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Review

Thermodynamics of specific protein–RNA interactions✪

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Description of the recognition specificity between proteins and nucleic acids at the level of molecular interactions is one of the most challenging tasks in biophysics. It is key to understanding the course and control of gene expression and to the application of the thus acquired knowledge in chemotherapy. This review presents experimental results of thermodynamic studies and a discussion of the role of thermodynamics in formation and stability of functional protein–RNA complexes, with a special attention to the interactions involving mRNA 5′ cap and cap-binding proteins in the initiation of protein biosynthesis in the eukaryotic cell. A theoretical framework for analysis of the thermodynamic parameters of protein–nucleic acid association is also briefly surveyed. Overshadowed by more spectacular achievements in structural studies, the thermodynamic investigations are of equal importance for full comprehension of biopolymers' activity in a quantitative way. In this regard, thermodynamics gives a direct insight into the energetic and entropic characteristics of complex macromolecular systems in their natural environment, aqueous solution, and thus complements the structural view derived from X-ray crystallography and multidimensional NMR. Further development of the thermodynamic approach toward interpretation of recognition and binding specificity in terms of molecular biophysics requires more profound contribution from statistical mechanics.

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Abbreviations: DSC, differential scanning calorimetry; dsRNA, double-stranded RNA; ITC, isothermal titration calorimetry; m^7G, 7-methylguanosine; m^7GDP, 7-methylguanosine-5′-diphosphate; m^7GTP, 7-methylguanosine-5′-triphosphate; m^7GpppG, P^1-7-methylguanosine-5′-P^3-guanosine-5′-triphosphate; SPR, surface plasmon resonance; ssRNA, single-stranded RNA.

After the sequencing of the human genome and the genomes of other organisms, a key task of molecular biology is characterization of the proteome, i.e. all proteins encoded by the genomes. Structural proteomics (genomics) aims at high-throughput determination of three-dimensional protein structures by means of X-ray diffraction (crystallography), nuclear magnetic resonance and computer structure prediction based on sequence homology with proteins of known 3D fold (Chance *et al.*, 2002; Yee *et al.*, 2002). The main goal is to construct molecular models for the whole protein families that cooperate in metabolic processes by forming functional complexes with one another, with ribo- and deoxyribonucleic acids, and with various drugs. As a result, pharmacological agents (antibacterial, antiviral and anticancer) are being to designed for precisely chosen and well characterized protein receptors. Structural proteomics requires cooperation of many well equipped and financially supported laboratories organized in LSF (Large Scale Facility) centres.

Knowledge of 3D structures of proteins and protein complexes with other metabolites by no means constitutes the full information about their biological activity. First, large biomolecules in solution are not rigid (Karplus & McCammon, 1981; Robinson & Drobny, 1995). During formation of a functional complex, conformational changes of the constituents (induced fit) occur in addition to changes of interaction with the surrounding water and ions inside the molecular solvation shell, referred to as preferential hydration and osmotic stress (Parsegian *et al.*, 1995). Kinetic and dynamic studies of proteins and nucleic acid fragments are performed by means of experimental spectroscopic methods, stopped-flow fluorescence (Blachut-Okrasinska *et al.*, 2000; Johnson, 1992; Wallis *et al.*, 1995), time resolved fluorescence (Brauns *et al.*, 1999; Millar, 2000), nuclear magnetic resonance (NMR) relaxation (Fischer *et al.*, 1997; Robinson & Drobny, 1995), and computer molecular dynamics (MD) simulations (Karplus & Petsko, 1990; van Gunsteren & Berendsen, 1990). Second, formation of molecular associates is ruled by electrostatic interactions between the charges distributed in the interacting molecules and between the molecules and the surrounding water. The analysis of the physical foundations of these interactions is necessary to understand the stabilization energy of the complex. The energies of individual stabilization contacts are close to the thermal fluctuation energy RT about 2.5 kJ/mol at room temperature. Easily formed and broken, it is formation of several such specific contacts that stabilize the functional complex in solution and enables its disruption on changing environmental parameters and/or binding regulatory molecules. The overall stability of a complex is dictated by standard Gibbs free energy change ΔG° that involves both enthalpic and entropic contributions, and thus requires a thermodynamic approach. Biochemical processes can be enthalpy and/or entropy driven in different temperature ranges. In spite of the rapid development of single molecule spectroscopy (Weiss, 1999) and manipulation techniques, i.e. atomic force microscopy AFM (Stolz *et al.*, 2000), and optical (Simmons *et al.*, 1996) and magnetic tweezers (Gosse & Croquette, 2002), most worked out research methods and models concern macroscopic sets of molecules (statistical ensembles), where thermodynamic parameters are of primary importance.

In this review some thermodynamic aspects of specific recognition and binding of proteins and nucleic acids will be summarized with a detailed analysis of the association between mRNA 5′ termini (cap) and various cap-binding proteins, i.e. eIF4E that is engaged in the processes of translation initiation in the eukaryotic cell. Structural studies are reported only to complete the necessary information about the interactions in the cap-binding centres. It will be shown that thermodynamic analysis of the interactions inside protein complexes, and between the complexes and

surrounding aqueous medium, although lagging behind the structural achievements (Patikoglou & Burley, 1997), is prerequisite to understanding and describing quantitatively, in physical terms, the biological functioning of large molecular systems. Together with 3D molecular structures and dynamics of conformational rearrangements, thermodynamics significantly contributes to building molecular models of the gene expression processes.

