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Thermodynamics of GTP and GDP Binding to Bacterial Initiation Factor 2 Suggests Two Types of Structural Transitions

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During initiation of messenger RNA translation in bacteria, the GTPase initiation factor (IF) 2 plays major roles in the assembly of the preinitiation 30S complex and its docking to the 50S ribosomal subunit leading to the 70S initiation complex, ready to form the first peptide bond in a nascent protein. Rapid and accurate initiation of bacterial protein synthesis is driven by conformational changes in IF2, induced by GDP–GTP exchange and GTP hydrolysis. We have used isothermal titration calorimetry and linear extrapolation to characterize the thermodynamics of the binding of GDP and GTP to free IF2 in the temperature interval 4–37 °C. IF2 binds with about 20-fold and 2-fold higher affinity for GDP than for GTP at 4 and 37 °C, respectively. The binding of IF2 to both GTP and GDP is characterized by a large heat capacity change (-868 ± 25 and -577 ± 23 cal mol^{-1} K^{-1}, respectively), associated with compensatory changes in binding entropy and enthalpy. From our data, we propose that GTP binding to IF2 leads to protection of hydrophobic amino acid residues from solvent by the locking of switch I and switch II loops to the γ-phosphate of GTP, as in the case of elongation factor G. From the large heat capacity change (also upon GDP binding) not seen in the case of elongation factor G, we propose the existence of yet another type of conformational change in IF2, which is induced by GDP and GTP alike. Also, this transition is likely to protect hydrophobic groups from solvent, and its functional relevance is discussed.

Keywords: IF2; translation initiation; GTPases; isothermal titration calorimetry; G nucleotides

Initiation of bacterial protein synthesis is promoted by an interplay between the three initiation factors (IFs) IF1, IF2, and IF3, among which IF2 belongs to the superfamily of translational GTPases.[1,2] The conformational IF2 switches induced by hydrolysis of IF2-bound GTP on the ribosome and GDP–GTP swapping on free IF2 are the key regulators that drive the rapid[3] and accurate[4] sequence of functional events defining the initiation of messenger RNA (mRNA) translation in bacteria.

During initiation, IF1, IF2, and IF3 recruit initiator tRNA (fMet-$tRNA^{fMet}$) to the mRNA-bound small (30S) ribosomal subunit.[5,6] After completion of the

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Abbreviations used: IF, initiation factor; cryo-EM, cryo-electron microscopy; SAXS, small-angle X-ray scattering; ITC, isothermal titration calorimetry; EF-G, elongation factor G.

preinitiation 30S complex containing mRNA, initiator tRNA, IF1, IF2·GTP, and IF3, the large (50S) ribosomal subunit rapidly docks to the 30S subunit upon formation of the 70S initiation complex. Rapid formation of the 70S initiation complex occurs also with the nonhydrolyzable GTP analogue GDPNP, but not with GDP, replacing GTP on IF2. In the presence of GDPNP on IF2, the ribosome is frozen in an intermediate state before the transfer of fMet from the initiator to the first elongator aminoacyl-tRNA in protein synthesis.[7] It is known from cryo-electron microscopy (cryo-EM) that in such a stalled 70S initiation complex initiator, tRNA is in a P/I site. At this site, different from the hybrid P/E site,[8] fMet-tRNA^{fMet} is in close contact with IF2·GTP (GDPNP)[9–11] and A-site-bound IF1.[12] We propose that it is in this configuration of the native 70S initiation complex that GTP is hydrolyzed to GDP, causing rapid dissociation of IF2 and IF1 from the ribosome.[7] Interestingly, GDP-bound IF2 has no contact with the initiator tRNA, which is in the hybrid P/E state.[13,14]

In a different view of initiation,[14] release of inorganic phosphate after hydrolysis of the IF2-bound GTP leads to the conversion of an initial labile 70S initiation complex into its stable conformation, with fMet-tRNA^{fMet} positioned at the P/I site. Furthermore, after hydrolysis of the IF2-bound GTP, IF2·GDP remains on the ribosome during A-site accommodation of the incoming aminoacyl-tRNA after hydrolysis of EF-Tu-bound GTP in ternary complex with aminoacyl-tRNA.[14,15] However, the slow release of IF2·GDP from the ribosome was observed in a heterologous system with ribosomes from *Escherichia coli* and in IF2 from the thermophile bacterium *Geobacillus stearothermophilus* (formerly *Bacillus stearothermophilus*). Since thermophile proteins often display anomalously high target binding affinities at suboptimal temperatures, the biological relevance of this result is difficult to assess.

