

# Direct Discrimination between Models of Protein Activation by Single-Molecule Force Measurements

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**ABSTRACT** The limitations imposed on the analyses of complex chemical and biological systems by ensemble averaging can be overcome by single-molecule experiments. Here, we used a single-molecule technique to discriminate between two generally accepted mechanisms of a key biological process—the activation of proteins by molecular effectors. The two mechanisms, namely induced-fit and population-shift, are normally difficult to discriminate by ensemble approaches. As a model, we focused on the interaction between the nuclear transport effector, RanBP1, and two related complexes consisting of the nuclear import receptor, importin  $\beta$ , and the GDP- or GppNHP-bound forms of the small GTPase, Ran. We found that recognition by the effector proceeds through either an induced-fit or a population-shift mechanism, depending on the substrate, and that the two mechanisms can be differentiated by the data.

## INTRODUCTION

Experimental analysis of complex systems by conventional methods is often limited by ensemble averaging. This is particularly problematic for biological systems, where the size and complexity of the reactants render them highly inhomogeneous. This inhomogeneity, which persists to the molecular level and often carries functional importance, cannot usually be revealed by ensemble approaches. Single-molecule measurements provide a natural solution to this problem. Because the molecules are probed one at a time, the distribution of the molecular properties can be determined directly rather than inferred, and information hidden in ensemble-averaged results is unveiled.

A central process in biology is the activation of proteins by other proteins, protein domains, or small ligands. Signaling pathways, enzyme activity, and the activation and inactivation of genes all depend on the switching of proteins between alternative functional states. The first model for activation was introduced by Koshland (1958) and was later extended by Koshland, Nemethy, and Filmer to a sequential model of allostery (Koshland et al., 1966). In this model, called induced-fit, the binding of the effector induces a conformational change in the target protein. The resulting change in conformation alters the properties of the protein, and consequently, leads to a change in its activity. The population-shift model, on the other hand, ascribes changes in protein activity to a redistribution of pre-existing conformational isomers. According to this model (known also as the pre-equilibrium or conformational selection model), protein structure is regarded as an ensemble of conformations existing in equilibrium. The ligand binds to

one of these conformations, i.e., the one to which it is most complementary, thus shifting the equilibrium in favor of this conformation. This mode of activation is embodied in the MWC (Monod-Wyman-Changeux) model of allostery (Monod et al., 1965). For recent reviews on the activation of proteins by molecular effectors, see James and Tawfik (2003) and Kumar et al. (2000).

Presently, there is very little experimental evidence that distinguishes between the induced-fit and population-shift models of activation (for notable exceptions, see James et al., 2003; and Volkman et al., 2001). This is because the presence of multiple conformations is difficult to ascertain by conventional methods, even if all conformations are represented in the ensemble in a significant amount. If the populations are skewed, an accurate probing of the ensemble becomes even more problematic. Consequently, there is a natural tendency to interpret the results as an indication for an induced-fit, explaining the broad popularity of this model.

In this work, we studied the activation of two related protein complexes at the single-molecule level, using dynamic force spectroscopy. We show that the data obtained from the measurements allow discrimination between the two modes of activation by comparing the distributions of forces required to unbind the complexes in the presence and in the absence of the effector.

## MATERIALS AND METHODS

### Proteins

His-tagged human importin  $\beta$  was expressed and purified as described in Nevo et al. (2003). His-tagged Ran (human) was purchased from Cytoskeleton (Denver, CO) or was obtained from the soluble phase of *Escherichia coli* BL21 (DE3; trxB) induced with 0.25 mM IPTG for 8 h, at 30°C. Human RanBP1 was purchased from Cytoskeleton. All proteins were ~95% pure, as determined by silver staining. Loading of Ran with GDP or GppNHP was made following Nevo et al. (2003). Proteins were analyzed for

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structural integrity by circular dichroism, and for binding, by ELISA, native gel electrophoresis, and sizing chromatography.

## Tip and surface immobilization

Immobilization of Ran and imp $\beta$  over the AFM tip and the surface (mica) was made via short polyethylene glycol (PEG) linkers, as described previously in Nevo et al. (2003).