METHODOLOGY OF THE THERMODYNAMIC APPROACH

Formation of molecular complexes involving proteins and nucleic acids is crucial for biological activity of these biopolymers. According to a fundamental paradigm in molecular biology structure determines molecular activity *in vivo* (see, e.g., Cho *et al.*, 1994). Due to the complexity of multicomponent molecular complexes most studies have concentrated on some "half-model" systems, composed of two or a small number of protein fragments and synthetic nucleic acid oligomers, directly engaged in the intermolecular binding. Up to date, the structures of more than 250 DNA–protein complexes (Garvie & Wolberger, 2001; Jones *et al.*, 1999) and 20 RNA–protein (Arnez & Cavarelli, 1997; Draper, 1995; 1999) complexes are known at atomic resolution. Significant development of the leading method in the field, X-ray diffraction on molecular crystals (X-ray crystallography), led to the determination of supramolecular structures of bacterial ribosome components at about 3 Å resolution: the 30S small ribosomal subunit from *Thermus thermophilius* (Carter *et al.*, 2000; Schluenzen *et al.*, 2000; Wimberly *et al.*, 2000), and 50S large ribosomal subunit from *Haloarcula marismortui* (Ban *et al.*, 2000). The crystal structure of the complete *Thermus thermophilius* 70S ribosome was also reported (Yusupov *et al.*, 2001), although at a lower resolution of 5.5 Å. The crystallographic data can now be combined with single-particle electron microscopy imaging to reconstruct cellular structures, like U1 snRNP (Stark *et al.*, 2001). Numerous structural investigations of large biomolecules have been also performed using multidimensional NMR (Riek *et al.*, 2002), after inventing new pulse sequences, TROSY (transverse relaxation-optimized spectroscopy) (Pervushin *et al.*, 1997) and CRINEPT (cross-correlated relaxation-enhanced polarization transfer) (Riek *et al.*, 1999), and application of new technical achievements like 900 MHz spectrometers equipped with cryoprobes for signal detection.

Resolution of the 3D molecular structures reveals the stabilizing patterns inside the macromolecules and their complexes, which include hydrogen bonding, salt bridges between positively and negatively charged groups, π-π and cation-π stacking, van der Waals contacts, and hydrophobic interactions of aliphatic molecular parts (Jayaram *et al.*, 1999; Reyes & Kollman, 2000; Meyer *et al.*, 2003). Total Gibbs free energy (ΔG°), enthalpy (ΔH°), entropy (ΔS°), and heat capacity (ΔC_p^o) can be derived from spectroscopic and/or calorimetric measurements (see below). Theoretical assessments of binding free energies of biopolymers (and association equilibrium constants) are also feasible, usually within the force field approximation (Gilson *et al.*, 1997; Hermans & Wang, 1997; Karplus & Petsko, 1990; Lesyng & McCammon, 1993; Luo & Sharp, 2002). Various approaches have been applied for entropy calculations in protein folding and binding (Amzel, 1997; Karplus *et al.*, 1987; Lee *et al.*, 1994; Schafer *et al.*, 2002). However, at issue is the widely utilized parsing of ΔG° into contributions from individual, specific interactions (Chaires *et al.*, 1996; Chaires, 1997a; Jayaram *et al.*, 1999; Schneider, 1991), i.e. hydrogen bonding, van der Waals contacts etc., from deeming it meaningful (Boresch *et al.*, 1994) to proving its unreliability (Mark & van Gunsteren, 1994). The reasoning of Mark and van Gunsteren is based on the fact that even if

the energy of a macroscopic system can be approximated as a linear combination of particular terms, it is in general not possible to express similarly the total free energy due to the entropic term. Nevertheless, the free energy parsing gives meaningful physical insight into macromolecular processes as long as the theoretically or experimentally data thus obtained are interpreted with care (Boresch *et al.*, 1994).

The stability of specific and non-specific complexes (AB...X) of reactants (A, B,..., X) is defined by standard free energy change (ΔG°) referred to the one molar concentrations. The ΔG° value is evaluated from the equilibrium association constant (K_{as}) expressed in terms of the concentrations of the interacting molecules and the absolute temperature (T):

$$\Delta G^\circ = -RT \ln K_{as} \quad (1)$$

$$K_{as} = \frac{[AB...X]}{[A]\cdot[B]\cdot \ldots \cdot [X]} \quad (2)$$

The observed equilibrium constant thus defined differs from the thermodynamic equilibrium constant that depends only on pressure and temperature. Participation of ions (polyelectrolyte effect), water (preferential hydration) and other buffer components, which can associate with the complex and with the reactants, as well as neglecting of the activity coefficients describing nonideality of the solution, result in additional dependence of the observed K_{as} on pH, ionic strength, osmotic stress and other solution variables (Record, *et al.*, 1991). Due to the weak van der Waals forces, proteins and nucleic acid oligomers attract one another nonspecifically with K_{as} of the order of $10^4\ M^{-1}$ or less for binary associates. Highly specific complexes, e.g. lac repressor–operator, attain K_{as} of $10^{11}\ M^{-1}$ (Ha *et al.*, 1989) that corresponds to ΔG° of over 60 kJ/mol at room temperature. Such a value was also postulated from a wide survey of experimental data (Kuntz *et al.*, 1999) for the tightest, noncovalent binding of ligands to macromolecular targets, although designing of femtomolar inhibitors was reported recently for isoleucyl tRNA synthetase (Brown *et al.*, 2000). Measurements of K_{as} as a function of temperature yield the standard entropy (ΔS°) and the standard van't Hoff enthalpy (ΔH°_{VH}) of the association from the van't Hoff dependence of $\ln K_{as}$ on $1/T$. In the case of a non-linear van't Hoff plot complex formation and other processes like protein folding are characterized by a nonzero value of the standard molar heat capacity change under constant pressure ΔC°_p (Spolar & Record, 1994; Sturtevant, 1977).

