP73-Fl-ARE13 binding affinity by fluorescence anisotropy. Binding reactions containing the Fl-ARE13 RNA substrate (0.2 nM) and a titration of TTP73 peptide were assembled as described under “Experimental Procedures.” A, total fluorescence intensity (λex = 490 nm; λem = 535 nm) was monitored as a function of TTP73 concentration in binding reactions containing ZnCl2 (5 μM) to detect any change in the fluorescence quantum yield of the Fl-ARE13 RNA substrate following peptide binding. B, fluorescence anisotropy was measured for reactions containing (solid circles) or lacking (open circles) 5 μM ZnCl2. Samples lacking ZnCl2 included EDTA (0.5 mM). TTP73-dependent changes in the anisotropy of the Fl-ARE13 substrate (+Zn2+) were resolved by nonlinear regression using Equation 2 (solid line). A residual plot (bottom) was prepared to detect any bias for data subsets in the regression solution.

**Fig. 3.** Off-rate analysis of TTP73-Fl-ARE13 complexes. TTP73-Fl-ARE13 complexes were prepared by incubation of TTP73 peptide (20 nM) with Fl-ARE13 substrate (0.2 nM). At equilibrium, the fluorescence anisotropy of the mixture was measured (t = 0). Following addition of a 5000-fold excess of unlabeled ARE13 substrate, anisotropy measurements were taken as a function of time (solid circles), with each point representing the mean ± S.D. of three independent reactions. Data were analyzed by nonlinear regression using a first-order exponential decay function (solid line). Specificity is shown using a 5000-fold excess of unlabeled Rβ substrate as competitor, which was unable to displace the Fl-ARE13 ligand (open circles).

**Fig. 4.** Sensitivity of TTP73-Fl-ARE13 binding equilibria to ionic strength. The affinity of TTP73 peptide binding to the Fl-ARE13 RNA substrate was assayed across a range of 50–500 mM KCl. Association constants (K) were calculated at each tested concentration of KCl based on nonlinear regression of a minimum of 20 data points using Equation 2 and are plotted as log(K ± 95% confidence interval) versus −dlog([KCl]) for each experiment. An index of ionic sensitivity for the TTP73-Fl-ARE13 binding equilibrium was calculated as −dlog(K)/dlog([KCl]) (solid line).
for Fl-ARE-AU3 < Fl-ARE-AU3A and Fl-ARE-UA3A < Fl-ARE-
AU3A). A cytidine residue may not substitute for either adeny-
late within the AUUUA motif (cf. Fl-ARE-AU3 versus Fl-
ARE-AU3A; Fl-ARE-CU3A versus Fl-ARE-UA3A). However, TTP73 also dis-
played strong affinity for substrates containing AU 4A, and
Fl-ARE-AU4A 1.6
between two adenylate residues
inhibits TTP73 peptide binding relative to poly(U) substrates
containing the canonical ARE motif AU3A. However, TTP73 also
displayed strong affinity for substrates containing UA3A, and
intermediate affinity for those containing AU3A and UA3A,
relative to substrates containing a single adenylate residue (i.e.
K<sub>off</sub> for Fl-ARE-AU3A > Fl-ARE-AU3A > Fl-ARE-AU3A > Fl-ARE-
AU3A > Fl-ARE-UA3A, Fl-ARE-UA3A). By contrast, placement
of a single uridylate between two adenylate residues
inhibits TTP73 peptide binding relative to poly(U) substrates
containing a single A (cf. Fl-ARE-AUA versus Fl-ARE-AU3A and
Fl-ARE-UA3A). Together, these data indicate that U-rich
RNA sequences containing the motif AU3A (n = 2–5) may serve
as moderate to high affinity (K<sub>off</sub> <20 nM at 25 °C) TTP-binding
sites.