No crystal structures of free IF2 exist to date, and there is a nuclear magnetic resonance reconstruction only of its C1 subdomain.[16] Crystal structures do, however, exist for the IF2 orthologue aIF5B in the free apo, GDP, and GTP conformations, which differ little from each other.[17] A recent small-angle X-ray scattering (SAXS) reconstruction of free IF2[18] suggests a more compact conformation of its guanine-nucleotide-free apo form than of its GDP-bound, GDPNP-bound, and GDPCP-bound forms in the 70S initiation complex, as seen by cryo-EM.[9,13,19]

In the present work, we have studied the binding of GTP or GDP to IF2 by isothermal titration calorimetry (ITC). We have obtained complete sets of thermodynamic parameters (standard enthalpy, standard entropy, Gibbs standard free energy, and change in heat capacity) for the equilibrium interaction between IF2 and GDP or GTP in the temperature range 4–25 °C. Our results on IF2 are reminiscent of ITC data previously obtained for the translocating GTPase elongation factor G (EF-G), in particular regarding the putative ordering of the switch I and switch II peptide loops upon GTP, but not GDP, addition to the apo form of the factor.[20] There is, however, an unexpected difference between IF2 and EF-G: the addition of either GDP and GTP to the apo form of IF2, but not of EF-G, induces yet another type of conformational change in the factor in which hydrophobic amino acid residues become protected from the solvent. We discuss the intriguing functional implications of this unexpected structural transition.

Effect of temperature on IF2 interactions with G nucleotides

We used ITC to estimate the affinities of GDP and GTP for IF2 at 4, 10, 15, 20, and 25 °C. The results from this temperature interval, limited by protein aggregation above 25 °C (data not shown), are summarized in Table 1 as association (K_a) and

Table 1. Thermodynamic parameters of IF2 binding to GDP and GTP, as determined by ITC

Ligand	T (°C)	K_a[a] (M^{-1})	K_d[b] (μM)	ΔH^{oc}[c] (kcal/mol)	$T\Delta S^{od}$[d] (kcal/mol)	ΔG^{oe}[e] (kcal/mol)
GDP	4	1.2×10^6	0.8	−0.55	7.16	−7.71
GDP	10	9.5×10^5	1.1	−3.31	4.43	−7.74
GDP	15	7.9×10^5	1.3	−6.22	1.55	−7.77
GDP	20	7.3×10^5	1.4	−9.53	−1.67	−7.86
GDP	25	6.1×10^5	1.6	−12.50	−4.62	−7.88
GDP[f]	37	2.2×10^5	4.5	−19.23	−11.68	−7.55
GTP	4	6.3×10^4	15.9	−1.10	4.98	−6.08
GTP	10	1.1×10^5	9.1	−6.12	0.41	−6.53
GTP	15	1.3×10^5	7.7	−11.3	−4.56	−6.73
GTP	20	1.4×10^5	7.1	−14.81	−7.91	−6.90
GTP	25	1.5×10^5	6.7	−19.3	−12.25	−7.05
GTP[f]	37	9.4×10^4	10.6	−29.78	−22.76	−7.02

All measurements were performed two to four times in phosphate buffer (5 mM K_2HPO_4, 10% glycerol, 1 mM DTT, 95 mM KCl, and 5 mM $MgCl_2$, pH 7.5).

[a] K_a, association equilibrium constant; standard deviation did not exceed ±20%.

[b] K_d, dissociation equilibrium constant; calculated as $1/K_a$.

[c] ΔH^o, standard enthalpy change; standard deviation did not exceed ±10%.

[d] $T\Delta S^o$, standard entropy change; calculated from the equation $\Delta G^o = \Delta H^o - T\Delta S^o$.

[e] ΔG^o, $G^o = -RT\ln K_a$.

[f] These values were backcalculated from the linear plots of ΔH^o and ΔS^o (Fig. 2a and b).

dissociation (K_d) equilibrium constants. A typical set of ITC data for GDP and GTP binding to IF2 in phosphate buffer at 25 °C is shown in Fig. 1, including "raw" calorimetric data for the ligand-into-protein titration (upper parts) and the binding curves (lower parts). The latter was fitted to a model with one guanine nucleotide binding site per IF2 molecule.