The K_{as} values are routinely determined using various titration methods. Detection of characteristic changes of the measured parameters upon formation of a complex involving proteins and nucleic acids entails protein intrinsic fluorescence quenching (Eftink, 1997; Laws & Contino, 1992; Niedzwiecka *et al.*, 2002a), fluorescence anisotropy changes of a probe attached to an oligonucleotide (Fidalgo *et al.*, 2002), changes of NMR chemical shifts (Cameron & Fielding, 2001; Fielding, 2000; Niedzwiecka-Kornas *et al.*, 1999) or translational diffusion coefficients (Derrick *et al.*, 2002) of the interacting molecules, equilibrium dialysis with radioactively labelled ligand (Ha *et al.*, 1989), competitive displacement of bound chromophoric or radiolabelled ligand (Wang, 1995), and isothermal calorimetry titration (ITC) (Forstner *et al.*, 1999; Wiseman *et al.*, 1989; Oberfelder & Lee, 1985). Other methods include surface plasmon resonance (SPR) (Fivash *et al.*, 1998; Szabo *et al.*, 1995; von der Haar *et al.*, 2000) and gel electrophoresis (Talbot & Altman, 1994a; Werner, 1991). When a surface-based SPR experiment is performed with care, the equilibrium and kinetic constants match those acquired in solution, e.g. by calorimetry or fluorescence titration (Day *et al.*, 2002). In addition to K_{as} the ITC technique provides directly the enthalpy of association, ΔH°_{cal}

(Fisher & Singh, 1995; Haun *et al.*, 1995; Niedzwiecka *et al.*, 2002b). Values and temperature dependence of the heat capacity change ΔC_p^o are directly obtained by means of differential scanning calorimetry (DSC) (Krupakar *et al.*, 1999; Rosgen & Hinz, 2002).

Differences between calorimetric (ΔH_{cal}^o) and van't Hoff (ΔH_{vH}^o) enthalpy is a well-known phenomenon in the case of departure from the all-or-none (cooperative) model in a phase transition of macromolecules, like protein folding/unfolding or the helix-to-coil transition of nucleic acids (see, e.g., Chaires & Sturtevant, 1986; Wu & Sugimoto, 2000). For intermolecular association the observed differences in the enthalpy estimates have given rise to numerous empirical and theoretical analyses. The discrepancies were ascribed to the contributions of usually unknown molecular transitions or coupled processes other than the net complex formation (Liu & Sturtevant, 1995; Liu & Sturtevant, 1997; Naghibi *et al.*, 1995), and/or erroneous apparent values of ΔH_{vH}^o and ΔC_p^o arising from the experimental noise (Chaires, 1997b; Rouzina & Bloomfield, 1999). An explanation ascribing the effect to experimental flaws rather than to going beyond simple one-to-one binding model ("linked equilibrium") was recently proposed on the basis of ITC measurements for the Ba^{2+}/18-crown-6 ether and 2′CMP/RNaseA association (Horn *et al.*, 2001). However, such discrepancies can arise in an "open" binding system linked with proton ionization equilibrium (Horn *et al.*, 2002; Niedzwiecka *et al.*, 2002b).

Molar heat capacity under constant pressure is defined by the variance of the internal energy E distribution as:

$$C_p = \left(\frac{\partial H}{\partial T} \right)_P = \frac{\left\langle (E - \langle E \rangle)^2 \right\rangle}{kT^2} \qquad (3)$$

where $\langle A \rangle$ means the ensemble average of the quantity A. The heat capacity occupies a central role in the determination of stabilization of molecular complexes (Ha *et al.*, 1989; Spolar & Record, 1994; Sturtevant, 1977) or protein folding (Gomez *et al.*, 1995; Murphy *et al.*, 1990; Murphy *et al.*, 1992; Spolar *et al.*, 1992). Most of such processes are characterized by negative values of ΔC_p^o. For protein–DNA interactions large, negative ΔC_p^o was proposed to be a distinctive feature of site-specific recognition (Spolar & Record, 1994). However, some observations showed the existence of negative ΔC_p^o even in nonspecific binding (Kozlov & Lohman, 1999; Oda & Nakamura, 2000). Consequently, the standard enthalpy ($\Delta H°$) and entropy ($T\Delta S°$) of association are strongly temperature dependent (steep linear functions) passing through zero at the characteristic temperatures T_H and T_S, respectively. The former corresponds to the maximum in K_{as} from the nonlinear van't Hoff plot. The nearly parallel variation of $\Delta H°$ and $T\Delta S°$ with temperature results in compensation of one by another to yield the standard free energy of association ($\Delta G°$) which is relatively temperature invariant:

$$\Delta G^o = \Delta C_p^o [T - T_H - T \ln(T/T_S)] \qquad (4)$$

$$\Delta H^o = \Delta C_p^o [T - T_H) \qquad (5)$$

$$\Delta S° = \Delta C_p^o \ln(T/T_S) \qquad (6)$$

The enthalpy-entropy compensation results in an essential change in the nature of the thermodynamic driving force of association, from entropy-driven and enthalpy-opposed below T_H, through enthalpy- and entropy-driven between T_H and T_S, to enthalpy-driven and entropy-opposed above T_S. ΔC_p^o is nearly temperature independent in the physiological range and contains contributions from several sources (Murphy & Freire, 1992; Murphy, 1999; Spolar & Record, 1994; Sturtevant, 1977).