Specific RNA Substrates Induce a Significant Negative Change in Heat Capacity upon TTP73-RNA Complex Formation—Contributions of enthalpy and entropy to the stability of TTP73-RNA complexes were evaluated by the temperature dependence of equilibrium binding constants. A van’t Hoff plot of ln(K) versus 1/T for TTP73 binding to the Fl-ARE<sub>13</sub> substrate was clearly nonlinear (Fig. S4a), indicating that ∆H<sub>T</sub> varied with temperature. Nonlinear regression using Equation 4 indicated that formation of the TTP73-Fl-ARE<sub>13</sub> RNP was accompanied by a large negative change in heat capacity (Table III). Solutions of ∆H<sub>T</sub> and T∆S<sub>T</sub> versus T revealed that changes in enthalpy and entropy accompanying TTP73-Fl-ARE<sub>13</sub> complex formation are strongly temperature-dependent (Fig. 5B). Below 19 °C (292 K), TTP73 binding to the Fl-ARE<sub>13</sub> substrate is driven entirely by favorable changes in entropy (∆S<sub>T</sub> > 0), which compensate for unfavorable enthalpic changes (∆H<sub>T</sub> > 0). Between 19 and 24 °C (297 K), both entropy and enthalpy contribute to complex stability, whereas above 24 °C, formation of the TTP73-Fl-ARE<sub>13</sub> RNP is driven entirely by enthalpy, which must overcome unfavorable entropic changes in this

A. RNA Recognition by Tristetraprolin Zinc Finger Domain

B. System

Fig. 5. Temperature dependence of TTP73-Fl-ARE<sub>13</sub> binding equilibria. The binding affinity of the TTP73 peptide for the Fl-ARE<sub>13</sub> RNA substrate was assayed by fluorescence anisotropy across a temperature range of 10–41 °C. Association constants (K) at each temperature (T) were derived from nonlinear regression of a minimum of 20 data points using Equation 2. A, a van’t Hoff plot of ln(K) versus 1/T. Points are based on the regression solutions of K ±5% confidence interval of each independent binding experiment. Nonlinear regression of this plot using Equation 4 yielded the change in heat capacity (∆C<sub>P</sub> at each specified temperature Ts, enthalpic (∆H<sub>T</sub>, solid line) and entropic (∆S<sub>T</sub>, dashed line) contributions to the free energy of TTP73-Fl-ARE<sub>13</sub> complex formation as a function of temperature were calculated using Equations 5 and 6, respectively. Enthalpy and entropy contribute no energy to ∆G<sub>T</sub> at their respective critical temperatures, Ts<sub>H</sub> and Ts<sub>S</sub>, indicated by the intersection of these functions with ∆G<sub>T</sub> = 0 (dotted line). The total change in free energy upon peptide-RNA complex formation (open circles) was calculated at assayed temperatures using ∆G<sub>T</sub> = −RTln(K).

By contrast, no significant change in heat capacity accompanied formation of the TTP73-Fl-ARE<sub>13</sub> complex (∆C<sub>P</sub><sub>obs</sub> = 0.0 ± 0.3 kcal/mol, data not shown). Together, these experiments indicated that RNA substrates containing a single AUUUA motif were able to direct a change in heat

