Characterization of the hinges of the effector loop in the reaction pathway of the activation of *ras*-proteins. Kinetics of binding of beryllium trifluoride to V29G and I36G mutants of Ha-*ras*-p21

STEVEN KUPPENS,¹ JOSÉ FERNANDO DÍAZ,² AND YVES ENGELBORGHES¹

¹Laboratory of Biomolecular Dynamics, Katholieke Universiteit Leuven, Celestijnenlaan 200D, B-3001 Heverlee, Belgium

²Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, C/ Velazquez 144, 28015 Madrid, Spain

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Abstract

This work experimentally confirms the pathway of activation of Ha-*ras*-p21, which was calculated by the method of Targeted Molecular Dynamics (TMD) (Díaz JF, Wroblewski B, Schlitter J, Engelborghs Y, 1997a, *Proteins Struct Funct Genet* 28:434–451). The process can be studied experimentally by analyzing the binding of BeF₃[−] to the GDP complex of the active fluorescent mutant Y32W (Díaz JF, Sillen A, Engelborghs Y, 1997b, *J Biol Chem* 272:23138–23143). Two mutants, V29G and I36G, have been constructed at both sides of the effector loop of the active fluorescent mutant. This was done to check the proposed reaction pathway and to provide further insight into the mechanism of the activation of *ras* proteins. Both mutations accelerate the conformational isomerization with two orders of magnitude, demonstrating convincingly the role of these residues as hinges of the effector loop in one or more of the transitions of the conformational change. These results provide experimental support to the pathway calculated by TMD analysis.

Keywords: conformational change; G-proteins; molecular dynamics; molecular switch; stopped flow

Ha-*ras*-p21 is a small protein (189 residues) belonging to the group of the guanine-binding proteins (G-proteins) (Bourne et al., 1990, 1991). Like all the other G-proteins, the membrane-bound Ha-*ras*-p21 is a molecular switch that controls the growing, proliferation, and differentiation processes of the cell (Barbacid, 1987). The *ras*-p21 proteins bind guanine nucleotides complexed with a magnesium ion. The physiological state of the protein depends on the nature of the bound nucleotide. In the active form the protein has GTP bound, and to change to the inactive state the γ -phosphate of the GTP has to be hydrolyzed and released. After hydrolysis the GDP remains bound to the protein, which changes its conformation to the inactive form. The bound GDP can be exchanged for GTP allowing the protein to switch back to the active form and to close the cycle (Wittinghofer & Pai, 1991). Both processes, the hydrolysis and the exchange, which determine the conformational

state of the protein, are controlled by external proteins, the GTPase activating proteins (GAPs) in the case of the hydrolysis (Adari et al., 1988), the guanine exchange factors (GEFs), and guanine release proteins (GNRPs) in the case of the nucleotide exchange (McCormick, 1994).

The structures of the active and the inactive form have been crystallographically resolved (Pai et al., 1990; Tong et al., 1991), but very little is known about the internal rearrangements involved, the driving forces, and the amino acids that are the hinges of the conformational transition.

The system is extremely important because point mutations of the *ras*-genes can be found in 30% of the human tumors. Because of this the *ras*-genes were identified as proto-oncogenes (Barbacid, 1987).

The biological relevance of the system as well as the small size of the protein make it a useful model system to study the mechanism of conformational transitions in proteins. In our previous papers, computational methods, i.e., molecular dynamics (MD) (Díaz et al., 1995) and targeted molecular dynamics (TMD) (Schlitter et al., 1994; Díaz et al., 1997a) have been applied to find possible hinges and levers of the conformational transition as well as to find a possible pathway for the activation and deactivation of the molecule. Using the TMD technique it is possible to correlate the successive conformational changes of certain residues and parts

Reprint requests to: Yves Engelborghs, Laboratory of Biomolecular Dynamics, Katholieke Universiteit Leuven, Celestijnenlaan 200D, B-3001 Heverlee, Belgium; e-mail: yves.engelborghs@fys.kuleuven.ac.be.

