FEBS Letters 347 (1994) 133–136

FEBS 14135

Energetic aspects of intramolecular coupling between the nucleotide binding site and the distal switch II region of the yeast RAS2 protein

Emmanuele De Vendittis[a], Ottavio Fasano[b,*]

[a]*Dipartimento di Biochimica e Biotecnologie Mediche, Universita' di Napoli Federico II, via S. Pansini 5, I-80131 Napoli, Italy*
[b]*Dipartimento di Biologia Cellulare e dello Sviluppo, Universita' di Palermo, via Archirafi 22, I-90123 Palermo, Italy*

Received 5 May 1994

Abstract

We have studied the interaction of the yeast RAS2 protein with guanine nucleotides using energetic parameters for the dissociation of RAS·nucleotide complexes. The results indicated that a Gly → Ser substitution at position 82 led to an altered interaction with GppNHp and, to a lesser extent, also with GDP. It was also possible to conclude that structural perturbation of Gly^{82} can stimulate nucleotide release by decreasing the energetic barrier for nucleotide dissociation. This, together with the observation that residues 80 and 81 are involved in the response of RAS to nucleotide exchange factors without affecting GDP binding per se, suggests a potential mechanism for exchange factor-stimulated GDP release.

Key words: RAS; *Saccharomyces cerevisiae*; GDP exchange factor; *CDC25*; *SCD25*

1. Introduction

RAS proteins are involved in the conversion of chemical energy into vectorial processes in evolutionarily distant organisms such as yeast and vertebrates (for reviews see [1–4]). The specificity of interaction of RAS proteins with distinct macromolecular effectors is determined by a non-covalently bound GTP or GDP molecule. The conversion of RAS between GTP- and GDP-bound states is regulated by effectors that stimulate either the GTPase activity (GAPs) or the nucleotide exchange reaction (GEFs). This, together with the irreversibility of the GTP hydrolytic step, causes RAS to alternatively interact with distinct elements during the signal transduction cycle [5–9]. Two regions of the RAS molecule, denominated 'switch I' and 'switch II', displaying different conformations in the GTP- and GDP-bound states, are the major determinants of the specificity of the interaction with effectors. A model for the conformational transition of the switch II region of RAS (residues 67–84, yeast RAS2 coordinates) upon conversion from the GTP- to the GDP-bound form has been proposed by Stouten et al. [10].

Recently, it has been shown that distal amino acid residues of the switch II region, including the critical Arg^{80}, play a role in determining the response of the RAS2·GDP complex to the SCD25 nucleotide exchange factor [11]. It was also shown that an Arg^{80} → Asp substitution did not affect the GDP off-rate in the absence of the exchange factor. The finding by Kavounis et al. [12] that a Gly^{82} → Ser substitution increased the rate of Gpp(NH)p release indicated that structural alterations of the distal switch II region were transmitted to the nucleotide binding site, as also suggested by genetic, crystallographic, and NMR data [8,13–15]. At the temperature used, the effect of the Gly^{82} → Ser substitution on the Gpp(NH)p off-rate was selective, since no significant modification of the GDP off-rate was found. In this paper, we show that a Gpp(NH)p-specific effect can be observed only in a narrow temperature interval. Using energetic parameters for the dissociation of RAS·nucleotide complexes for evaluating RAS·nucleotide interactions, we show that the replacement of Gly^{82} by Ser decreases the energetic barrier for nucleotide release. The structural alteration induced by the Gly → Ser substitution could share features with that induced by GDP dissociation factors to physiologically enhance the rate of recycling of RAS·GDP.

2. Materials and methods

2.1. Purification of wild-type and mutated RAS2 proteins

Pure, biologically active wild-type and mutated RAS2 proteins were obtained as described by Kavounis et al. [12]. Bacterial strains and induction conditions were also as described by the same authors.

