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Time-resolved FTIR studies provide activation free energy, activation enthalpy and activation entropy for GTPase reactions

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Abstract

GTPases, which catalyze the hydrolysis of GTP to GDP and P_i, play a key role in the regulation of many biological processes. In this work, we quantify the activation parameters ΔG_0^*, ΔH_0^* and ΔS_0^* for the hydrolysis reaction of GTP in water, in water with Mg^{2+} ions and in Ras. Ras belongs to the superfamily of small GTPases (guanine nucleotide-binding proteins; GNBPs). Surprisingly, we find that in all cases, the activation energy consists mainly of enthalpic contributions. Additionally, the small entropic contributions in water and in Ras are similar, so that $\Delta\Delta S^*$ is close to 0. Thus the entropic contributions are only minor in GTPase catalysis and the enthalpic contributions from electrostatic interactions are key to the catalysis. The protein induced change in charge distribution of GTP can be monitored by time-resolved difference FTIR spectroscopy. For Ras the main effect due to protein binding is a charge shift towards the β-phosphate of GTP. This seems to have the main contribution to the catalytic mechanism. Because the G-domain of Ras is highly conserved in GNBPs, we propose that the finding here holds for all GNBPs.

1. Introduction

Guanine nucleotide-binding proteins (GNBPs) are key in many processes such as cell growth, cell differentiation, vesicular trafficking, cytoskeletal reorganization, protein syntheses and nucleocytoplasmic transport [1]. In an eukaryotic cell 100–150 different GNBPs are found [2]. They act as molecular switches. Bound to GDP they are inactive, bound to GTP they are active, transducing the signal. The GTP hydrolysis, initiated by a nucleophilic attack of water at the γ-phosphate, turns the signal off [3]. Therefore, the catalysis of the intrinsic slow GTP hydrolysis by Ras and its activation by GAP · Ras (GTPase activating protein) is crucial for signal transduction. If the catalysis of GTP hydrolysis is malfunctioning by oncogenic mutations in Ras, uncontrolled cell growth and finally cancer results [1]. Ras is the prototype of the family of GNBPs, which contain a structurally highly conserved G-domain, and is extensively studied by various methods, including X-ray [4–6], NMR [7,8], theoretical methods [9–12] and FTIR [13–18].

The structures of the active form (Ras · GTP) [5], the inactive form (Ras · GDP) and the transition state analog (GAP · Ras · GDP · AlF4) [4] were milestones in the understanding of the GTPase mechanism. The active form Ras · GTP is measured at 100 K [5] or with the non-hydrolysable GPPNHP [3] as the nucleotide, where the key bond is substantially altered. Complementary, time resolved FTIR can resolve the GTPase reaction in real time at room temperature in solution. Fig. 1(a) is showing the absorbance changes in the infrared during the intrinsic GTPase reaction of Ras together with the structural model of Ras. The global multiexponential kinetic analysis [19] gives a single exponential for the time course of this reaction. In Fig. 1(b) the absorbance changes in the infrared during the GAP-catalyzed

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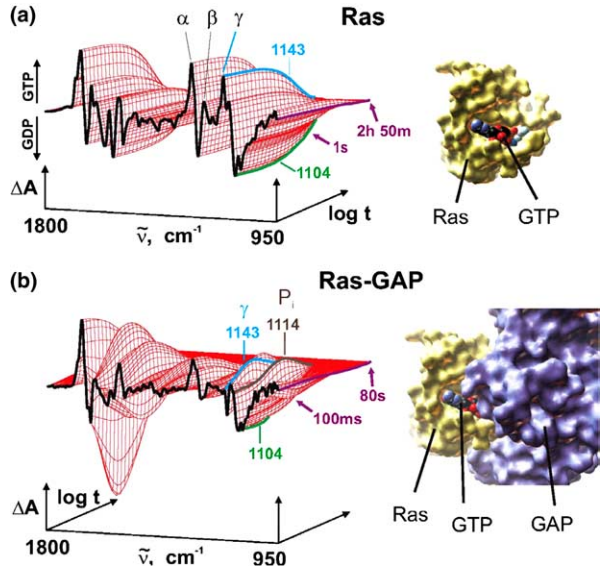