Abbreviations: DT transition, GDP- to GTP-bound transition; DTE, dithioerythritol; GAPs, GTPase activating proteins; GDP, 5'-guanosine diphosphate; GEFs, guanine exchange factors; GNRPs, guanine release proteins; GTP, 5'-guanosine triphosphate; MD, molecular dynamics; PCR, polymerase chain reaction; ps, picosecond; SDS-PAGE, sodium dodecyl sulfate–polyacryl amide gel electrophoresis; TMD, targeted molecular dynamics.

of the molecule with transient maxima of the energy during the transition. In this way it became clear that the conformational transition between both states of the protein is not a single process but a succession of different microtransitions that complete the process like domino pieces falling one after another. It is possible to determine the residues that may constitute energy barriers for a certain microtransition and so to design mutants that may stabilize or destabilize the transition state of one particular microtransition.

To check the theoretical calculations, it is necessary to design an experimental system that allows the measurement of the speed of the conformational transition. The main problem with the natural system is that in physiological conditions the rate limiting step of the conformational change is not the transition itself but the hydrolysis of the γ -phosphate in the case of the deactivation, and the interchange of the nucleotide in the case of the activation (Adari et al., 1988; Krenzel et al., 1990; Neal et al., 1990; Antonny et al., 1991; Lowy & Willumsen, 1993; Moore et al., 1993). The obvious solution for this problem is to use a phosphate analogue that would be able to bind or dissociate from its site, so activating or deactivating the protein without the steps of hydrolysis or nucleotide exchange (Bigay et al., 1987). Unfortunately, the *ras*-like G-proteins have been described not to bind or be activated by phosphate analogues (Kahn, 1991). Recently, Mittal et al. (1996) described that in the presence of GAP, Ha-*ras*-p21 can be activated by the phosphate analogue aluminum tetrafluoride. In this case the GAP provides an additional residue to the active center (Ahmadian et al., 1997) that stabilizes the transition state of the hydrolysis reaction. This transition state is mimicked by the aluminum tetrafluoride-GDP-p21 complex. It was therefore logical to assume that beryllium trifluoride, which is an analogue of the ground state of the γ -phosphate, would be able to bind the Ha-*ras*-p21 GDP complex and activate it by forming an analogue of the p21-GTP complex. In our previous work (Díaz et al., 1997b) using an active mutant of Ha-*ras*-p21 (Y32W) that contains a tryptophan as a fluorescent label in the effector loop, we have demonstrated that beryllium trifluoride is able to bind to its site in the protein with a millimolar dissociation constant ($K_d = 8.13$ mM at 25 °C) and to induce the conformational transition toward a GTP-like state (judged on the equality of the fluorescence properties of W32 in the GTP and the GDP-BeF₃⁻ complexed state) (Díaz et al., 1997b). On the basis of the experimental results, we have postulated a reaction mechanism for the binding of the beryllium trifluoride complex and the subsequent conformational transition. The model is a two-step reaction with the first step being the binding of the BeF₃⁻ complex to the GDP-bound form of the protein. The second step is the conformational transition of the protein:

This model was able to explain the following kinetic data obtained with the stopped-flow measurements. In pseudo first order conditions, a bi-exponential fluorescence decrease is observed. The first exponential gives a rate constant that increases linearly with the concentration of BeF₃⁻ and therefore can be attributed to the direct binding of the ion. The value of the bimolecular rate constant ($146 \text{ M}^{-1} \text{ s}^{-1}$ at 25 °C), however, is much too small to be attributed to a simple diffusion controlled binding step.

The second exponential gives a rate constant that increases in a nonlinear way with the BeF₃⁻ concentration, allowing the determination of k_{+2} , k_{-2} , and K_1 .

To link the calculated pathway obtained from the TMD technique to the kinetic data and to obtain further information about the mechanism of the conformational transition, two mutants at both sides of the effector loop have been constructed. According to the calculated TMD pathway (Díaz et al., 1997a), the effector loop plays a key role in the transition by transmitting the recognition signal from the P-loop, which binds the phosphate chain of the nucleotide, to the switch II region. NMR experiments also demonstrated that the structure of the effector loop (residues 30–38) is ill defined because of a high dynamic mobility (Kraulis et al., 1994). The two mutated amino acids Val29 and Ile36 play the role of a hinge in the movements of the effector loop. They show important dihedral transitions during the conformational changes, which may contribute to the weighted average activation energy. (The actual energy profile consists of a sequence of many small peaks which are very difficult to attribute to specific residues.) Their substitution by glycines should lower the activation energy of the microtransitions and should speed up the overall conformational transition. These mutants have been experimentally constructed and studied in this work.