Table II

| RNA substrate | K at 25 °C | n | K<sub>off</sub> at 25 °C | ∆G<sub>T</sub> at 25 °C |
|---------------|-----------|---|-------------------|-----------------|
| Fl-ARE<sub>13</sub> | 2.8 ± 0.5 | 4 | 3.6 | −11.5 |
| Fl-ARE<sub>9</sub> | 3.3 ± 0.4 | 3 | 3.0 | −11.6 |
| Fl-ARE<sub>3</sub> | 0.52 ± 0.07 | 3 | 19 | −10.5 |
| Fl-U<sub>13</sub> | 0.063 ± 0.002 | 2 | 280 | −8.9 |
| Fl-ARE-AU3A | 3.1 ± 0.4 | 2 | 3.2 | −11.6 |
| Fl-ARE-AU3U | 0.18 ± 0.01 | 2 | 56 | −9.9 |
| Fl-ARE-AU3C | 0.12 ± 0.02 | 3 | 83 | −9.7 |
| Fl-ARE-AU3G | 0.36 ± 0.07 | 3 | 28 | −10.3 |
| Fl-ARE-UU3A | 0.19 ± 0.02 | 2 | 54 | −9.9 |
| Fl-ARE-CU3A | 0.11 ± 0.02 | 2 | 93 | −9.6 |
| Fl-ARE-GU3A | 0.36 ± 0.03 | 2 | 25 | −10.3 |
| Fl-ARE-UG3A | 0.079 ± 0.005 | 2 | 130 | −9.4 |
| Fl-ARE-UC3A | 0.085 ± 0.009 | 2 | 120 | −9.4 |
| Fl-ARE-UG | 0.064 ± 0.005 | 2 | 160 | −9.3 |
| Fl-ARE-AU3A | 0.55 ± 0.06 | 2 | 18 | −10.6 |
| Fl-ARE-AU3A | 1.6 ± 0.2 | 3 | 6.4 | −11.2 |
| Fl-ARE-UA3A | 0.58 ± 0.04 | 2 | 17 | −10.6 |

* Mean ± σ<sub>n</sub> for n independent experiments where n > 2, or mean ± spread for n = 2. * K<sub>off</sub> estimated as 1/K using mean value of K. Errors are hyperbolic about K<sub>off</sub> but significant differences may be inferred by comparison of K values. * Calculated as ∆G<sub>T</sub> = −RTln(K).
RNA Recognition by Tristetraprolin Zinc Finger Domain

| RNA substrate | \( \Delta C_{\text{P,obs}}^{0} \) kcal/mol | \( T_{\text{d}}^{0} \) K | \( T_{\text{s}}^{0} \) K | \( \Delta H^{0} \), 37 °C kcal/mol | \( T_{\text{d}}^{0} \), 37 °C K mol K kcal | \( T_{\text{s}}^{0} \), 37 °C K mol K kcal |
|---------------|----------------------------------|----------------|----------------|---------------------------------|---------------------------------|---------------------------------|
| Fl-ARE\(_{13}\) | -2.5 ± 0.2 | 292.0 ± 0.5 | 296.5 ± 0.4 | -46 ± 5\(^{a}\) | -35 ± 4\(^{a}\) | 26 ± 2 \(^{c}\) |
| Fl-ARE\(_{9}\) | -2.5 ± 0.2 | 287.3 ± 0.8 | 292.1 ± 0.5 | -57 ± 7\(^{a}\) | -46 ± 5\(^{c}\) | 26 ± 2 \(^{c}\) |
| Fl-ARE\(_{A}\) | ~0 | NA\(^{d}\) | NA\(^{d}\) | -26 ± 2 \(^{c}\) | -16 ± 2 \(^{c}\) | NA\(^{d}\) |
| Fl-ARE-AU\(_{A}\) | -2.0 ± 0.3 | 289 ± 2 | 294.4 ± 0.9 | -43 ± 8\(^{a}\) | -32 ± 6\(^{a}\) | NA\(^{a}\) |
| Fl-ARE-AU\(_{U}\) | ~0 | NA\(^{d}\) | NA\(^{d}\) | -29 ± 2 \(^{c}\) | -20 ± 2 \(^{c}\) | ND\(^{a}\) |
| Fl-ARE-UU\(_{A}\) | -3.4 ± 0.5 | 280 ± 2 | 283 ± 1 | ND\(^{a}\) | ND\(^{a}\) | ND\(^{a}\) |

\(^{a}\) Values calculated from nonlinear regression solutions of Equation 4 are shown in Figs. 5A and 6. Values are quoted as the regression solution ± S.E. of regression, calculated by PRISM version 3.0, and equivalent to approximately one-half of the 95% confidence interval.

\(^{b}\) Values were calculated using Equation 5 and incorporating errors from \( \Delta C_{\text{P,obs}}^{0} \) and \( T_{\text{d}}^{0} \).