Fig. 1. (a) Time resolved infrared absorbance difference spectra during the intrinsic Ras-catalyzed GTPase reaction. The global fit analysis [19] provides a single exponential function and shows the change from Ras · GTP towards Ras · GDP as seen by the vibration at 1143 cm^{-1}. (b) Time resolved infrared absorbance difference spectra during the GAP-catalyzed GTPase reaction of Ras. The global fit is a sum of three exponential functions, giving rise to two intermediates as seen at 1143 and 1114 cm^{-1}, respectively. At 1143 cm^{-1} appearance of GTP from caged-GTP and GTP hydrolysis is observed. At 1114 cm^{-1} the protein bound P_i appears and is released in the rate limiting step to the external bulk medium. Compared with the intrinsic reaction, it is catalyzed by several orders of magnitude. Additionally the structural models of Ras and Ras · GAP bound to GTP are shown [45].

GTPase reaction of Ras are shown. GAP accelerates the GTPase reaction of Ras by a factor of 10^5. The global multiexponential kinetic analysis provides a sum of three exponential functions for the GAP-catalyzed GTPase reaction of Ras. The first describes the appearance of GTP from our precursor caged-GTP (see, for example, 1143 cm^{-1}). With the second rate, GTP disappears and protein bound P_i appears (1114 cm^{-1}) as an intermediate. The latter is released from the protein in the rate limiting step, described by the third rate [13]. The GTP bands are assigned using group specific isotopically labeled GTP. Thereby, the IR frequency of the labeled group is downshifted (change of μ in formula 1). The isolated α, β and γ-asymmetric stretching vibrations are assigned [14,15]. The frequency ν of a diatomic vibration can be calculated by

$$\nu = \frac{1}{2\pi}\sqrt{\frac{k}{\mu}}, \tag{1}$$

where k is the force constant and μ is the reduced mass of the involved atoms. A change in the charge distribution leads to a change in bond order, which is connected with a change in k. Thus frequency shifts of isolated vibrations indicate changes in the charge distribution [20]. The band positions for Ras · GTP are remarkably

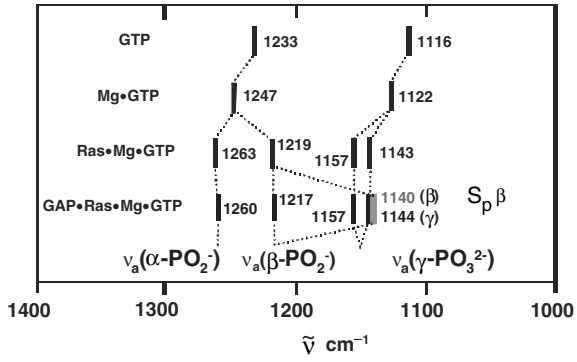


Fig. 2. The frequency shifts of GTP with different binding partners, assigned by means of isotopically labeled GTP [13–15,17,46]. The asymmetric stretching vibration of β-phosphate is downshifted by Ras binding and much further downshifted by binding of Ras · GAP.

different from the vibrations of GTP in water. The following information (see also Fig. 2) is deduced:

1. The GTP bands are much sharper due to Ras binding, because the conformational phase space of GTP is constricted by the protein.
2. The vibrations of α-, β- and γ-phosphate are highly coupled in water but decoupled within the protein. This is due to the different environment of these groups within the binding pocket and due to a specific conformation induced by the protein, which is less susceptive to coupling.
3. α- and γ-bands are upshifted in Ras but the β-band is downshifted compared to GTP in water. Positioning of the positively charged Mg and Lys16 draws negative charge towards the β-oxygens [14]. This decreases the bond order of the β-PO bonds and leads to the downshift. The environment is thus already more product like. In GDP, each β-oxygen consists of a formal partial charge of −2/3 compared to −1/2 in GTP.
4. The only effect of GAP binding on GTP and GDP is a large downshift of the $S_p\beta$ vibration. This indicates that even more negative charge is shifted towards β-phosphate.