For processes with large, negative ΔC_p^o the hydrophobic contribution to $\Delta G°$ can be esti-

mated by means of the "hydrocarbon model" (Baldwin, 1986). The thermodynamic characteristics of transfer of hydrocarbons from water to pure liquids are similar to those of protein–DNA interaction or protein folding (Ha *et al.*, 1989). In the case of each hydrocarbon the observed entropy ΔS^o converges to zero at 386 K, on the assumption that ΔC_p^o is temperature-independent. Hence, at temperature T_S the entropy of the intermolecular association obeys the relation (Patikoglou & Burley, 1997; Spolar & Record, 1994):

$$\Delta S^o = 0 = \Delta S^o_{HE}(T_S) + \\ + \Delta S^o_{rt} + \Delta S^o_{PE} + \Delta S^o_{other} \qquad (7)$$

where ΔS^o_{HE}, ΔS^o_{PE}, and ΔS^o_{other} denote the entropic terms that result from hydrophobic effect, reduction in the rotational and translational degrees of freedom, polyelectrolyte effect and other processes accompanying the association, respectively. A body of evidence has been compiled that large, negative ΔC_p^o is dominated by the hydrophobic effect, i.e. burial of water-accessible nonpolar surface (ΔA_{np}) associated with conformational rearrangements of the interacting molecules, protein and nucleic acid, as well as water exchange. Additionally, burial of polar (ΔA_p) surface area contributes to ΔC_p^o with the positive sign. Both effects result in:

$$\Delta C_p^o = \alpha \cdot \Delta A_{np} - \beta \cdot \Delta A_p \qquad (8)$$

Different proportionality coefficients α and β are used by various research groups: α = 0.32 kcal · mol^{-1} · K^{-1} · Å^{-2}, β = 0.14 kcal · mol^{-1} · K^{-1} · Å^{-2} (Livingstone *et al.*, 1991; Spolar & Recordr, 1994), or α = 0.45 kcal · mol^{-1} · K^{-1} · Å^{-2}, β = 0.26 kcal · mol^{-1} · K^{-1}· Å^{-2} (Murphy & Freire, 1992; Murphy, 1999). However, the area-based models do not fully account for the ΔC_p^o values, e.g. poor agreement was found between the experimentally observed ΔC_p^o and that calculated from X-ray crystal structure for the binding of the DNA operator to the tryptophan repressor of *Escherichia coli* (Jin *et al.*, 1993). Additional contributions that do not scale with the surface area are long-range electrostatic interactions (Gallagher & Sharp, 1998) and tightening of soft internal modes at the polar interface of the complex (Ladbury *et al.*, 1994). Macromolecular equilibrium without intrinsic heat capacity changes, e.g. conformational rearrangements and ionic and hydration equillibria can give rise to non-zero (positive or negative) ΔC_p^o of ligand binding to the macromolecule (Baker & Murphy, 1996; Eftink *et al.*, 1983). Nonzero ΔC_p^o, and the resulting enthalpy-entropy compensation, have been also proposed to arise from other sources: quantum confinement effects, multiple weak interactions in cooperative order-disorder transition or simply as a consequence of the limited Gibbs "free energy window" afforded by the experimental techniques (Cooper *et al.*, 2001). The problem will be further discussed regarding the positive heat capacity change in the case of the eIF4E–mRNA 5′ cap interaction, below.

THERMODYNAMIC DESCRIPTION OF PROTEIN–RNA ASSOCIATION

Numerous papers report studies of interactions between small ligands and biopolymers, i.e. between nucleotides and proteins, and amino acids or peptides and RNA aptamers. The latter reports deal mainly with structural aspects that reveal key molecular interactions conferring high specificity on the aptamer-ligand association (for a review see Hermann & Patel, 2000). Large amount of structural data on molecular complexes involving proteins and nucleotides has been supplemented for the last ten years by papers containing thermodynamic analyses. The majority of them is devoted to enzyme–ligand interac-

tions, e.g. binding of ADP and ATPγS to preprotein translocase subunit SecA (den Blaauwen *et al.*, 1999), binding of ATP to dimeric muscle creatine kinase (Forstner *et al.*, 1999), interaction of Mg-ATP and Mg-ADP with nitrogenase iron protein (Lanzilotta *et al.*, 1999), guanosine mono-, di- and triphosphate binding to hGBP1 GTPase (Praefcke *et al.*, 1999), association of dCMP with thymidylate synthase of *Lactobacillus casei* and its Asn229Asp mutant (Tellez-Sanz *et al.*, 1997), interaction between AMP and two liver glycogen phosphorylases, *a* and *b*, (Garcia-Fuentes *et al.*, 1996b; Garcia-Fuentes *et al.*, 1996a), affinity of Mg-ATP for mutated β subunit of TF_1-ATPase (Odaka *et al.*, 1994), and binding of several mononucleotides to ribonuclease T_1 (Hu & Sturtevant, 1992). Some of the studies involve association between flavoproteins and flavine mononucleotides, FMN (Lostao *et al.*, 2000), association between IMP and inosine monophosphate dehydrogenase (Bruzzese & Connelly, 1997), forced two-state transition in bovine liver glutamate dehydrogenase upon binding of NADPH (Singh & Fisher, 1994), and binding of the anticancer agent 5-fluoro-dUMP to thymidylate synthase (Garcia-Fuentes *et al.*, 1995). The thermodynamic studies of nonenzymatic proteins included the ATP interactions with heat shock protein Hsp90 (Scheibel *et al.*, 1999), chaperonin GroEL (Terada & Kuwajima, 1999) and A3 adenosine receptors (Gessi *et al.*, 2001), and comparison of binding of biotin and bio-5′-AMP to a transcriptional repressor of biotin biosynthesis, BirA (Xu *et al.*, 1996). Most of the thermodynamic data, ΔG°, ΔH°, ΔS° and ΔC_p^o, have been gathered by means of sensitive calorimeters and interpreted in relevance to the complex stability, driving forces of the complex formation, enthalpy-entropy compensation (see Eqns. 4–6), structural and/or molecular surface changes, influence of pH and ionic strength, and stoichiometry and cooperativity of binding of several ligands. A comprehensive review of the earlier publications on the thermodynamic aspects of nucleotide binding to proteins can be found in a Beaudette and Langerman's publication in CRC *Critical Reviews in Biochemistry* (Beaudette & Langerman, 1980). Theoretical and empirical (combined) approaches for predicting the binding affinity of small ligands for proteins (rational drug design) including microcalorimetry methods were recently reviewed by Gholke and Klebe in *Angewandte Chemie* (Gohlke & Klebe, 2002).