## Dynamic force spectroscopy

Measurements were carried out at room temperature in 50 mM Tris (pH 7.5)/150 mM NaCl, using a PicoSPM AFM (Molecular Imaging, Phoenix, AZ). Spring constants of the cantilevers (TM Microscopes, CA) were 0.02–0.03 N/m, as determined by the thermal noise method (Hutter and Bechhoefer, 1993). Force-distance cycles were performed at amplitudes of 100 nm and at frequencies ranging from 0.2 to 20 Hz; loading rates indicated in the text were corrected for linkage compliance. The number of rupture events acquired at each loading rate was between 50 and 700. Specificity of binding was verified by blocking experiments using free importin  $\beta$  or Ran or by adding a nonspecific protein (lysozyme; in experiments involving RanBP1). Data were processed as described in Baumgartner et al. (2000), using Matlab v6.1. Data shown in the text are presented as Gaussian fits of histograms. Fitting was carried out using nonlinear least squares analysis made over all band parameters;  $r^2$  values were typically >0.95. Analysis of the data according to their calculated probability density functions gave similar results.

## RESULTS AND DISCUSSION

RanBP1 is a small cytosolic protein that contains a conserved Ran-binding domain (RanBD). It functions in the disassembly of RanGTP-containing transport complexes that exit from the nucleus through the nuclear pores by priming these complexes for GTP hydrolysis, stimulated by the GTPase-activating protein RanGAP1 (Richards et al., 1995; Bischoff and Gorlich, 1997; Floer et al., 1997; Kutay et al., 1997, 1998; Lounsbury and Macara, 1997; Gorlich and Kutay, 1999). One such complex is that formed between RanGTP and the nuclear import receptor importin  $\beta$  (imp $\beta$ ). Unlike other transport complexes, efficient dissociation of this complex requires, in addition to RanBP1, the presence of importin  $\alpha$ —a cargo adaptor of imp $\beta$  (Bischoff and Gorlich, 1997; Floer et al., 1997). In the absence of importin  $\alpha$ , the addition of RanBP1 was actually found to lead to a small ( $\sim 3$ -fold) increase in the association of RanGTP with imp $\beta$  (Villa Braslavsky et al., 2000). A similar positive, but far more pronounced, effect is observed when Ran is loaded with GDP. In contrast to RanGTP, which binds avidly to imp $\beta$ , RanGDP has an extremely low affinity to the transport receptor. However, in the presence of RanBP1, stable trimeric RanBP1-RanGDP-imp $\beta$  complexes are readily formed (Chi et al., 1996; Villa Braslavsky et al., 2000).

The aforementioned interactions are summarized in Fig. 1, which shows the results obtained from binding assays of the three proteins, using native gel electrophoresis. Loaded with a nonhydrolysable GTP analog (GppNHp), Ran effectively binds to imp $\beta$  as well as to RanBP1. When mixed together,

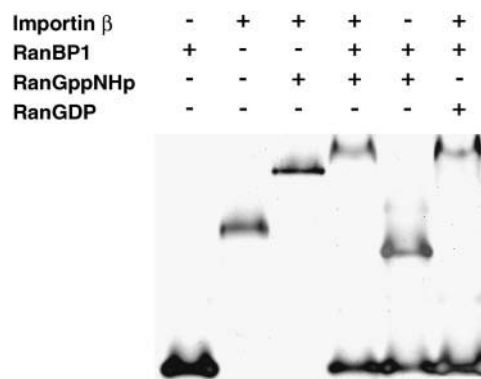


FIGURE 1 Binding of imp $\beta$  to GDP- and GppNHp-loaded Ran in the presence and absence of RanBP1. Proteins were incubated for 30 min at room temperature in a reaction buffer containing 50 mM Tris (pH 7.5), 200 mM NaCl, and 1 mM DTT. After incubation, the mixtures were loaded onto a 10% native polyacrylamide gel. Proteins were present in equimolar amounts, except RanBP1, which was added in excess.

the three proteins associate to form a ternary complex in which Ran is simultaneously bound to imp $\beta$  and RanBP1. Such a trimeric complex is also formed when imp $\beta$  is incubated with Ran loaded with GDP in the presence of RanBP1.