Entropy is often taken as the major factor in enzyme catalysis [21,22]. The idea is, that within the protein, many degrees of freedom are "frozen" and that by this the reaction is guided towards the products. This theory was recently questioned by Warshel [23].

The measure for the amount of enzyme catalysis is the difference of the activation free energy of the enzyme catalyzed reaction and the reaction in water,

$$\Delta\Delta G^* = \Delta G^*_{\text{enzyme}} - \Delta G^*_{\text{water}}, \tag{2}$$

which is composed of $\Delta\Delta H^*$ and $\Delta\Delta S^*$. Warshel did molecular dynamics calculations on enzyme catalyzed reactions and compared them to the corresponding reac-

tion in solution. He found that $\Delta\Delta S^*$ is small, because many of the motions that are free in the reactants state are still free in the transition state and the binding to the enzyme does not completely freeze the motion of the reacting fragments. Further he pointed out, that the binding entropy is different from the entropy of activation in water.

Experimentally, the individual ΔH^* and ΔS^* can be determined by Arrhenius plots. This method is frequently used in chemistry and also applications to biological systems are found in the literature [24,25]. A frequent problem is the separation of individual steps in a complex kinetic system, i.e. for GTPases, one has to use single turnover conditions, because otherwise the nucleotide release could be the rate determing step. To achieve this, we start with a 1:1 complex of Ras and caged-GTP, which after irradiation results Ras · GTP [26]. Monitoring the phosphate bands of both, Ras · GTP and the products Ras · GDP + P_i will give us the kinetics of the hydrolysis reaction, separated from other reactions like the substrate binding.

In this paper we determine the entropic and enthalpic contributions of the activation energy for the Ras catalyzed GTP hydrolysis and we compare these results with the corresponding reaction in water and in water with Mg^{2+} ions.

2. Experimental and theoretical methods

Samples for the hydrolysis rate of GTP in water were prepared by dissolving its trilithium salt in water (bidest), resulting a pH of 5.5. No buffer or other ions were added. The concentration was 1 mM. For the Mg samples, 100 mM $MgCl_2$ were added to the above solution.

The hydrolysis rate was measured by HPLC. A C18 reversed phase column (ODS-Hypersil, 5 μm, 250 mm * 5 mm) was used with a flow rate of 1.2 mlmin^{-1}. The mobile phase was a mixture of water and acetonitrile 87:13 (v/v), 50 mM potassiumphosphate (pH 6.5) and 5 mM tetrabutylammoniumbromide (as ion pair reagent), resulting a good separation of G, GMP, GDP, GTP. The detection was done by measuring the UV absorption at 254 nm. Because the absorption stems from the guanosine moiety of the molecules, the absorption coefficients are very similar for the four species. Thus the GTP ratio was determined by the integration of the GTP absorption divided by the sum of all four integrated peaks.