Results

Binding of BeF₃⁻ to the GDP-bound form of the double mutants

Using the difference in fluorescence of Trp32 of Y32W-Ha-*ras*-p21 in the GDP-bound state and in the BeF₃⁻-GDP-bound state (GTP-like) (Díaz et al., 1997b), it is possible to titrate the protein with BeF₃⁻ and obtain the apparent binding constant. Figure 1 shows the titration curve of GDP-p21 (V29G-Y32W) at 15 °C. The curves were fitted to Equation 2. The apparent binding constants obtained are listed in Tables 1 and 2, and can be seen to agree within experimental error with the kinetically measured ones, except at 45 °C where the deviations are bigger.

Fig. 1. Titration curve of 10 μM GDP-p21 (V29G-Y32W) with BeF₃⁻ at 15 °C. The filled circles are the experimental data points; the solid line is the fitting to Equation 2.

Table 1. Results of the global kinetic analysis of binding of BeF_3^- to V29G–Y32W–Ha-ras-p21

	15 °C	25 °C	35 °C	45 °C
K_1 (M^{-1})	2.9 ± 0.5	4.7 ± 1.1	8.7 ± 0.5	25.1 ± 3.2
k_{+2} (s^{-1})	422.7 ± 7.6	482 ± 12	516.8 ± 4.4	520.0 ± 3.8
k_{-2} (s^{-1})	4.699 ± 0.048	9.052 ± 0.064	14.069 ± 0.050	17.753 ± 0.093
K_2	90.0 ± 2.6	53.2 ± 1.7	36.7 ± 0.4	29.3 ± 0.4
K_{app} (M^{-1})	264 ± 54	255 ± 69	328 ± 23	761 ± 108
K_{app} (M^{-1}) ^a	256 ± 23	238 ± 26	292 ± 49	447 ± 49
K_{app} (M^{-1}) ^b	90 ± 7	113 ± 11	140 ± 12	211 ± 15
K_{app} (M^{-1}) ^{a,b}	92 ± 3	123 ± 8	152 ± 10	228 ± 15

^aResults from the equilibrium measurements.^bApparent binding constants for Y32W–Ha-ras-p21 (Díaz et al., 1997b).

Kinetics of binding of BeF_3^- to the GDP-bound form of the double mutants

The kinetics of binding of BeF_3^- to Ha-ras-p21 were measured using a stopped flow device as described in Díaz et al. (1997b). Figure 2 shows the change of fluorescence of GDP-p21(V29G–Y32W) upon the binding of BeF_3^- as a function of time. At the same concentration of BeF_3^- the time scale is 100 times shorter than with the single Y32W mutant. In contrast to the single mutant, the curves can be fitted to a single exponential. The rate constant determined from the fitting depends in a nonlinear way on the concentration of BeF_3^- (Fig. 3). The nonlinearity indicates that the binding phenomenon has to be dissected in two steps, as with the reference protein. The reason that, in this case, the same mechanism does not lead to two observable exponentials must be due to the fact that the first step is too fast to be measured and/or to the absence of a fluorescence change upon initial binding to the double mutant. Figure 4 shows the fluorescence decrease upon the binding of BeF_3^- to the protein GDP-p21(I36G–Y32W). Here the time dependence of the fluorescence decrease has to be described again by a sum of two exponentials.

The amplitude of the second exponential is, however, very small. Moreover, the rate constant obtained from the first exponential shows the same nonlinear concentration dependence as described before (Fig. 5). Therefore, the first exponential process is attrib-

uted to the second step of the overall binding mechanism, and the second exponential, in this case, is attributed to a third step, i.e., an additional isomerization step that is not present in the single and the V29G double mutant. The rate constant k_{+3} and k_{-3} can be obtained from the fitting to the model extended with a third step.