\(^{c}\) Values were calculated using Equation 6 and incorporating errors from \( \Delta C_{\text{P,obs}}^{0} \) and \( T_{\text{s}}^{0} \).

\(^{d}\) Not applicable, given \( \Delta C_{\text{P,obs}}^{0} \sim 0 \).

\(^{e}\) Values were calculated from linear regression solutions of Equation 3 as shown in Fig. 6.

\(^{f}\) Not determined, since the Fl-ARE-UU\(_{A}\) RNA substrate does not show detectable binding to the TTP73 peptide at 37 °C.

(Fl-ARE-AU\(_{U}\)) completely abrogated the change in heat capacity upon TTP73 binding (\( \Delta C_{\text{P,obs}}^{0} = -0.4 \pm 0.4 \) kcal/mol, data not shown), permitting a linear solution for \( \ln(K) \) versus \( 1/T \). However, association of TTP73 with the 5’ A–U substitution mutant substrate (Fl-ARE-UU\(_{A}\)) yielded a large negative \( \Delta C_{\text{P,obs}}^{0} \) (Table III) but also displayed a severe temperature dependence that precluded any significant RNA binding activity above 30 °C (\( K_{d} > 500 \) nM). From these observations, we infer that the 3’-adenylate residue within the AUUUAA motif is essential for the observed change in heat capacity upon TTP73 binding. By contrast, the 5’-adenylate is dispensable for this change in heat capacity but strongly prevents binding at elevated temperatures, when enthalpic contributions to binding energy are required to overcome the entropic penalty of conformational change (i.e. Fig. 5B).

Conformational Changes Accompanying TTP73 Binding to a High Affinity RNA Substrate Do Not Involve Significant Alteration of RNA Structure—Changes in heat capacity resulting from TTP73 binding to high affinity RNA substrates Fl-ARE\(_{13}\), Fl-ARE\(_{9}\), and Fl-ARE-AU\(_{A}\) (Table III) are consistent with conformational changes in one or more components of these TTP73-RNA complexes. Although a previous NMR study (20) indicated that the inherently unfolded C-terminal zinc finger of TTP73 became ordered as a result of interactions with its target as a function of TTP73 peptide binding. It was unclear whether the RNA moiety also experienced significant conformational changes during peptide-RNA complex formation. To determine whether TTP73 may alter local RNA structure, FRET was employed to measure changes in the distance between the 5’ and 3’ termini of a high affinity RNA target as a function of TTP73 peptide binding.

The RNA substrate Fl-ARE-AU\(_{A}\)-Cy5 (Fig. 7A) incorporates a FRET donor (Fl) and acceptor (Cy5) at its 5’- and 3’-ends, respectively (Table I). The emission spectrum of this substrate in the absence of TTP73 revealed characteristic peaks for both Fl and Cy5 (667 nm) (Fig. 7B, solid line). Digestion of the RNA substrate with RNase A resulted in increased donor emission with a concomitant decrease in acceptor emission (Fig. 7B, dotted line), indicating that the distance (\( r \)) between the 5’- and 3’-ends of the intact Fl-ARE-AU\(_{A}\)-Cy5 RNA substrate was within the detection limit of FRET. Based on Fl emission from RNA substrates Fl-ARE-AU\(_{A}\)-Cy5 (\( F_{\text{D}} \), Fig. 7C, solid line) and Fl-ARE-AU\(_{A}\)-Cy5 (\( F_{\text{D}} \), Fig. 7D, solid line), \( E_{\text{FRET}} \) for the unbound Fl-ARE-AU\(_{A}\)-Cy5 substrate was calculated as 0.34 ± 0.01 by Equation 10. However, addition of TTP73 (500 nM) did not appreciably alter the emission spectra of ARE-AU\(_{A}\) substrates containing the donor fluorophore alone (Fl-ARE-AU\(_{A}\); Fig. 7D) or the donor-acceptor pair (Fl-ARE-AU\(_{A}\)-Cy5; Fig. 7E). Samples spanning a titration of TTP73 peptide concentrations yielded no significant change in \( E_{\text{FRET}} \) (Fig. 7F).
The moiety may release energy by quantum emission (by nonquantum events, including excitation of Cy5 by FRET. Addition of TTP73 peptide (500 nM). Cy5 (as described under “Experimental Procedures.” Each point indicates measured by FRET. 