2.2. Interaction of purified RAS proteins with guanosine nucleotides

Radioactive RAS2·nucleotide complexes were prepared by pre-incubating purified RAS2 proteins (complexed with GDP) with excess [^{3}H]GDP or [^{3}H]Gpp(NH)p for 30 min at 30°C in 40 mM Tris-HCl, pH 7.5, 0.5 mM dithiothreitol, 100 mM KCl, 1 mM EDTA. EDTA was added to facilitate the exchange of bound, unlabelled nucleotide with free, radioactive nucleotide. The reaction was terminated by dilution (to decrease EDTA concentration) and by the addition of $MgSO_4$ up to

*Corresponding author. Fax: (39) (91) 616 5665.

Abbreviations: GEF, guanine nucleotide exchange factor; Gpp(NH)p, guanyl-5′-yl imidodiphosphate; EDTA, [ethylenediamino]tetraacetic acid.

SSDI 0014-5793(94)00521-V

5 mM. The rate of dissociation of nucleotides from the corresponding RAS2·nucleotide complexes was measured by adding at zero time a 100-fold excess of unlabelled GDP. The displacement of the radioactive nucleotide was followed at appropriate time intervals by loading aliquots of the reaction mixture on Sephadex G-50 columns, as described in [12,16]. Semilogarithmic plots of residual RAS·nucleotide complex vs. time were linear [12]. Determination coefficients for linear interpolation of the experimental data were better than 0.990.

2.3. Determination of energetic parameters

Energies of activation were calculated from the Arrhenius equation:

$$k_{off} = A \cdot \exp(-E/RT)$$

where k_{off} = rate constant for dissociation of the nucleotide from Ras, A = Arrhenius constant, a term including collision frequency and steric factor, E = energy of activation, R = gas constant (8.314 $J \cdot K^{-1} \cdot mol^{-1}$), T = absolute temperature. The enthalpy of activation (ΔH^*) was calculated from the equation $\Delta H = E - RT$; the entropy of activation (ΔS^*) from $\Delta S^* = R \cdot \ln(\Delta h N_A / RTe)$, in which h is the Planck constant (6.624×10^{-34} $J \cdot s^{-1}$), N_A is the Avogadro number (6.023×10^{23} molecules $\cdot$ mol^{-1}), and e the base of natural logarithms. The Gibbs energy change of activation (ΔG^*) was calculated from $\Delta G^* = \Delta H^* - T \cdot \Delta S^*$.

3. Results

We purified a truncated, biologically active fragment of the yeast RAS2 protein over-expressed in *E. coli* [12]. The same procedure was used for the purification of a mutated form of the RAS2 protein with a Gly → Ser substitution at position 82. Since the purification procedure was carried out in the presence of excess GDP, the RAS2 protein was obtained in the GDP-bound form. We prepared defined amounts of RAS · [^{3}H]Gpp(NH)p complex by pre-incubating RAS · GDP with a tenfold excess of [^{3}H]Gpp(NH)p in the absence of Mg^{2+} ions and in the presence of EDTA (see section 2). The reaction was terminated by the addition of Mg^{2+} and by separation of the complex from the free nucleotide by gel-filtration. The Gpp(NH)p off-rate was determined by adding at zero time a 100-fold excess of unlabelled nucleotide to the purified RAS · [^{3}H]Gpp(NH)p complex. Following incubation in water baths at different temperatures the residual complex was determined at appropriate time intervals by analytical gel-filtration at 0°C (see section 2). It should be noted that the slow rate of nucleotide dissociation minimised the error introduced by dissociation taking place during the separation on analytical columns. The semilogarithmic plot of the residual complex vs. time was linear and allowed the determination of k_{off} of the reaction (see section 2.2.). We measured the Gpp(NH) k_{off} at different temperatures (Table 1). The corresponding Arrhenius plot is shown in Fig. 1A.

The same procedure was used to evaluate the GDP off-rate from a preformed RAS · [^{3}H]GDP complex at different temperatures (Table 1). The Arrhenius plot is shown in Fig. 1B. Derived energetic parameters for nucleotide dissociation are reported in Table 2. Mg^{2+} and SO_4 ions affected the GDP k_{off} (Table 3), however, the effect was similar for wild-type and mutated RAS protein (Table 3).