In addition to the binding constant estimates obtained from the shape of the binding curves, we also estimated the change in standard enthalpy ΔH^o upon binding of a guanine nucleotide to IF2 by monitoring the heat added to the reaction chamber or removed from the reaction chamber to keep the temperature unaltered upon ligand addition. The change in Gibbs standard free energy ΔG^oupon ligand binding to IF2 was estimated from the equilibrium association constant K_a through the relation $\Delta G^o = -RT\ln K_a$. Since ΔG^o is related to

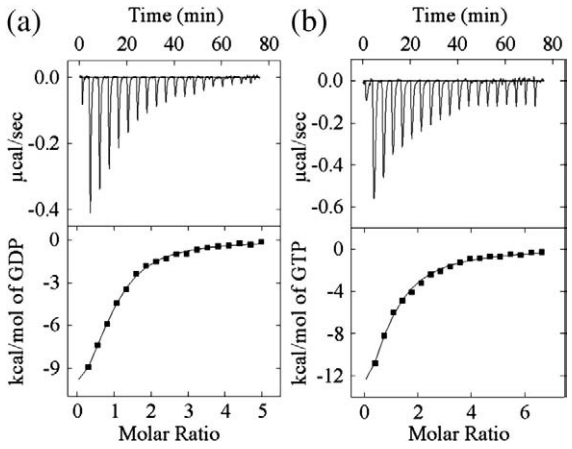

Fig. 1. ITC titration curves (top) and binding isotherms (bottom) for IF2 interaction with GDP (a) and GTP (b) at 25 °C and pH 7.5. The 6His-tagged *E. coli* IF2 from the plasmid described in Forster *et al.*[21] was overexpressed as described for the nontagged IF2 in Antoun *et al.*[22] and purified essentially in the same way with addition of the Ni-NTA purification step before the rest of the chromatographic procedures. All guanine nucleotides were purified in a MonoQ column (Amersham Bioscience), as described by Zavialov *et al.*[23] The thermodynamic parameters of IF2 binding to GDP and GTP were measured using a MicroCal VP-ITC instrument (MicroCal, Northampton, MA), as described previously.[24] Experiments were carried out at 4, 10, 15, 20, and 25 °C in phosphate (5 mM K_2HPO_4, 10% glycerol, 1 mM DTT, 95 mM KCl, and 5 mM $MgCl_2$, pH 7.5) buffer. Fifteen-microliter aliquots of ligands were injected into the 1.42-ml cell containing the IF2 solution to achieve a complete binding isotherm. Protein concentration in the cell ranged from 5 to 40 µM, and ligand concentration in the syringe ranged from 90 to 600 µM. The heat of dilution was measured by injecting the ligand into the buffer solution or by additional injections of ligand after saturation; the values obtained were subtracted from the heat of reaction to obtain the effective heat of binding. The resulting titration curves were fitted using MicroCal Origin software. Affinity constants (K_a), binding stoichiometry, and enthalpy variations (ΔH) were determined by a nonlinear regression fitting procedure.

ΔH^o and the change ΔS^o in standard entropy upon ligand binding through $\Delta G^o = \Delta H^o - T\Delta S^o$, where T is the absolute temperature, the estimates of ΔG^o and ΔH^o allowed us in each case to estimate also ΔS^o. The estimates of ΔG^o, ΔH^o, and ΔS^o for GDP and GTP binding to IF2 at 4, 10, 15, 20, and 25 °C are summarized in Table 1. Using the linear behavior of the entropy and enthalpy functions (Fig. 2a and b; see the text below), we used extrapolation to estimate ΔG^o, ΔH^o, and ΔS^o along with the equilibrium binding constants K_d and K_a also at 37 °C (Table 1).

We observed a ~20-fold higher IF2 affinity for GDP ($K_a = 1.2\times10^6$ M^{-1}) than for GTP ($K_a = 6.3\times10^4$ M^{-1}) at 4 °C, but our extrapolated data suggest a more similar IF2 affinity for GDP ($K_a = 2.2\times10^5$ M^{-1}) and GTP ($K_a = 9.4\times10^4$ M^{-1}) at 37 °C. At 25 °C, the IF2 affinity is ~4-fold higher for GDP than for GTP, in reasonable agreement with the previously reported ~9-fold higher IF2 affinity for GDP at 25 °C.[25,26] The significant temperature variations in opposite directions of the K_a values for IF2 binding to GTP and GDP underscore the importance of affinity recording throughout the whole physiologically relevant temperature interval. We note, in particular, that the affinities of IF2 for the two guanine nucleotides are similar at the optimal growth temperature for *E. coli*, but quite different at the lower temperatures generally used for biochemical measurements.[26,27] A similar temperature trend was observed for GTP and GDP binding to EF-G from *E. coli*, with higher EF-G affinity for GDP than for GTP at 4 °C, but higher EF-G affinity for GTP at 37 °C.[20] The temperature dependence of the guanine nucleotide affinities for IF2 is physiologically relevant, but does not contain information about (putative) conformational changes in the factor upon GTP or GDP binding. The thermodynamic binding parameters available from ITC, in contrast, contain information about structural transitions.