Similarly to structural studies, the number of publications on the thermodynamics of protein–RNA interactions lags behind those concerning protein–DNA complexes. The latter topic has been reviewed several times in relation to sequence-specific protein–DNA recognition (Oda & Nakamura, 2000; Patikoglou & Burley, 1997; Plum & Breslauer, 1995) as well as the role of water in protein–DNA association (Schwabe, 1997). ProNIT, an electronically accessible Thermodynamic Database for Protein–Nucleic Acid Interactions (Sarai *et al.*, 2001), which contains thermodynamic data on interactions between proteins and nucleic acids, is mainly devoted to protein–DNA complexes. According to a 1995 review by Draper (1995) "thorough thermodynamic analyses of [protein–RNA] recognition mechanism have yet to be performed". Some specific thermodynamic aspects related to RNA have been surveyed recently, e.g. RNA folding due to formation of complexes with proteins (Weeks, 1997), verification of the allosteric three-state model of the elongation cycle in the translation process (Nierhaus *et al.*, 1992), and mechanisms of translation and mRNA decay in yeast (McCarthy, 1998).

Most of the publications that deal with the kinetic and energetic properties of protein–RNA interactions limit the thermodynamic description to merely free energy of the complex stability and its consequences for the binding mechanism. Several examples are as follows. A thermodynamic scheme for binding of a 153 nucleotide fragment R153 of 23S rRNA and ATP to *E. coli* DbpA protein re-

vealed cooperativity, lost upon removal of a necessary structural element, helix 89, from R153 (Polach & Uhlenbeck, 2002). The thermodynamic contributions of the amino-acid side chain and the tRNA body to the overall binding affinity for the elongation factor Tu (EF-Tu) were shown to be independent of, and compensate for, each other, when the tRNA was correctly acetylated (LaRiviere *et al.*, 2001). A series of protein mutations and RNA modifications were used to evaluate the thermodynamic basis for the improved affinity of the specific RNA hairpin for bacteriophage MS2 coat protein (Johansson *et al.*, 1998). A minimal kinetic and thermodynamic framework (Lorsch & Herschlag, 1998) for the RNA-activated ATPase function was established for the translation initiation factor eIF4A, an ATP-dependent helicase that unwinds secondary structures in the 5′-untranslated regions of eukaryotic mRNA during translation initiation. Synthetic nucleotide analogues provide opportunity to evaluate the importance of individual functional groups on RNA in the thermodynamic stability of protein–RNA complexes (Elliott *et al.*, 2001), the role of the 2′-hydroxyl being probably the most thoroughly investigated (Baidya & Uhlenbeck, 1995; Batey *et al.*, 2001; Pleiss & Uhlenbeck, 2001).

More thorough thermodynamic analyses of protein-RNA association require studies on the temperature-dependence of the binding. Interaction of *Neurospora crassa* mitochondrial tyrosyl–tRNA synthetase (CYT-18) with a small RNA intron fragment (P4-P6 RNA) was shown to be enthalpy-driven and entropy-opposed (Caprara *et al.*, 2001). Thermodynamic studies together with tracing RNA conformational changes induced by Mg^{2+} suggested a model in which the binding of magnesium ions to some parts of the RNA induced specific phosphodiester-backbone geometry that was necessary for the CYT-18 binding. Investigations of the association between an A+U rich element (ARE) of tumor necrosis factor α mRNA and the protein chaperone Hsp70 by gel mobility shift and fluorescence anisotropy assays (Wilson *et al.*, 2001) indicated that the binding was driven entirely by enthalpy at physiological temperatures. Hence, the principal stabilization mechanism was ascribed to burial of hydrophobic surfaces. A thermodynamic and functional analysis of the formation of the ternary complex composed of tRNA and two proteins, tRNA synthetase and Trbp111, showed that sandwiched tRNA retains its native structure (Nomanbhoy *et al.*, 2001). Complete sets of thermodynamic parameters, $\Delta H°$, $\Delta S°$, and $\Delta G°$ were obtained and analysed in relation to the mechanisms of recognition between interferon-induced protein kinase (PKP) and bulged dsRNA (Zheng & Bevilacqua, 2000), between Q β-replicase and its RNA template molecules (Werner, 1991), and between TRAP (tryptophan RNA-binding attenuation protein) and *trp* leader RNA (Baumann *et al.*, 1996). The thermodynamic parameters in the latter case, i.e. regulation of the tryptophan biosynthetic genes in *Bacillus subtilis*, led to an unexpected observation that the interaction between TRAP and *trp* leader RNA is higly enthalpy unfavourable ($\Delta H° = +66.5$ kJ · mol^{-1}) and completely entropy-driven, $\Delta S°$ of +406 J · mol^{-1} · K^{-1}. Thermodynamic description was linked to analyses of ionic strength influence on the association between *E. coli* single-stranded binding (SSB) protein and poly(U) (Lohman *et al.*, 1996), between *E. coli* C5 protein and M1 RNA (Talbot & Altman, 1994b), and between *E. coli* ribosomal protein S8 and 16S rRNA (Mougel *et al.*, 1986). Similar studies were performed for the interaction between phage R17 coat protein and its 21-nucleotide binding site (Carey & Uhlenbeck, 1983). The results gave insight into the charge effect on the binding affinity.