Table 1
Effect of temperature on the rate of dissociation of [^{3}H]GDP and [^{3}H]Gpp(NH)p from wild-type (wt) and mutated RAS2 proteins

Protein	Temperature (°C)	k_{off} GDP (10^3 min^{-1})	k_{off} Gpp(NH) (10^3 min^{-1})
RAS2 (wt)	0	0.077 ± 0.004	0.099 ± 0.012
	10	0.387 ± 0.037	0.620 ± 0.031
	20	1.332 ± 0.032	2.684 ± 0.226
	30	7.746 ± 0.424	18.491 ± 1.705
RAS2-G82S	0	0.104 ± 0.006	0.476 ± 0.017
	10	0.397 ± 0.027	1.989 ± 0.276
	20	1.350 ± 0.063	6.667 ± 0.850
	30	6.144 ± 0.381	35.973 ± 3.018

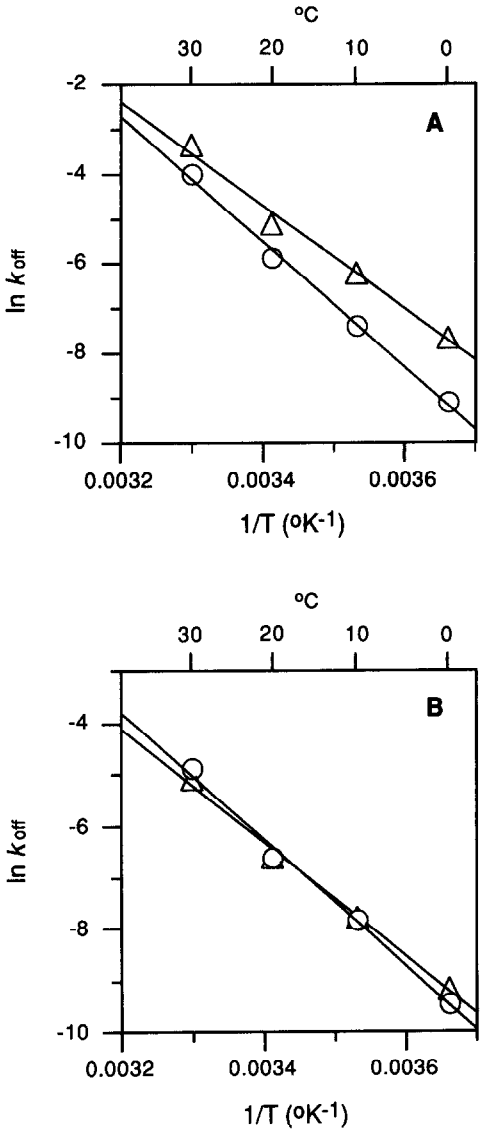

Fig. 1. Arrhenius plots of Gpp(NH)p (A) and GDP (B) k_{off} from wild-type RAS2 (○) and mutated RAS2-G82S (△) proteins. Apparent dissociation rate constants are from Table 1.

Table 2
Energetic parameters for the dissociation of guanosine nucleotides from the corresponding RAS·nucleotide complexes

Protein	Nucleotide	E (kJ/mol)	A (min^{-1})	ΔH^{*a} (kJ/mol)	ΔS^{*a} (kJ/mol K)	ΔG^{*a} (kJ/mol)
RAS2 (wt)	GDP	102.9	$3.6\ 10^{15}$	100.5	11.0	97.4
RAS2-G82S	GDP	92.3	$4.4\ 10^{13}$	89.9	−25.6	97.1
RAS2 (wt)	Gpp(NH)p	116.1	$1.6\ 10^{18}$	113.7	61.7	96.2
RAS2-G82S	Gpp(NH)p	96.0	$1.0\ 10^{15}$	93.6	0.3	93.5

[a]Calculated at 10°C as described in section 2.

4. Discussion

A previous kinetic analysis indicated that the Gpp(NH)p off-rate from a purified RAS2-G82S protein was faster than from the wild-type RAS2 protein [12]. The data reported in the present paper support the conclusion that the altered Gpp(NH)p off-rate caused by the G82S substitution could reflect a specific structural alteration of the nucleotide binding site, rather than a denaturation-prone protein conformation. In fact, the difference between the wild-type and the mutated protein was more evident at 0°C than at 30°C (see Fig. 1A). Moreover, at least in a narrow temperature interval, the GDP off-rate was unaffected by the G82S substitution (Fig. 1B).