Wild-type full-length H-Ras was prepared from *Escherichia coli* [27] CK600K with the *ptac-ras* plasmide. The FTIR measurements were performed similar as described in [14,15]. The sample solutions for the measurements with Ras · caged nucleotides contained 12 mM Ras · caged GTP, 200 mM Mes (pH 6.0), 20 mM $MgCl_2$, 20 mM DTT and 0.1% glycol. DTT was added to scavenge the reactive photolysis byproduct 2-nitrosoacetophenone [28]. The FTIR measurements were performed using the fast scan technique [29] with a scanner velocity of 100 kHz in the single sided fast return mode. The spectrometer used was an IFS 66v/s (Bruker). Photolysis of caged-GTP was performed by a series of 50 flashes at a repetition rate of 250 Hz with an LPX 240 XeCl-excimer laser (Lambda Physics, Göttingen, Germany) at 308 nm. The degree of conversion of the caged compound was greater 90%. The sample temperature was measured directly at the sample holder by a thermocouple element. Difference spectra were processed with the OPUS software (Bruker) between 1800 and 900 cm^{-1}. The progress of GTPase can be monitored best as a difference of the two ΔA of the asymmetric GTP- and GDP-stretching vibration of α-PO_2^- at 1263 and 1236 cm^{-1}. The advantage of taking two reference points close to each other instead of monitoring the trace of a single wavenumber is an annihilation of baseline drifts.

3. Results and discussion

The rates of GTP hydrolysis in water and in water with Mg^{2+} ions were measured in the temperature range from 334 to 354 K. The conversion was determined by ion-pair HPLC. The decay of GTP with time t was single exponential in all cases and was fitted by

$$[\mathrm{GTP}] = [\mathrm{GTP}]^0 \cdot \mathrm{e}^{-kt}. \tag{3}$$

The resulting values for the reaction rate constant k were used for the Arrhenius plots shown in Fig. 3. From a linear regression the values for the activation energy E_a and logarithm of the pre-exponential factor ln(A) were obtained (see formula in Fig. 3).

According to transition state theory (TST) the reaction rate k relates to the free energy of activation by

$$k^{\mathrm{TST}} = \left(\frac{k_B T}{h}\right) \cdot \mathrm{e}^{-\Delta G_0^*/RT}, \tag{4}$$

where k_B is the Boltzman constant, T the temperature, h the Planck constant, R the gas constant and ΔG_0^* the free energy of activation [30,31]. In solution often Kramers theory is applied, because here multiple passages across the transitions state are possible due to viscous damping by non-reactive degrees of freedom [32]. The deviation from transition state theory is described by the transmission coefficient κ.

$$k = \kappa \cdot k^{\mathrm{TST}}. \tag{5}$$

Our reactions will be in the overdamped regime, where the prefactor is inversely proportional to the viscosity [33]. In Kramers theory, the curvature of the one dimensional reaction coordinate (ω) replaces $k_B T/h$ leading to:

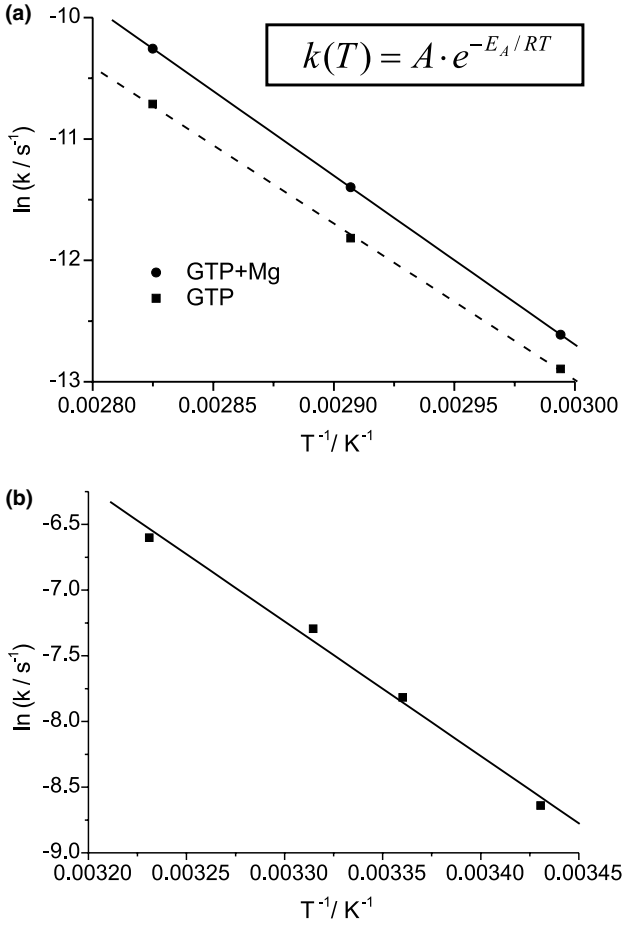


Fig. 3. (a) Linear regressions of the plots of $\ln(k)$ versus $1/T$ for the hydrolysis reaction of GTP in water (dashed line) and GTP in water/Mg^{2+} (solid line). (b) Linear regression of the plot of $\ln(k)$ versus $1/T$ for the hydrolysis reaction of GTP in Ras.

$$k = \left(\frac{\omega\eta(T_0)}{\eta(T)}\right) \cdot e^{-\Delta G_0^*/RT}. \quad (6)$$

In our temperature range, the viscosity η of water changes only by a factor of ~2. Further, because a well defined bond is broken and the changes in the system are far smaller than e.g. in protein folding, we assume, that our prefactor comes close to k_BT/h. Note, that even a deviation by a factor of 100 would change $T\Delta S$ by less than 3 kcal mol^{-1} and our general conclusions will still be valid. Thus, we assume $\kappa \sim 1$ and we will discuss our results within the framework of transition state theory.

Since the standard volume of activation is negligible for reactions in solution, from E_a the activation enthalpy ΔH^* can be calculated by means of

$$\Delta H_0^* = E_a - RT, \quad (7)$$

and the activation entropy can be calculated by

$$\Delta S_0^* = R\left(\ln(A) - \ln\left(\frac{k_BT}{h}\right) - 1\right) \quad (8)$$

(see [30]). The results are shown in Table 1.

Further, the temperature dependence of the GTPase reaction of Ras was investigated. Here, time-resolved FTIR was used for the measurements. A typical temporal evolution of a spectrum is shown in Fig. 1(a). The advantages of FTIR compared to HPLC for the Ras catalyzed reaction are the higher time-resolution and the spectral information which is obtained, i.e. one can determine Ras bound nucleotide from unbound nucleotide. The temperature was varied between 292 and 310 K. The corresponding Arrhenius plot is shown in Fig. 3(b). The results and the calculated (formula 7 and 8) thermodynamic parameters are summarized in Table 1.

Fig. 4 shows the standard free energy along the GTP hydrolysis reaction coordinate with and without Ras at standard conditions (25 °C). The ΔG_0^* for both reactions are taken from this work. The additional energies are obtained from the K_D values for Ras + GTP [34] and Ras + GDP [35] and the K_{eq} for the hydrolysis reaction [36] by

$$\Delta G_0^R = -RT \ln K. \quad (9)$$

Further, the barrier for GTP dissociation from Ras · GTP was calculated by

$$\Delta G_0^* = -RT \ln\left(\frac{kh}{k_BT}\right), \quad (10)$$

using the k_{off} from [34]. The barrier for the GDP association was calculated by the same formula using the apparent rate constant for k_{on} as described in [35].

For the reaction in water applies $\Delta G_0^* = 27.9$ kcal mol^{-1}. At room temperature it has a high enthalpy contribution ($\Delta H_0^* = 25$ kcal mol^{-1}) and a small entropy contribution ($T\Delta S_0^* = 2.8$ kcal mol^{-1} at 25 °C). This result is in line with a measurement of another triphosphate [37] and calculated barriers by Wang et al. [38].