Analysis of thermodynamic data

When ΔH^o was plotted against temperature in the GDP and GTP cases, straight lines with negative slopes were obtained (Fig. 2a). From these, the change in heat capacity upon complex formation $\Delta C_p = d(\Delta H)/dT$ was estimated to be -577 ± 23 cal mol^{-1} K^{-1} in the GDP case and -868 ± 25 cal mol^{-1} K^{-1} in the GTP case. In both cases, ΔC_p was virtually constant in the temperature interval (4–25 °C), meaning that plots of ΔS^o *versus* the logarithm of the temperature (integrated van't Hoff equation[28]) should give straight lines, with slopes estimating the corresponding ΔC_p values. From such plots (Fig. 2b), we estimated ΔC_p as -573 ± 18 cal mol^{-1} K^{-1} in the GDP case and as -811 ± 25 cal mol^{-1} K^{-1} in the GTP case. As should be, these estimates correspond well to those obtained from the enthalpy changes (Fig. 1a). Linear graphs (Fig. 1a and b) reflect constant heat capacity change in the reaction in the studied interval. The unchanging heat capacity change and basal thermodynamic relations lead to linear rela-

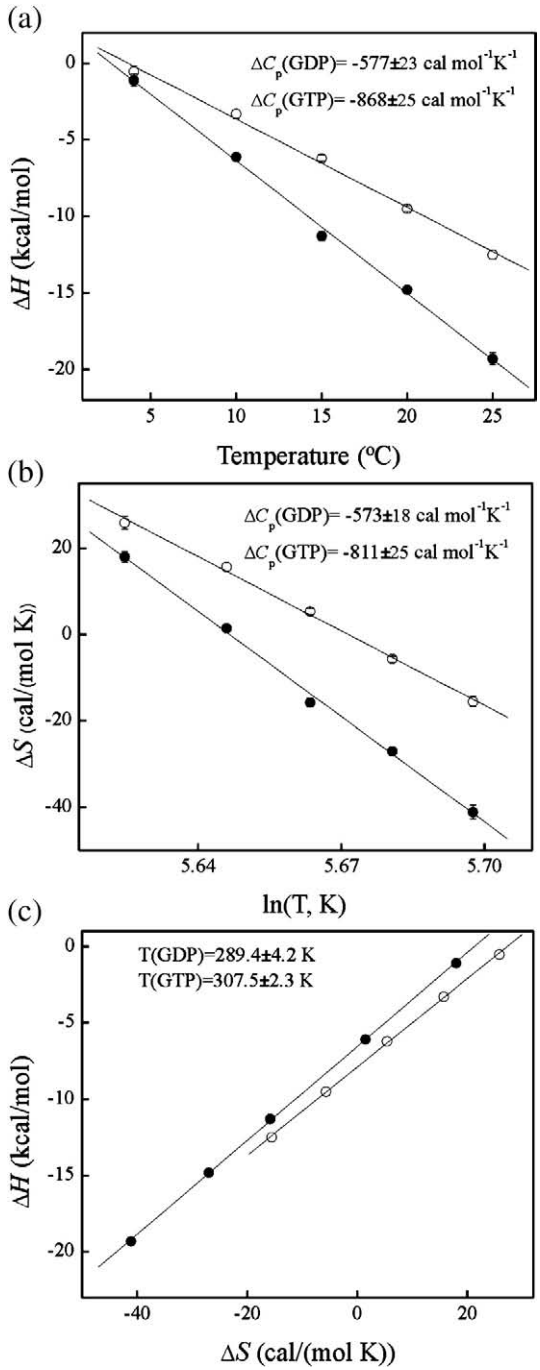

Fig. 2. Temperature dependence of the entropy and enthalpy of IF2 interactions with GTP and GDP. (a) Enthalpy of binding of GDP (empty circles) and GTP (filled circles) to IF2 as a function of the temperature (°C) at pH 7.5. (b) Entropy of binding of GDP (empty circles) and GTP (filled circles) to IF2 as a function of the logarithm of the temperature (K) at pH 7.5. The ΔC_p values obtained from the enthalpy plots are similar to those obtained from the entropy plots. (c) Enthalpy–entropy compensation effect for GDP (empty circles) and GTP (filled circles) binding to IF2.