Thermodynamic parameters of the binding of BeF_3^- to the Ha-ras-p21 mutants

Having the kinetic constants determined at different temperatures (Tables 1, 2), it is possible to obtain the thermodynamic parameters of the equilibrium constants and the activation energies of the kinetic constants. Table 3 shows the activation energy and pre-exponential factors of the individual kinetic reactions. Table 4 shows the enthalpy and entropy changes of the reaction steps. An enthalpic and entropic pathway can then be described (Figs. 6, 7).

Discussion

V29 and I36 are hinges of the conformational change of activation

Using the computational technique of TMD (Schlitter et al., 1994), we calculated a molecular reaction pathway between the active and

Table 2. Results of the global kinetic analysis of binding of BeF_3^- to Y32W–I36G–Ha-ras-p21

	15 °C	25 °C	35 °C	45 °C
K_1 (M^{-1})	4.6 ± 0.6	4.0 ± 0.2	9.5 ± 2.2	17.9 ± 0.4
k_{+2} (s^{-1})	133 ± 1	300 ± 3	260 ± 2	269.5 ± 2.1
k_{-2} (s^{-1})	4.943 ± 0.067	11.01 ± 0.13	13.4 ± 0.3	16.54 ± 0.40
K_2	26.9 ± 0.6	27.2 ± 0.6	19.4 ± 0.6	16.3 ± 0.5
k_{+3} (s^{-1})	1.446 ± 0.089	2.98 ± 0.14	5.46 ± 0.75	9.41 ± 0.91
k_{-3} (s^{-1})	2.82 ± 0.14	7.05 ± 0.18	16.7 ± 1.2	15.62 ± 0.90
K_3	0.51 ± 0.06	0.42 ± 0.03	0.33 ± 0.07	0.60 ± 0.10
K_{app} (M^{-1})	191 ± 38	158 ± 15	255 ± 84	485 ± 56
K_{app} (M^{-1}) ^a	173 ± 30	152 ± 18	240 ± 19	331 ± 71

^aResults from the equilibrium measurements.

Fig. 2. Fluorescence decrease observed upon the binding of BeF_3^- to GDP-p21(V29G-Y32W) ($30 \mu\text{M}$) at 15°C and 7.85 mM BeF_3^- . The inset shows the residuals of the fitting with the experimental points with one exponential for V29G.

Fig. 4. Fluorescence decrease observed upon the binding of BeF_3^- to GDP-p21(Y32W-I36G) ($30 \mu\text{M}$) at 15°C and 52.21 mM BeF_3^- . The inset shows the residuals of the fitting with the experimental points: (a) with one exponential and (b) with two exponentials for I36G.

the inactive form of Ha-ras-p21 (Díaz et al., 1997a). In this paper a model is presented for a transition process in several steps, initiated by the binding or removal of the γ -phosphate of the nucleotide in its site, affecting the conformation of the P-loop, which transmits the signal to the other parts of the molecule. The energy profiles of the calculated trajectories show very noisy curves with a lot of transition steps, which are difficult to attribute to specific residues. Therefore, we also looked for dihedral transitions observed during the DT transition (Díaz et al., 1997a). Based on this plot, residue V29 shows a substantial dihedral transition around 100 ps of the DT pathway. Residue I36 also shows one major dihedral transition coincident with the starting of the last peak in

the energy profile of the transition, at ps 150. The only way to validate the proposed pathway was to mutate the residues that may be involved in such a transition and to study the behavior of the mutated molecule. Since V29 and I36 had been proposed to act as hinges of the conformational transition, they were mutated into glycine with the intention of increasing their conformational freedom. The mutation of V29G and of I36G should lower the energy of at least one transition. As predicted, the rate constant of the conformational change k_{+2} of V29G is 128 times bigger than that of the wild-type, while the rate constant in I36G is 80 times faster as well. These results support, therefore, the TMD method as a way to study conformational changes in proteins.

Fig. 3. Dependence of the observed rate constant of binding of BeF_3^- to GDP-p21(V29G-Y32W) ($30 \mu\text{M}$) on the concentration of BeF_3^- at 35°C . The solid line is the theoretical curve following the model of an initial conformational change followed by the binding of the ligand as described in Results.

Fig. 5. Dependence of the observed rate constant of binding of BeF_3^- to GDP-p21(Y32W-I36G) ($30 \mu\text{M}$) on the concentration of BeF_3^- at 35°C . The solid line is the theoretical curve following the model of an initial conformational change followed by the binding of the ligand (see Results).