D and C, 20 min, 25 °C). Positions of Fl and Cy5 emission peaks are indicated. At its 5’-end and Cy5 at the 3’-end. Following excitation at 485 nm, the Fl moiety may release energy by quantum emission (λ_{max} = 518 nm) or by nonquantum events, including excitation of Cy5 by FRET. B, emission spectra of the Fl-ARE-AU3U-Cy5 RNA substrate (2 nm) prior to (solid line) and following (dotted line) digestion with RNase A (1 μg/ml, 20 min, 25 °C). Positions of Fl and Cy5 emission peaks are indicated. C and D, emission spectra of the Fl-ARE-AU3U (C) and Fl-ARE-AU3U-Cy5 (D) RNA substrates prior to (solid line) and following (dotted line) addition of TTP73 peptide (500 nM). E, values of E_{FRET} for the Fl-ARE-AU3U-Cy5 RNA substrate as a function of TTP73 concentration, solved as described under “Experimental Procedures.” Each point indicates the mean ± spread of two independent experiments.

consistent with inter-fluorophore distances of 52–54 Å for all cases, based on E_{FRET} values from 0.31 to 0.35. Evaluation of TTP73 binding to the Fl-ARE-AU3U-A-Cy5 substrate by changes in Fl anisotropy verified that peptide binding was not inhibited by the 3’-Cy5 modification (data not shown). Because the distance between the RNA 5’ and 3’ termini does not detectably change as a result of TTP73 peptide binding, we conclude that gross alterations in RNA conformation do not substantially contribute to changes in system heat capacity accompanying TTP73-RNA complex formation. These findings do not, however, preclude the possibility that minor, localized alterations in RNA structure may contribute to changes in heat capacity upon TTP73 binding to selected RNA substrates.

**DISCUSSION**

AREs direct the rapid decay of many cellular mRNAs, yet the sequence diversity of these mRNA-stabilizing and destabilizing sequences and the plethora of proteins known to interact with them suggest that competitive or combinatorial binding events may contribute to the regulation of ARE function. The former possibility is supported by observations that several ARE-binding factors, including the mRNA-stabilizing factor HuD (17), the mRNA-binding domain of TTP (this work), Hsp70 (39), and the mRNA-stabilizing factor HU (21) all bind AU-rich RNA substrates through moderately dynamic mechanisms, with dissociative half-times varying from 10 to 120 s. By contrast, some other sequence-specific nucleic acid-binding proteins, including U1A (40) and the TATA-binding protein (41), exhibit significantly slower dissociation kinetics (t_{1/2} > 20 min). Accordingly, it is conceivable that cellular control of ARE-directed mRNA decay kinetics may include modulation of the cytoplasmic concentration, RNA binding activity, or even the RNA-binding dynamics of selected ARE-binding proteins.

Although many proteins associate with AU-rich mRNA substrates, emerging data indicate that different ARE-binding factors display a spectrum of distinct but overlapping RNA sequence preferences. From comparisons of thermodynamic parameters describing binding of the TTP73 peptide with both high and low affinity RNA substrates, we have shown that high affinity interactions between the TTP zinc finger domain and ARE-like RNA substrates involve several features. First, the two adenylate residues contained within the canonical AUUUUA motif are required for high affinity binding, but some variation is permitted in the spacing between these residues. Second, uridylylate residues flanking the AUUUUA motif contribute to peptide binding. Together, these RNA substrate requirements for high affinity TTP73 binding account for the following results: (i) in vitro RNA selection experiments using recombinant TTP, where a preference for AUUUUA motifs flanked by uridylylate residues was observed (19); (ii) the ability of AREs from different mRNAs to compete for TTP binding activity in cell extracts (18). In the latter study, only RNA competitors containing one or more AUUUUA or AUUUUA motifs flanked by uridylylate residues were able to effectively displace TTP from a high affinity ARE substrate.