We first address the action of RanBP1 on the complex formed between imp $\beta$  and RanGppNHp. Using dynamic force spectroscopy (DFS), we previously showed that this complex alternates between two conformations of different adhesion strengths (Nevo et al., 2003). In this methodology (Fig. 2), which we also applied here, the binding partners are immobilized onto a cantilevered tip, used in the atomic force microscope (AFM; Binnig et al., 1986), and to a hard surface, typically mica. The proteins are immobilized on the

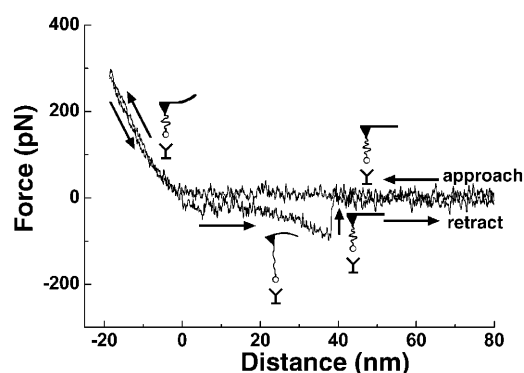


FIGURE 2 Mechanical unbinding of single imp $\beta$ -Ran pairs. Ran and imp $\beta$  were immobilized onto AFM cantilevered tip and mica, respectively, through short polymer (PEG) linkers as described in Nevo et al. (2003). The tip was then repeatedly brought to and retracted from the surface, and the interaction force was measured by following the cantilever deflection, using the AFM setup. The trace shows a representative force-distance cycle recorded (at 5 Hz) for RanGDP-imp $\beta$  in the presence of RanBP1. Unbinding is indicated by the sharp spike in the retraction curve; the parabolic delay antecedent the spike reflects the extension of the polymer linkers.

surfaces through short polymer linkers to allow for unconstrained recognition and to minimize rebinding of the molecules following rupture (Hinterdorfer et al., 1996). To achieve single-molecule recognition, both the ligand and the receptor are immobilized on the surfaces at low densities, e.g., between 200 and 500 molecules per square micron. For a typical AFM tip radius of 20–50 nm, these densities correspond to  $\sim 1$  molecule per effective tip area. Measurements are conducted by force-distance cycles, where the cantilever is repeatedly brought to, and retracted from, the surface. The interaction force is measured by following the cantilever deflection. This setup allows measurements of adhesion forces down to the pico-Newton (pN) range and detection of states with a fractional occupancy as low as a few percent.

Owing to its two bound states, RanGppNHp-imp $\beta$  gives rise to unique, bimodal distributions of unbinding forces (Nevo et al., 2003). The dashed lines in Fig. 3 A show one such distribution. The two states of the complex are represented by two partially overlapping force populations, of which the higher-strength population is  $\sim 2^{1/2}$  times larger than the lower-strength population. This situation is reversed when RanBP1 is added to the solution after which the higher-strength population is diminished and the lower-strength population predominates the ensemble (Fig. 3 A,

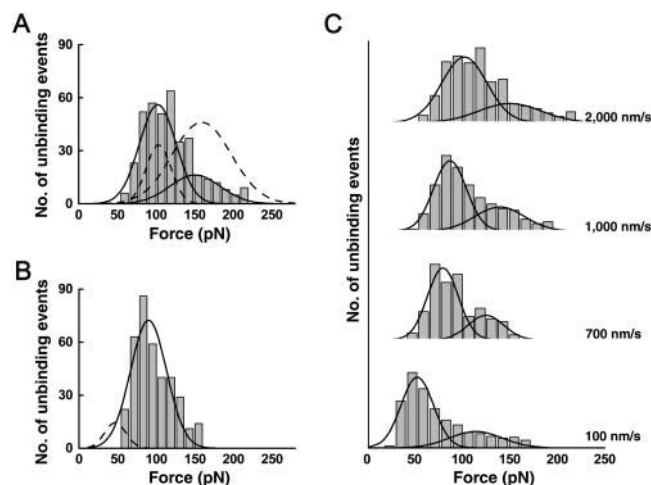


FIGURE 3 Unbinding force distributions of RanGppNHp-imp $\beta$  and RanGDP-imp $\beta$  complexes in the absence (*dashed lines*) and presence (*solid line*) of RanBP1. (A and B) Distributions of unbinding forces recorded at 20 Hz. Data are presented as Gaussian fits of histograms; the width of the bins represents the thermal noise of the cantilever. For clarity, histograms are shown only for Ran-imp $\beta$  pairs unbound in the presence of RanBP1. In this and the following figure, data shown for pairs dissociated in the absence of RanBP1 (*dashed lines*) were taken from Nevo et al. (2003). The marked increase in the number of unbinding events recorded for RanGDP-imp $\beta$  in the presence of RanBP1 reflects the ability of the latter to facilitate association of RanGDP with imp $\beta$  (Villa Braslavsky et al., 2000, and see text). (C) Unbinding force distributions recorded for RanGppNHp-imp $\beta$  in the presence of RanBP1 at different loading rates. Albeit significantly skewed toward the low-strength conformation, the ensemble still reveals two populations (*t*-test *p*-value,  $3 \times 10^{-5}$ ).