Table 3. Activation energy and pre-exponential factors of conformational change reactions of Ha-ras-p21 (V29G-Y32W and Y32W-I36G mutants)

	Y32W mutant ^a	V29G-Y32W mutant	Y32W-I36G mutant
Ea ₊₂	52 ± 1 kJ mol ⁻¹	5.2 ± 1.5 kJ mol ⁻¹	15 ± 11 kJ mol ⁻¹
LnA ₊₂	22.3 ± 0.9	8.3 ± 0.6	11.4 ± 4.4
Ea ₋₂	44 ± 4 kJ mol ⁻¹	33.5 ± 5.1 kJ mol ⁻¹	28.9 ± 7.8 kJ mol ⁻¹
LnA ₋₂	18.1 ± 0.7	15.6 ± 2.0	13.8 ± 3.1
Ea ₊₃	—	—	47.5 ± 1.0 kJ mol ⁻¹
LnA ₊₃	—	—	20.2 ± 0.5
Ea ₋₃	—	—	45.3 ± 12.6 kJ mol ⁻¹
LnA ₋₃	—	—	20.2 ± 5.0

^aData from Díaz et al. (1997b).

Comparison of the thermodynamic parameters of individual steps of the transition between the single and the double mutants

When the enthalpy and entropy profiles of the pathway of the different mutants are compared, a striking pattern appears (Figs. 6, 7). For the single mutant, the two steps are accompanied by an enthalpy increase and both steps are clearly entropy driven. Between the initial and the final complex, a big energy barrier is found.

For the double mutants a different situation is observed. The enthalpy and entropy increase of the first step is much bigger than for the Y32W mutant. The second step is accompanied by an entropy decrease and is clearly enthalpy driven. The activation barrier between the initial and the final complex is strongly reduced compared to the Y32W mutant. These results indicate that the mutations also cause some rearrangements in the binding site such that the strong enthalpy increase, which takes place during the activation process in the Y32W mutant, is now associated with the whole binding step itself. The role of the residues I36G and V29G is qualitatively the same, but quantitatively different.

General conclusions

In this paper we have studied, using fluorescence stopped flow techniques, the conformational change induced in Ha-ras-p21 upon

the binding of BeF₃⁻ to the GDP-protein complex. The pathway of the conformational change calculated by the TMD method predicted that the mutations V29G and I36G should show much faster kinetics and that the residues V29 and I36 play the role of a hinge in the overall conformational change. The experimental results obtained confirm this prediction and demonstrate that TMD is a valuable tool to construct reaction pathways of conformational changes in proteins.

Materials and methods

The beryllium sulfate and potassium fluoride used to prepare the stock solution of beryllium fluoride were from Merck (Darmstadt, Germany) in analytical grade; all other chemicals employed were analytical grade or better.

The beryllium fluoride stock solution was prepared in buffer A (64 mM Tris, 50 mM HCl, 1 mM NaN₃, 1 mM dithioerytrol (DTE)), containing 1 mM MgCl₂, 0.75 M KF, and 0.25 M BeSO₄, the pH was later adjusted to 7.2. The effective concentration of beryllium trifluoride was calculated using the effective complexing constants of the beryllium fluoride system at a given final concentration of the Be²⁺ and F⁻ ions (Mesmer & Baes, 1969), the beryllium fluoride concentration of the stock turned out to be 175.7 mM.

The two double mutants (V29G-Y32W and Y32W-I36G) were constructed by long-range PCR (Cheng et al., 1994) of the ptac-ras plasmid (Tucker et al., 1986) containing the Y32W mutation. *Escherichia coli* cells of the WK6 line (Δ(lac-proAB) galE strA nal^r [F' lacI^q lacZΔM15 pro AB⁺]) (Zell & Fritz, 1987) were transformed with the obtained plasmids. The mutant proteins were purified as described previously (Díaz et al., 1997b), their purity was checked by SDS-PAGE (Laemmli, 1970), and their activity was tested by measuring their ability to bind GDP.