Two topics concerning protein–RNA interactions have gained a considerable interest among various research groups: recognition between U1 protein and small nuclear RNA (snRNA) in mRNA splicing, and specific binding of mRNA 5′ terminus, the so-called cap

structure, to various cap-binding proteins. The latter subject will be discussed in detail in the next paragraph (see below). The U1A protein is one of the family of RNA binding proteins that contain RNA binding domain(s) (RBD) also called RNA recognition motif (RRM) (for a review see Varani & Nagai, 1998). Two thermodynamics-related research areas were exploited by K.B. Hall and her group at Washington University, i.e. affinity and thermodynamics of the association between the RBD1 of the human U1A protein and stem/loop II of U1 snRNA (Hall & Stump, 1992; Williams & Hall, 1996), as well as the application of the pairwise coupling theory to determine the energetics between the two elements (Kranz & Hall, 1998; Kranz & Hall, 1999). Van't Hoff plots of BRD1 association with the normal RNA hairpin and the 1XL RNA containing hexaethylene glycol are accompanied by large, negative apparent heat capacity changes, ΔC_p^o = –13.0 kJ/mol · K and ΔC_p^o = –18.0 kJ/mol · K, respectively, due to the burial of hydrophobic groups on the surface of the β-sheet BRD. Accordingly, the thermodynamic properties of this protein–RNA system are similar to those of protein–DNA (Ha *et al.*, 1989). Determination of the salt dependence of the K_{as} suggested that at least 8 ion-pairs were formed upon formation of the complex. The two- and three-dimensional thermodynamic cycles for the local interactions in terms of the pairwise coupling theory showed indirect coupling between Tyr13 and the C-terminal tail, mediated through the bound RNA. Combination of thermodynamic pairwise coupling and backbone dynamics derived from ^{15}N-relaxation and ^{1}H-^{15}N-NOE (nuclear Overhauser effect) provided further evidence for local cooperative interactions between Tyr13, Gln54 and Phe56 that directly affected the RNA binding. The system was also analysed by means of molecular dynamics simulation (Pitici *et al.*, 2002; Reyes & Kollman, 2000). The results from the MD studies combined with structural and thermodynamic data indicated that the induced fit of the U1A protein upon the binding of RNA involves a non-native thermodynamic substate while the conformational change of the RNA involves a distortion of the native structure to an unstable form (Pitici *et al.*, 2002). MD simulations were also applied to answer a more general question on the reduction of the entropic cost of induced fit in protein–RNA recognition (Ribas *et al.*, 1996).

THERMODYNAMIC ASPECTS OF mRNA 5′ cap–PROTEIN RECOGNITION

Translation initiation, a multi-step and highly regulated process of formation of large protein–mRNA complexes, determines the overall rate of protein biosynthesis in eukaryotes (Dever, 2002; Gingras *et al.*, 1999). Eukaryotic mRNA differs from its prokaryotic counterpart by the presence of *cis*-acting elements that stimulate translation (Londei, 1998; Sachs *et al.*, 1997; Shatkin *et al.*, 1982): the 5′-terminal cap, the 3′-terminal poly(A) tract, and, in a small subset of viral and cellular mRNAs, the internal ribosome entry sequence (IRES). Messenger RNA 5′-terminus in most organisms consists of 7-methylguanosine linked by a 5′-to-5′ triphosphate bridge to the next nucleoside (m^7GpppN), guanosine, adenosine, cytidine or uridine. A significant fraction of cellular and viral RNAs is additionally methylated at the ribose 2′-hydroxyl of the first (N) or the first and second nucleosides. The translation initiation starts by recognition of the cap structure by the 25 kDa eukaryotic initiation factor eIF4E (Raught & Gingras, 1999). The 4E protein is a member of the eIF4F complex that also includes eIF4A, a 46 kDa RNA helicase, and eIF4G (154–180 kDa), which serves as the central organizing protein in recruitment of mRNA. The 43S complex of eIF3-eIF2-GTP-(Met-tRNA)–eIF1A–(40S ribosomal subunit) recruits mRNA to form the 48S initiation complex. It scans until the start-

ing AUG codon is encountered. Then, replacement of the initiation factors by the 60S ribosome subunit makes it possible to form the first peptide bond.

The molecular structures of several eIF4E–cap complexes have been resolved by crystallography: murine eIF4E(28-217) with m^7GDP (Marcotrigiano *et al.*, 1997) and m^7GpppG (Niedzwiecka *et al.*, 2002a), full length human eIF4E with m^7GTP and m^7GpppA (Tomoo *et al.*, 2002), two ternary complexes of murine eIF4E(28-217) with m^7GDP and a synthetic peptide, one corresponding to the eIF4E recognition sequence of eIF4G and the other corresponding to the eIF4E recognition sequence of 4E-BP1 (Marcotrigiano *et al.*, 1999). The solution structure of yeast eIF4E bound to m^7GDP was resolved for the double labelled $^{13}C/^{15}N$ protein by multidimensional NMR (Matsuo *et al.*, 1997). The cap is located in a narrow slot, formed like a "hand" from an antiparallel β-sheet and three loops, and stabilized by the cation-π sandwich stacking of 7-methylguanine in between two tryptophan indole rings, Trp102 and Trp56 (Fig. 1). Additionally, the nucleic base forms three Watson–Crick-like hydrogen bonds with the Glu103 carboxyl group and peptide chain NH of Trp102, and a van der Waals contact with Trp166. Positively charged arginines and lysines interact through hydrogen bonds and/or salt bridges with the phosphate chain of the cap analogue, depending on the eIF4E type and the length of the cap phosphate chain. Recognition of the cap by sandwich cation-π stacking between the protein aromatic side chains is shared by other cap-binding proteins, viral methyltransferase VP39 (Hodel *et al.*, 1997; 1998), and nuclear cap-binding complex CBP80/20 (Calero *et al.*, 2002; Mazza *et al.*, 2002).