With Ras $\Delta G_0^* = 22.1$ kcal mol^{-1}, again with a high enthalpy ($\Delta H_0^* = 19.8$ kcal mol^{-1}) but a low entropy ($-T\Delta S_0^* = 2.3$ kcal mol^{-1} at 25 °C) contribution. Surprisingly, there is no determination of ΔH^* and ΔS^* for the Ras catalyzed GTPase reaction in the literature to our knowledge. The calculated barrier of more than 40 kcal mol^{-1} on the potential energy surface from

Table 1
Summary of the data obtained by the Arrhenius plots and the derived standard thermodynamic parameters (25 °C) in kcal mol^{-1}

	$\ln(A)$	E_a	ΔH_0^*	ΔS_0^*	$-\Delta S \cdot T$	ΔG_0^*
Water	25.7	25.6	25.0	−0.0095	2.8	27.9
Water/Mg^{2+}	29.1	27.7	27.1	−0.0027	0.8	27.9
Ras	26.6	20.3	19.8	−0.0078	2.3	22.1

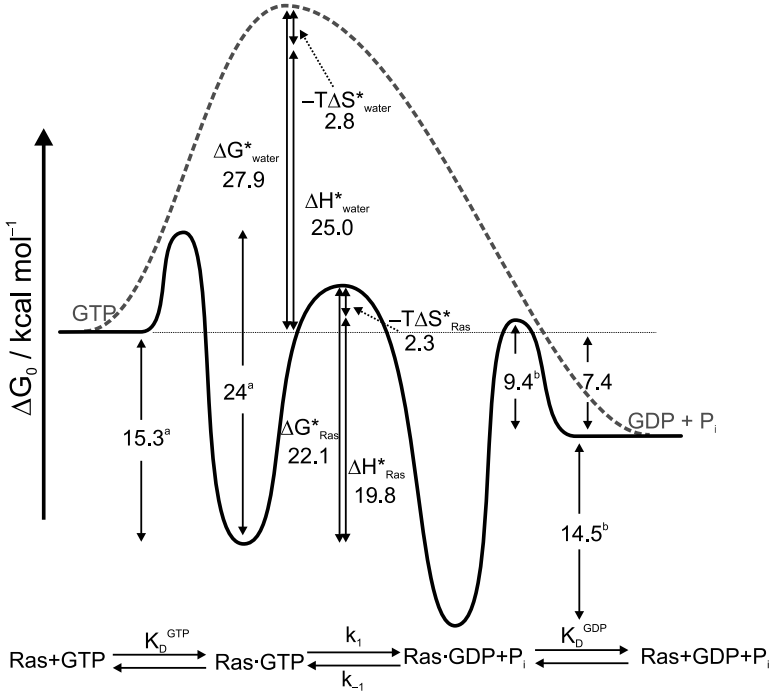


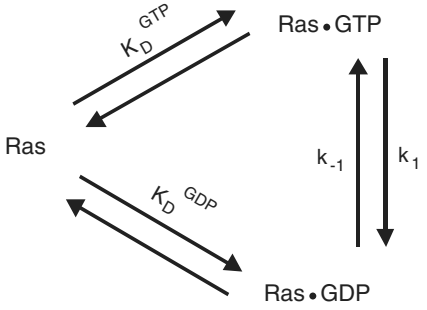
Fig. 4. The standard free energy during the hydrolysis of GTP in water (dashed line) and Ras catalyzed (solid line). The reaction coordinate shows one GTPase cycle according to Scheme 1: The first step is the association of Ras and GTP, the second is the hydrolysis and the third the dissociation of Ras and GDP. The enthalpic and the entropic contributions to the activation free energy of the rate determing hydrolysis step is depicted. [a] from [34] and [b] from [35].

Futatsugi et al. [10,39] is far from our experimental ΔH^* and even higher then the barrier in water without catalysis.

Note, that the rate limiting barrier is the difference in free energy of the Ras · GTP transition state with the Ras · GTP complex and not with the separated Ras + GTP.