The difference in Gpp(NH)p off-rate was paralleled by a different value of the activation energy for Gpp(NH)p dissociation for the two proteins (116.1 vs. 96.0 kJ/mol for the wild-type and for the RAS2-G82S protein, respectively, see Table 2). The activation energies for nucleotide dissociation were more informative than dissociation rate constants when comparing the GDP-binding properties of the wild-type and mutated protein. In fact, the observation that the GDP off-rates of the RAS2 and RAS2-G82S proteins were apparently identical at 10°C [12] seemed to indicate that the GDP-bound conformation was not sensitive to structural perturbation of residue 82. However, a significant difference between the wild-type and the RAS2-G82S protein was found when considering the activation energies for GDP dissociation (102.9 vs. 92.3 kJ/mol, respectively, Table 2). This apparent paradox can be explained by the fact that the Arrhenius plots for the two proteins, even though showing different slopes, intersect at a temperature value of about 16°C (Fig. 1B). The differences in the GDP k_{off} value between the wild-type and the mutated RAS2-G82S protein were retained at different Mg concentrations (Table 3). Even though the differences between activation energies and activation entropies (Table 2) for GDP dissociation from wild-type and RAS2-G82S protein were small, we think that they were significant because they could be reproduced in several experiments and with different protein preparations. Moreover, also in the case of GppNHp, the slope of the Arrhenius plot was more informative than the k_{off} value at a single temperature in revealing differences between wild-type and mutated proteins (Fig. 1A).

Table 3
Effect of $MgSO_4$ on the rate of dissociation of GDP from wild-type (wt) and mutated RAS2 proteins at 0°C

Protein	$MgSO_4$ (mM)	k_{off} GDP ($10^3\ min^{-1}$)	$k_{0.5}/k_5$
RAS2 (wt)	0.5	0.147	1.88
	5	0.078	
RAS2-G82S	0.5	0.231	2.22
	5	0.104	

Both activation entropies and activation enthalpies for nucleotide dissociation were reduced by the G82S substitution (Table 2). However, the introduction of an unfavourable entropic factor by the mutation was counteracted by a smaller ΔH^* value. As a consequence, the rate of nucleotide release from the RAS2-G82S protein was faster than that from the wild-type protein at low temperatures (Fig. 1).

A likely explanation for our data is that the G82S substitution influences the conformation of the nucleotide binding site. The effect of a single amino acid substitution on a site that is more than 20 Å away is possibly exerted through the intervening L_4-α2-L_5 region, acting as a linker between nucleotide-proximal and -distal residues [8]. If this interpretation is correct, the conformational flexibility of the linker region is expected to attenuate the effects of structural alterations of the distal switch II region on nucleotide-proximal amino acids and hence on nucleotide off-rates. Thus, the smaller effect of the G82S substitution on the GDP vs. GppNHp off-rate could reflect the greater conformational mobility of the L_4-α2-L_5 region in RAS·GDP compared to RAS·GppNHp [17,18]. Recently, residues 68–70 (yeast RAS2 protein coordinates), 73–74, 76, and 80–85 within the L_4-α2-L_5 region have been shown to be important for the stimulation of the GDP off-rate by GEFs [11,19–22]. Our data suggest that at least some of these residues might not have equivalent roles. In fact, mutagenic alteration of residues 80 and 81 strongly impaired GEFs' action while not affecting GDP binding in the absence of GEFs [11]. The recessive nature of mutations

leading to amino acid substitutions at positions 80 and 81 (M.G. Mirisola and O. Fasano, unpublished) indicates that these residues might be important for GEF binding to the GDP-bound conformation of RAS and GEF-stimulated nucleotide release. On the other hand, the effect of the G82S substitution on GDP and Gpp(NH)p off-rates in the absence of GEFs suggests that G82 is critical for determining a proper conformation of the Gpp(NH)p-, and, to a lesser extent, of the GDP-bound state. The partial response of the RAS2-G82S protein to a GDP exchange factor in vitro might therefore reflect a reduced ability to assume the GTP-bound conformation [12,22].