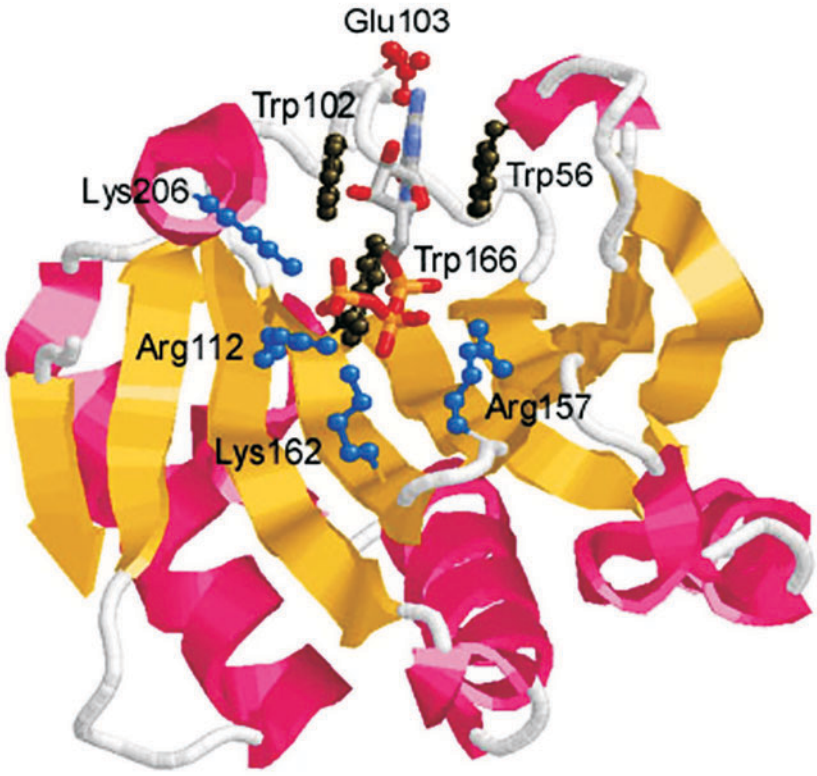

Figure 1. Crystal structure of murine eIF4E in complex with m^7GpppG (Niedzwiecka *et al.*, 2002a), with marked amino acids in direct stabilizing contacts with the cap analogue.

The second nucleoside G is not visible in the electron density map.

Description of the 43S initiation complex formation in terms of association (dissociation) constants derived from fluorescence anisotropy measurements and the corresponding free energy changes ($\Delta G°$) appeared as early as middle 90s (Parkhurst *et al.*, 1994). Similarly, a fragmentary thermodynamic approach to translation regulation in yeast was reported (Koloteva *et al.*, 1997), based on the determination of binding affinity of IRP1 (iron regulatory protein 1) for the iron-responsive element (IRE) in the 5′-untranslated region of mRNA. Development of the thermodynamic description of the interactions involving the mRNA 5′ cap structure was hampered by a lack of precise values of the association constants. The first two communications on thermodynamic parameters of the eIF4E–m^7GTP and eIF4E–m^7GpppG association (Carberry *et al.*, 1989; Shen *et al.*, 2001) were misleading and contradictory to each other due to lack of precise values of the association constants K_{as}, e.g. the binding of the latter cap analogue was postulated to be enthalpy driven ($\Delta H° = -36.4$ kJ · mol^{-1}, $\Delta S° = -2.3$ J · mol^{-1} · K^{-1}) by one group (Shen *et al.*, 2001), and entropy driven ($\Delta H° = +34.0$ kJ · mol^{-1}, $\Delta S° = +219$ J · mol^{-1} · K^{-1}) by the other (Carberry *et al.*, 1989).

A new fluorescence time synchronized titration method (Niedzwiecka *et al.*, 2002a) provided precise and absolute values of equilibrium association constants K_{as}, without previous experimental and numerical sources of errors. The new methodology gave rise to reliable parsing of $\Delta G°$ into several components, i.e. anchoring of the cap to eIF4E through the phosphate groups, and subsequent cooperative sandwich cation-π stacking and hydrogen bonding of 7-methylguanine. Similarly, the van't Hoff plots of ln K_{as} *vs.* temperature for the binding of m^7GTP (Niedzwiecka *et al.*, 2002a) and of m^7GpppG (Niedzwiecka *et al.*, 2002b) to murine eIF4E resulted in a proper thermodynamic description of the association. The strong specific interaction between m^7GTP and eIF4E is unambiguously connected with a high enthalpy of association, $\Delta H° = -74.3$ kJ · mol^{-1}, and negative entropy change (entropy-opposed), $\Delta S° = +98.7$ J · mol^{-1} · K^{-1}. The less strong binding of m^7GpppG is characterized by nonlinear van't Hoff relation (Fig. 2) leading to an unexpected, large positive heat capacity change $\Delta C_p^o = +1.94$ kJ · mol^{-1} · K^{-1} with the critical temperatures, $T_H = 327.1$ K and $T_S = 307.4$ K. The

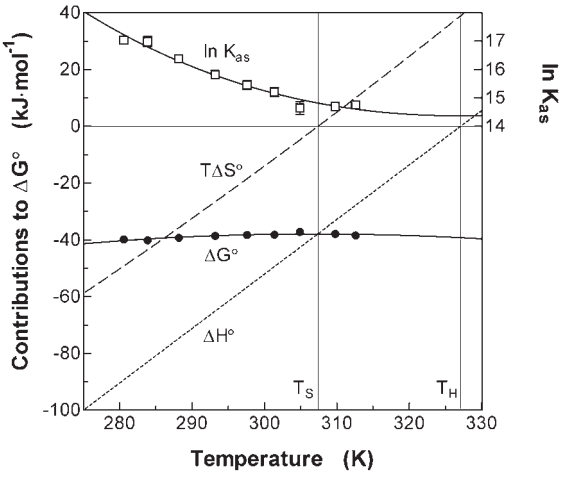

Figure 2. Plot of ln K_{as} *vs.* temperature (T), and the temperature dependence of enthalpy ($\Delta H°$), entropy ($T\Delta S°$), and free energy ($\Delta G°$), for the binding of m^7GpppG to murine eIF4E (Niedzwiecka *et al.*, 2002b).