Within biological systems, the energy of an exergonic substrate binding process will rapidly be given to the environment and thus the rate determing barrier will be $\Delta G^*_{\mathrm{Ras}}$ (Fig. 4). A measure for the enzymatic acceleration is $\Delta\Delta G^*$ (formula 2), which is here $27.9-22.1 = 5.8$ [kcal mol^{-1}] at 25 °C. It is composed of $\Delta\Delta S^*$ and $\Delta\Delta H^*$. Our result clearly shows, that the main catalytic effect from the protein is enthalpic, since $\Delta\Delta H^*_0$ is $25.0-19.8 = 5.2$ [kcal mol^{-1}] but $-T\Delta\Delta S^*_0$ is only $2.8-2.3 = 0.5$ [kcal mol^{-1}]. Interestingly Mg^{2+} lowers the activation entropy more than Ras. This can be explained by specific interaction of Mg^{2+} with only the ground state of GTP. GTP is forced in distinct conformations, which lowers its entropy. Thus $-TS$ of the ground state is higher with Mg^{2+} than in water and accordingly ΔS^*_{Mg} gets smaller.

The question arises, what causes the large change in activation enthalpy between the GTP hydrolysis in water and the protein. Electrostatic interactions usually contribute most to the enthalpic term [40]. Vibrational spectroscopy is extremely sensitive in detecting changes



Scheme 1.

in charge distribution. Since a change in charge distribution will only slightly alter bond lengths, these effects are usually below the resolution of X-ray. For example a change of 0.001 Å of a PO bond will not be resolved by X-ray, but will give a shift of 4 cm^{-1} of a PO^- stretching vibration, which can be resolved by FTIR. This makes FTIR an extremely valuable tool, to find the key features for enzyme catalysis.

As shown in Fig. 2, binding of GTP to Ras and Ras · GAP indeed induces downshifts of the β-phosphate vibration, indicating shifts of negative charge towards β-phosphate [13,14]. This charge shift is quantified in QM/MM-calculations [41] to be 0.14 e according to the ESP method [42]. The Ras catalyzed GTP hydrolysis is relatively slow, to enable the regulation of signal transduction by GAP. GAP binding catalysis the hydrolysis by addi-

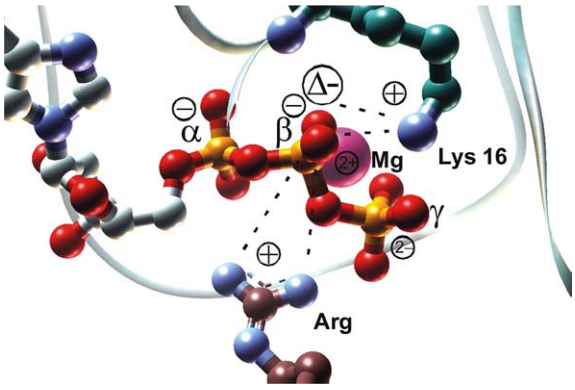


Fig. 5. Mg^{2+} and Lys16 from Ras and the arginine finger from GAP draw negative charge towards the β-phosphate of GTP. The main catalytic effect of Ras and GAP is enthalpic and seems to originate from this electrostatic interactions.

tional five orders of magnitude [43]. The only effect on GTP-bands due to GAP binding is a much larger downshift of the β-vibration, all other GTP and GDP vibrations are not affected. The downshift indicates a shift of even more negative charge towards β-phosphate. The analysis here shows that the charge shift in GTP seems the main contribution to the catalysis.

The interactions which induce the charge shift towards β-phosphate are shown in Fig. 5 [14]. Besides the positive charge of Lys16 and Mg^{2+} from Ras, the catalytic arginine finger provides a positive charge which pulls the negative charge towards β-phosphate in the GTP ground state. This contradicts the picture provided for transition state analogs in GTPases in which arginine interacts with the γ-phosphate or the P_i, respectively [44]. We propose an arginine movement towards β-phosphate in the ground state.

The main catalytic effect of Ras is enthalpic and originates from electrostatic interactions. Combination of structural models and time-resolved FTIR can explain the molecular basis of the thermodynamics of GTPases.

Acknowledgements

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