Acknowledgements: We would like to thank Prof. V. Bocchini for introducing us to thermodynamic aspects of protein–nucleotide interactions. This work was supported by grants from the Associazione Italiana per la Ricerca sul Cancro and from MURST 60%, with the collaboration of Centro di Oncobiologia Sperimentale.

References

[1] Tamanoi, F. (1988) Biochim. Biophys. Acta 948, 1–15.
[2] Gibbs, J.B. and Marshall, M.S. (1989) Microbiol. Rev. 53, 171–185.
[3] Broach, J.R. and Deschenes, R.J. (1990) Adv. Cancer Res. 54, 79–139.
[4] Boguski M.S. and McCormick, F. (1993) Nature 366, 643–654.
[5] Bourne, H.R., Sanders, D.A. and McCormick, F. (1990) Natur 348, 125–132.
[6] Lowy, D.R. and Willumsen, B.M. (1993) Annu. Rev. Biochem. 62, 851–891.
[7] Kaziro, Y., Itoh, Y., Kozasa, T., Nakafuko, M. and Satoh, T. (1991) Annu. Rev. Biochem. 60, 349–400.
[8] Pai, E. (1991) Curr. Biol. 1, 941–945.
[9] Polakis, P. and McCormick, F. (1993) J. Biol. Chem. 268, 9157–9160.
[10] Stouten, P.F.W., Sander, C., Wittinghofer, A. and Valencia, A. (1993) FEBS Lett. 320, 1–6.
[11] Verrotti, A.C., Crechet, J.B., Di Blasi, F., Seidita, G., Mirisola, M., Kavounis, C., Nastopoulos, V., Burderi, E., De Vendittis, E., Parmeggiani, A. and Fasano, O. (1992) EMBO J. 11, 2855–2862.
[12] Kavounis, C., Verrotti, A.C., De Vendittis, E., Bozopoulos, A., Di Blasi, F., Zahn, R., Crechet, J.B., Parmeggiani, A., Tsernoglou, D. and Fasano, O. (1991) FEBS Lett. 281, 235–239.
[13] Fasano, O., Crechet, J.B., De Vendittis, E., Zahn, R., Feger, G., Vitelli, A. and Parmeggiani, A. (1988) EMBO J. 7, 3375–3383.
[14] Jurnak, F., Heffron, S. and Bergman, E. (1990) Cell 60, 525–528.
[15] Miller, A.F., Papastavros, M.Z. and Redfield, A.G. (1992) Biochemistry 31, 10208–10216.
[16] De Vendittis, E., Zahn, R. and Fasano, O. (1986) Eur. J. Biochem. 161, 473–478.
[17] Milburn, M.V., Tong, L., de Vos, A.M., Brünger, A., Yamaizumi, Z., Nishimura, S. and Kim, S.-H. (1990) Science 247, 939–945.
[18] Schlichting, I., Almo, S.C., Rapp, G., Wilson, K., Petratos, K., Lentfer, A., Wittinghofer, A., Kabsch, W., Pai, E.F., Petsko, G. and Goody, R.S. (1990) Nature 345, 309–315.
[19] Mistou, M.Y., Jacquet, E., Poullet, P., Rensland, H., Gideon, P., Schlichting, I., Wittinghofer, A. and Parmeggiani, A. (1992) EMBO J. 11, 2391–2397.
[20] Mosteller, R.D., Han, J., and Broek, D. (1994) Mol. Cell. Biol. 14, 1104–1112.
[21] Howe, L.R. and Marshall, C.J. (1993) Oncogene 8, 2583–2590.
[22] Quilliam, L.A., Kato, K.K., Rabun, K.M., Hisaka, M.M., Huff, S.Y., Campbell-Burk, S. and Der, C.J. (1994) Mol. Cell. Biol. 14, 1113–1121.