*solid lines*). Note that although the relative size of the two force populations changes upon addition of the effector, their means do not. As a control, we replaced RanBP1 with lysozyme. The results were similar to those obtained for the complex in the absence of the RanBP1.

Next, we investigated the effect of RanBP1 on the RanGppNHp-imp $\beta$  interaction over a broad range of loading rates. This is a common practice in force spectroscopy measurements since the measured forces are not only contingent on the molecules themselves, but also depend on the loading rate  $r_f (= \Delta f / \Delta t)$  at which force is applied to the complex (Evans and Ritchie, 1997; Heymann and Grubmüller, 2000; Evans, 2001; Dudko et al., 2003). Specifically, the most probable force for unbinding  $f^*$  (taken as the maximum of the force distributions) is related to the loading rate through  $f^* \approx f_\beta \ln(r_f t_{\text{off}} / f_\beta)$ , where the force scale  $f_\beta$  is given by the ratio of thermal energy  $k_B T$  to a small ( $< 1$  nm) molecular length  $x_\beta$ , which marks the thermally averaged projection of the transition state along the direction of the force. Alteration of the loading rate was achieved by varying the speed of retraction, giving rise to loading rates spanning almost three orders of magnitude in scale. The results are shown in terms of the most probable force for unbinding, plotted against the logarithm of the loading rate.

In the absence of RanBP1, the strength spectrum of RanGppNHp-imp $\beta$  is characterized by two well-separated  $f^*$  versus  $\log(r_f)$  curves corresponding to energy barriers encoded in the lower-strength (*bottom*) and higher-strength (*top*) conformations of the complex (Fig. 4 A, *dashed lines*; Nevo et al., 2003). The addition of RanBP1 greatly diminished contributions from the high-strength conformation throughout the whole range of loading rates, leaving a small number of unbinding events recorded for this conformation (Fig. 3 C). The other, now largely predominating population, gave rise to a strength spectrum that was practically identical to the one produced by the low-strength conformation of the complex in the *absence* of the effector (Fig. 4 A, *solid line*). These results clearly support a model in which activation of RanGppNHp-imp $\beta$  is achieved by a dynamic shift between its two conformations. They also indicate that, in addition to its previously reported ability to promote association between RanGppNHp and imp $\beta$  (Villa Braslavsky et al., 2000), RanBP1 also facilitates their dissociation by shifting the equilibrium toward the low-strength conformation of the complex.

We now turn to the effect of RanBP1 on the interaction between imp $\beta$  and RanGDP. This form of Ran has a very low affinity to imp $\beta$ . Nevertheless, binding can still be detected by force spectroscopy measurements, which show that RanGDP associates weakly with imp $\beta$  to form a single bound state characterized by unimodal distributions of rupture forces (Figs. 3 B and 4 B, *dashed lines*; Nevo et al., 2003). In contrast to its effect on RanGppNHp-imp $\beta$ , RanBP1 led to a marked shift of the distributions obtained for RanGDP-imp $\beta$  to higher unbinding forces throughout the

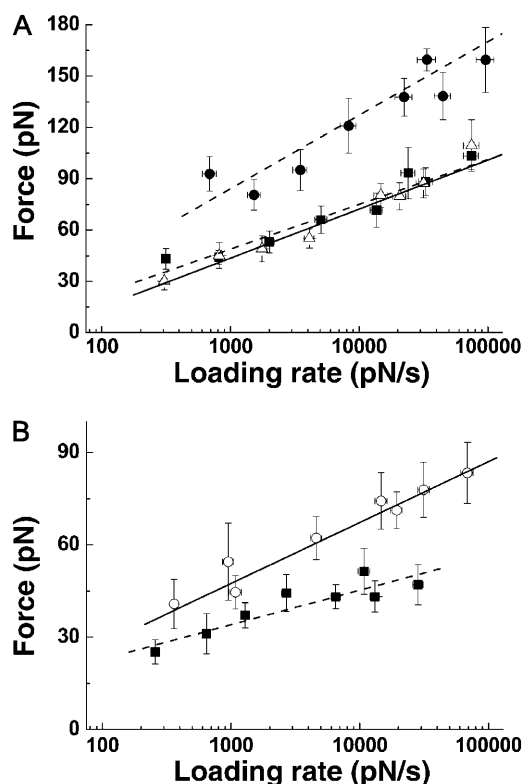


FIGURE 4 Force spectra of Ran-imp $\beta$  complexes in the presence and absence of RanBP1. Most probable unbinding forces were plotted against the logarithm of the loading rate. *A* and *B* show the force spectra obtained for RanGppNHp-imp $\beta$  and RanGDP-imp $\beta$  in the absence (*dashed lines*) and presence (*solid lines*) of RanBP1. Error bars represent standard deviation.