Recently, an NMR study using the tandem zinc finger domain of a TTP-related protein, TIS11d, indicates that both zinc fingers display similar structures upon binding an ARE substrate and interact via a 3’ → 5’ polarity with tandem AUUUUA motifs (36). Extrapolation of the TIS11d ARE complex model to the TTP73 peptide is supported by the strong binding affinity (K_{d} < 4 nM) and negative changes in heat capacity observed for binding events between TTP73 and all tested RNA substrates containing tandem AUUUUA motifs (FI-ARE-AU3U, FI-ARE-AU3U, and FI-ARE-AU3U). However, additional mechanistic details of ARE recognition by TTP may be extracted from binding studies involving mutant ARE substrates and support a model whereby conformational changes, most likely including the peptide C-terminal zinc finger, are coupled to the RNA substrate sequence and contribute to optimal RNA binding activity. First, high affinity RNA binding requires a conformational transition denoted by a change in heat capacity. Given (i) that the RNA substrate does not exhibit a significant structural change upon TTP73 binding (Fig. 7), and (ii) previous observations that structural ordering of the TTP73 C-terminal zinc finger requires association with high affinity RNA substrates (20), we conclude that conformational changes in the C-terminal zinc finger domain contribute significantly to the large negative change in heat capacity accompanying TTP73-RNA complex formation. Second, induction of conformational changes likely requires peptide-RNA contacts involving both zinc fingers. The N-terminal finger of TTP may interact independently with RNA substrates of the type UUUAUUU; however, binding affinity is very poor (K_{d} = 4.7 μM) (44). TTP73 binding to the FI-ARE-AU3U substrate yielded improved sta-
RNA Recognition by Tristetraprolin Zinc Finger Domain