The binding constants of beryllium trifluoride to the mutant proteins were measured in buffer A, 1 mM MgCl₂, 1 μM free GDP, pH 7.2, using the change in the fluorescent properties of the tryptophan in the effector loop upon binding of the complex (Díaz et al., 1997b). The fluorometer used was a Fluorolog 1691 (Spex Industries, Edison, New York). The excitation wavelength was 280 nm; the emission wavelength was 343 nm, both with slit apertures of 2 mm. The corrected fluorescence intensity was fitted to the following equation:

Table 4. Thermodynamic parameters of the individual steps of BeF₃⁻ binding to Ha-ras-p21 (V29G-Y32W and Y32W-I36G mutants)

	Y32W mutant ^a	V29G-Y32W mutant	Y32W-I36G mutant
ΔH ₁ ⁰	15 ± 2 kJ mol ⁻¹	53 ± 18 kJ mol ⁻¹	37.4 ± 12.0 kJ mol ⁻¹
ΔH ₂ ⁰	8 ± 3 kJ mol ⁻¹	-28.3 ± 3.3 kJ mol ⁻¹	-13.9 ± 9.4 kJ mol ⁻¹
ΔH ₃ ⁰	—	—	2.2 ± 6.8 kJ mol ⁻¹
ΔH _{ov} ⁰	23 ± 4 kJ mol ⁻¹	24.7 ± 10.7 kJ mol ⁻¹	25.7 ± 9.4 kJ mol ⁻¹
ΔH _{app} ⁰	22 ± 2 kJ mol ⁻¹	16 ± 7 kJ mol ⁻¹	18 ± 7 kJ mol ⁻¹
ΔS ₁ ⁰	78 ± 15 J mol ⁻¹ K ⁻¹	192 ± 61 J mol ⁻¹ K ⁻¹	141 ± 39 J mol ⁻¹ K ⁻¹
ΔS ₂ ⁰	38 ± 17 J mol ⁻¹ K ⁻¹	-62 ± 11 J mol ⁻¹ K ⁻¹	-20 ± 31 J mol ⁻¹ K ⁻¹
ΔS ₃ ⁰	—	—	0.7 ± 24 J mol ⁻¹ K ⁻¹
ΔS _{ov} ⁰	111 ± 19 J mol ⁻¹ K ⁻¹	131 ± 37 J mol ⁻¹ K ⁻¹	131 ± 33 J mol ⁻¹ K ⁻¹

^aData from Díaz et al. (1997b).^bData from equilibrium measurements.

Fig. 6. Standard enthalpy change during the pathway of binding of BeF_3^- . The reference state is the nonassociated mixture of protein and ion. State 1 corresponds to the initial complex, State 2 and 3 are the final complex states, and TS represents the transition state of the conformational change.

$$F_x = \frac{F_0 + F_1 \cdot [\text{BeF}_3^-] \cdot K_{app}}{1 + [\text{BeF}_3^-] \cdot K_{app}} \quad (2)$$

where F_x is the measured fluorescence intensity at a given concentration of beryllium trifluoride, F_0 the fluorescence intensity of the GDP-bound form of the protein, F_1 the fluorescence intensity of the $(\text{BeF}_3^- \text{-GDP})$ -bound form, and K_{app} the apparent binding constant.

The kinetics of binding of BeF_3^- to both mutants of Ha-ras-p21 were measured in buffer A, 1 mM MgCl_2 , 1 μM GDP, pH 7.2 containing different concentrations of BeF_3^- as described previously (Díaz et al., 1997b). The kinetic constants at a given temperature were obtained by fitting the whole set of curves at different concentrations of BeF_3^- with the programs KINSIM and FITSIM

Fig. 7. Standard entropy change during the pathway of binding of BeF_3^- . The reference state is the nonassociated mixture of protein and ion. State 1 corresponds to the initial complex, State 2 and 3 are the final complex states.

(Barshop et al., 1983), in which the reaction model is programmed. The calculations were performed in a Silicon Graphics Indy workstation (Silicon Graphics Computer Systems, Mountain View, California). This kind of global analysis also takes into account the amplitudes of the fast and slow phases, while the graphical analysis of the dependence of the kinetic constants on the concentration of the ligand only uses the information coming from the magnitude of the observed rate constants.

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