whole range of loading rates (Figs. 3 *B* and 4 *B*, *solid lines*). Such a behavior is expected for an induced-fit mechanism, where the binding of the effector induces the formation of a new structure in the complex, which results in higher stability.

The results obtained in this work demonstrate that single-molecule measurements can effectively discriminate between induced-fit and population-shift mechanisms of activation. The major limitation appears to be the inability to detect states with very low fractional occupancy (for the technique used here—lower than a few percent). In addition, the two binding mechanisms may coexist or occur consecutively (i.e., population-shift followed by local structural rearrangements). In such cases, only the predominant mechanism is likely to be exposed. These limitations, however, also exist in bulk approaches, which are further impaired by ensemble-averaging effects.

Within the limitations discussed above, our data indicate that recognition of RanGDP-imp $\beta$  by RanBP1 proceeds by an induced-fit process. The exact nature of the structural rearrangements triggered in RanGDP by RanBP1 is presently unknown, since the crystal structure of the complex is not available (for the structure of RanGppNHp-imp $\beta$ , see Vetter et al., 1999a). One region in RanGDP that probably

undergoes a large conformational change upon RanBP1 binding is the C-terminal region of Ran that serves as a major recognition site for RanBP1, but is thought to dock to a basic patch in Ran (Vetter et al., 1999b). [In RanGppNHp, the C terminus is probably displaced, since it is more exposed and appears to be bound less tightly to the rest of the protein (Vetter et al., 1999a,b, and references therein)]. The displacement of Ran's C-terminus by RanBP1 is believed to greatly facilitate association of RanGDP with imp $\beta$  (Villa Braslavsky et al., 2000). This effect is indeed reflected by the significant increase in the number of rupture events recorded for the pair in the presence of the effector (Fig. 3 *B*). However, structural changes in regions outside the C-terminal region of Ran are probably necessary to account for the pronounced effect of RanBP1 on the dissociation of RanGDP from imp $\beta$ .

On the other hand, the results obtained for the complex between imp $\beta$  and RanGppNHp fit very well to a model based on a dynamic shift between pre-existing alternative conformations. According to this model, RanBP1 binds to the lower-strength conformation of RanGppNHp-imp $\beta$ , thus shifting the equilibrium in favor of this conformation. A similar two-state allosteric behavior was demonstrated for the bacterial response regulator NtrC (Volkman et al., 2001) and calmodulin (Malmendal et al., 1999). Recently, a population selection model has been suggested for the recognition of the imp $\beta$ -binding (IBB) domain of importin  $\alpha$  by imp $\beta$  (Koerner et al., 2003).

How RanBP1 actually distinguishes between the two conformations of the RanGppNHp-imp $\beta$  complex is not clear at present. P-NMR studies have shown that RanGTP alternates between two conformations, of which only one could be detected in the presence of RanBP1 (Geyer et al., 1999). Although this effect was not obtained when Ran was loaded by GppNHp, it was observed for GppNHp-loaded Ras (Geyer et al., 1996), which is closely related to Ran. A potential target for discrimination is the basic patch of Ran, which interacts with an acidic loop connecting two centrally located helices of imp $\beta$ . The interface formed between Ran's basic patch and the acidic loop of imp $\beta$  may be accessible to an N-terminal extension present in RanBP1 or in the Ran-binding domains of RanBP2 (Vetter et al., 1999a). It has been suggested that the N-terminus of the Ran-binding domains, which is acidic, competes with the acidic loop of imp $\beta$  for the basic patch of Ran (Vetter et al., 1999a; Villa Braslavsky et al., 2000). If this prediction is correct, then the two conformations of RanGppNHp-imp $\beta$  may differ in their accessibility to the N-terminus of RanBP1, with the lower-strength conformation providing better access to the basic patch.

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