ΔC_p^o value was independently confirmed by isothermal titration calorimetry (Niedzwiecka *et al.*, 2002b). As a consequence, the nature of the thermodynamic driving forces changes with temperature, being enthalpy- and entropy-driven in the range of biological temperatures. The enthalpy-entropy compensation leaves constant free energy $\Delta G°$ of about –37 to 40 kJ · mol^{-1} within the whole temperature range. Both van't Hoff (ΔH^o_{VH}) and calorimetric (ΔH^o_{cal}) enthalpy values were in perfect agreement if protonation equilibrium in 7-methylguanine, coupled with the association, was taken into account. The positive ΔC_p^o relevant to intermolecular association is rarely observed (Hileman *et al.*, 1998; Luther *et al.*, 1986; Matulis *et al.*, 2000). However, it has been shown that the heat capacity change

could be strongly temperature- and ionic strength-dependent, even up to sign inversion (Oda *et al.*, 1998). Stabilization of the complex occurs by electrostatic interactions partially complemented by van der Waals and hydrophobic contacts. Hence, many charged and polar groups are removed from water, contributing positively to ΔC_p^o (see Eqn. 8). Kinetic studies of the eIF4E–m^7GpppG interaction by means of stopped-flow fluorescence spectroscopy and Brownian molecular dynamics simulations (Blachut-Okrasinska *et al.*, 2000) revealed a two-step character of the complex formation: diffusionally and electrostatically controlled encounter and internal rearrangement of the protein. The latter is accompanied by an uptake of about 65 water molecules (Niedzwiecka *et al.*, 2002a). Both effects, the preferential hydration and burial of charged and polar groups can make the heat capacity change positive. An additional positive contribution may come from the overall long-range electrostatic interactions upon the binding, dominated by rearrangement of water dipoles, redistribution of mobile ions, and the coupling between the dipolar and ionic terms (Gallagher & Sharp, 1998).

Parallel spectroscopic and calorimetry titration studies were performed for eIF4E from *Saccharomyces cerevisiae* (Kiraga-Motoszko *et al.*, 2003). The association equilibrium constants and the enthalpy of the association for m^7GTP derived from the two methods were in reasonably agreement, and showed significantly different affinities of the cap analogue for the yeast and mammalian proteins. This observation corresponds to the structural differences of the stacking between 7-methylguanine and two tryptophans in murine (Marcotrigiano *et al.*, 1997; Niedzwiecka *et al.*, 2002a) and yeast (Matsuo *et al.*, 1997) eIF4E, and relates to the thermodynamic (NMR) study of a model system of 7-methylguanosine and a synthetic dodecapeptide containing tryptophan (Niedzwiecka *et al.*, 2003). Both reports (Kiraga-Motoszko *et al.*, 2003; Niedzwiecka *et al.*, 2003) make the first attempt to analysis of the evolutionary changes of structural and energetic requirements in the eIF4E active centres.

PROGRESS ON THE WAY TO UNDERSTANDING MOLECULAR RECOGNITION

Thermodynamic characterization of intermolecular binding specificity is one of the fundamental goals of biophysical approach in molecular biology. Analysis of thermodynamic functions and parameters in terms of statistical physics, e.g. standard molar heat capacity, seems to be absolutely necessary here. Restriction to phenomenological thermodynamics of (linear) correlations between ΔH° and/or ΔS° and ΔC_p^o (see, e.g., Murphy *et al.*, 1990) as well as enthalpy-entropy compensation, ΔH° *vs.* ΔS° (see, e.g., Eftink *et al.*, 1983), although very helpful, is far from satisfactory. The problem is directly linked to the hydrophobic effect (Eftink *et al.*, 1983; Israelachvili & Wennerstrom, 1996) that still lacks a proper physical description. Recent attempts in this regard concentrate on development of good models of water structure (Madan & Sharp, 2003; Silverstein *et al.*, 1998; Tsai *et al.*, 2002).

Statistical mechanics allows straightforward calculations of the thermodynamic functions, including heat capacity, from the partition function (Boresch *et al.*, 1994; Freire, 1998; Gilson *et al.*, 1997; Luo & Sharp, 2002; Rosgen *et al.*, 1998). Predictions based on these calculations, e.g. related to protein folding/unfolding, can differ from the simple, more intuitive models derived from phenomenological thermodynamics (Rosgen *et al.*, 1998). Moreover, proper interpretation of experimental data like DSC curves is sometimes difficult without taking into account the principles of statistical thermodynamics (Rosgen & Hinz, 2002). A nice example of the application of a general statistical model to the widely discussed phenomenon of enthalpy-entropy compensation

shows benefits of the approach based on the sound knowledge of statistical thermodynamics (Sharp, 2001). The model provided a rigorous test for some extra-thermodynamic mechanism of the ΔH-ΔS linear relationship upon changing experimental variables that do not just follow the well-known thermodynamic laws or arise from experimental uncertainties.

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