tions between the standard enthalpy change (ΔH^o) and the standard entropy change (ΔS^o) in each equilibrium reaction.[29] Each line has a positive slope, ultimately determined by the temperature interval of the experiment (Fig. 2c), showing enthalpy–entropy compensation. This compensation leads to a small temperature variation in the Gibbs standard free energy ΔG^o of the reaction, in spite of large temperature variations in the reaction enthalpy (ΔH^o) and entropy (ΔS^o). The slope of the ΔH-*versus*-ΔS plot (Fig. 2c) is dubbed as compensation temperature (T_c),[29,30] which is 289.4 and 307.5 K for GDP and GTP cases, respectively.

The large decrease in heat capacity upon complex formation between IF2 and either GTP or GDP suggests that guanine nucleotide binding in each case greatly decreased the solvent-exposed area of nonpolar amino acids.[31] ΔC_p was larger for GTP binding to IF2 than for GDP binding to IF2, suggesting the existence of one set of structural rearrangements common to GDP and GTP binding and another set of rearrangements idiosyncratic to GTP binding to IF2 (see the text below).

Structural and functional implications for IF2 GTPase cycle

The heat capacity C_p is an important thermodynamic parameter for protein,[28] not the least because the heat capacity change upon complex formation $\Delta C_p = d(\Delta H)/dT$ provides a useful link between the thermodynamics of a protein and its structural rearrangements. For instance, a large negative ΔC_p signal is characteristic of a large reduction in the solvent-accessible area of a protein, typical of peptide chain folding or protein–protein complex formation.[32,33] The heat capacity change has been related to the change in solvent-accessible area through the empirical expression $\Delta C_p = 0.27\Delta A_{aromatic} + 0.4\Delta A_{nonaromatic}$, where $\Delta A_{aromatic}$ and $\Delta A_{nonaromatic}$ are the protected areas (in Å^2) due to aromatic and nonaromatic amino acid residues, respectively.[33] From the slopes of the two straight lines in Fig. 2a, we have estimated the heat capacity changes (constant in the temperature interval 4–25 °C) upon GTP ($\Delta C_p^{GTP} = -868$ cal mol^{-1}) and GDP ($\Delta C_p^{GTP} = -577$ cal mol^{-1}) binding to IF2. The difference in ΔC_p value for the GTP and GDP reactions is ~ -290 cal mol^{-1}, a value similar to the corresponding heat capacity difference between the GTP case and the GDP case previously reported for EF-G.[20] By translating this difference in heat capacity changes into solvent-accessible surface areas according to the formula above, we obtain the solvent-accessible area change ranging from 725 to 1074 Å^2. Assuming an area of $\sim$40 Å^2 per hydrophobic amino acid residue,[34] this would correspond to 18–27 amino acids. This differential surface area change we tentatively interpret as due to the ordering of switch 1 and switch 2 amino acid residues upon GTP—but not GDP—binding to IF2, in line with our previous interpretation of the differential heat capacity for GTP and GDP binding to EF-G.[20] The proposal regarding EF-G was recently corroborated in experiments monitoring the selective response in the proteolysis sensitivity of switch 1 to GTP and GDP binding.[35]

Although the *differences* in heat capacity change upon GTP and GDP binding are similar in the IF2 and EF-G cases, the heat capacity change upon GDP binding is numerically much smaller for EF-G than for IF2. This suggests that GDP binding to EF-G *does not* induce a conformational change in the factor,[20] meaning that the apo form and the GDP-bound EF-G form have very similar structures, as observed by SAXS[36] and X-ray crystallography.[37] Furthermore, GDP binding to IF2 *does* induce a conformational change, as revealed by the 1440–2140 $Å^2$ reduction in solvent-accessible area. Such a large structural difference between its apo form and its GDP form is not part of the classical model for the action of a GTPase,[1] but could be experimentally observed by X-ray crystallography and SAXS or nuclear magnetic resonance experiments.

The SAXS structure of free IF2 in the apo form, but not the structural effects of GTP or GDP binding to the factor, has been determined.[18] Such information would be particularly interesting, since the SAXS structure of IF2 differs significantly from available X-ray and cryo-EM reconstructions of IF2/eIF5B.[13,19,38]

The distinct apo form and GDP-bound form of IF2 could have a number of functional implications. One option is that rapid release of IF2 from the 70S initiation complex is greatly favored by the apo form in relation to the GDP-bound form of the factor, but further experiments will be necessary to clarify this intriguing issue.

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