REFERENCES

1. Ross, J. (1995) Microbiol. Rev. 59, 425–450
2. Gouhary, Y., and Brewer, G. (2001) Gene (Amst.) 265, 11–23
3. Wilusz, C. J., Worthington, M., and Peltz, S. W. (2001) Nature Rev. Mol. Cell. Biol. 2, 237–246
4. Chen, C.-Y. A., and Shyu, A.-B. (1995) Trends Biochem. Sci. 20, 465–470
5. Wilson, G. M., and Brewer, G. (1999) Prog. Nucleic Acids Res. Mol. Biol. 62, 257–291
6. Peng, S. S. Y., Chen, C.-Y. A., Xu, N., and Shyu, A.-B. (1998) EMBO J. 17, 3461–3470
7. Fan, X. C., and Steitz, J. A. (1998) EMBO J. 17, 3448–3460
8. Laflun, P., Chen, C.-Y. A., and Shyu, A.-B. (1999) Genes Dev. 13, 1884–1897
9. Lapucci, A., Denucci, M., Papucci, L., Witten, E., Tempestini, A., Bevilacqua, A., Nilson, A., Brewer, G., Schiavon, N., and Capaccioli, S. (2002) J. Biol. Chem. 277, 16139–16146
10. Sarkar, R., Xi, Q., He, C., and Schneider, R. J. (2003) Mol. Cell. Biol. 23, 6685–6693
11. Chen, C.-Y., Gherzi, R., Ung, S.-E., Chan, E. L., Rajmukers, R., Pruijn, G. J. M., Stoecklin, G., Moroni, C., Mann, M., and Karin, M. (2001) Cell 107, 451–464
12. Lai, W. S., Carballo, E., Thorn, J. M., Kennington, E. A., and Blackshear, P. J. (2000) J. Biol. Chem. 275, 17837–17837
13. Lai, W. S., Carballo, E., Strum, J. R., Kennington, E. A., Phillips, R. S., and Blackshear, P. J. (1999) Mol. Cell. Biol. 19, 4311–4323
14. Carballo, E., Lai, W. S., and Blackshear, P. J. (1998) Science 281, 1001–1005
15. Toker, G. A., Carballo, E., Lee, D. M., Lai, W. S., Thompson, M. P., Patel, D. D., Schenkenman, D. I., Gilkeson, G. S., Broxmeyer, H. E., Haynes, B. F., and Blackshear, P. J. (1996) Immunity 4, 445–454
16. Blackshear, P. J. (2002) Biochem. Soc. Trans. 30, 945–952
17. Park-Lee, S., Kim, S., and Laird-Offringa, I. A. (2003) J. Biol. Chem. 278, 39801–39809
18. Raghavan, A., Robison, R. L., McNabb, J., Miller, C. R., Williams, D. A., and Raghavan, A. (2001) J. Biol. Chem. 276, 6763–6772
19. Wilson, G. M., Sun, Y., Hu, L., and Brewer, G. (1999) J. Biol. Chem. 274, 492–503
20. Wilson, G. M., Stuphen, K., Chuang, K., and Brewer, G. (2001) J. Biol. Chem. 276, 8695–8704
21. Jameson, D. M., and Sawyer, W. H. (1995) Methods Enzymol. 246, 283–300
22. Weber, G. (1952) Biochem. J. 51, 155–167
23. Otto, M. R., Lillo, M. P., and Beechem, J. (1994) Biophys. J. 67, 2511–2521
24. Hall, K. B., and Kranz, J. K. (1995) Methods Enzymol. 259, 261–281
25. Ha, J.-H., Spolar, R. S., and Record, M. T. J. (1989) J. Mol. Biol. 209, 801–816
26. Clegg R. M. (1992) Methods Enzymol. 211, 353–388
27. Lakowicz, J. R. (1999) Principles of Fluorescence Spectroscopy, 2nd Ed., pp. 357–384, Kluwer Academic/Plenum, New York
28. Lakowicz, J. R. (1999) Principles of Fluorescence Spectroscopy, 2nd Ed., pp. 52–53, Kluwer Academic/Plenum, New York
29. Gryczynski, I., Kusba, J., and Lakowicz, J. R. (1997) J. Fluoresc. 7, 167–183
30. Klettermeier, D., and Millar, D. F. (2001) Methods (Orlando) 23, 240–254
31. Franke, A. D., Mattaj, I. W., and Rio, D. C. (1991) Cell 67, 1041–1046
32. Fisher, B. M., Ha, J.-H., and Raines, R. T. (1998) Biochemistry 37, 12131–12132
33. Sudlow, B. D., Martinez-Yamout, M. A., Dyson, H. J., and Wright, P. E. (2004) Natrue Struct. Mol. Biol. 11, 257–264
34. Draper, D. E. (1995) Annu. Rev. Biochem. 64, 593–620
35. Baumann, C., Otridge, J., and Gollnick, P. (1996) J. Biol. Chem. 271, 12269–12274
36. Wilson, G. M., Stuphen, K., Bolikal, S., Chuang, K., and Brewer, G. (2001) J. Biol. Chem. 276, 44459–44466
37. Katsamba, P. S., Myszka, D. G., and Laird-Offringa, I. A. (2001) J. Biol. Chem. 276, 21476–21481
38. DeMaria, C. T., and Brewer, G. (1996) J. Biol. Chem. 271, 12179–12184
39. Bergqvist, S., Williams, M. A., O'Brien, R. L., and Ladbury, J. E. (2004) J. Mol. Biol. 336, 829–842
40. Perese-Howard, G. M., Weil, P. A., and Beechem, J. M. (1995) Biochemistry 34, 8095–8107
41. Michael, S. L. J., Guerrero, A. L., and Berg, J. M. (2003) Biochemistry 42, 4626–4630

Acknowledgments—We thank Dr. Ignacy Gryczynski for helpful discussions. Additional support for the Center for Fluorescence Spectroscopy was provided by NCRR Grant P41 RR08119 from the National Institutes of Health.