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PAPER

Free energy evaluation of the p53-Mdm2 complex from unbinding work measured by dynamic force spectroscopy

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The complex between the tumor suppressor p53 and its down-regulator Mdm2 has been studied by dynamic force spectroscopy and the unbinding data have been analyzed in the framework of the Jarzynski theoretical approach. Accordingly, the unbinding equilibrium free energy has been determined from the work done along several non-equilibrium paths from the bound to the unbound state in the single molecule regime. An unbinding free energy of -8.4 kcal mol^{-1} has been found for the complex; such a value is in a good agreement with that measured both in the bulk by isothermal titration calorimetry and that obtained from theoretical computing at the single molecule level. The determination of the unbinding free energy, together with the knowledge of the dissociation rate constant and energy barrier width, as previously obtained by dynamic force spectroscopy, adds rewarding insights on the energy landscape for this complex which is currently at the focus of anticancer drug design.

Introduction

The transcription factor p53 is one of the major tumor suppressors in mammals, acting through the regulation of the expression of target genes involved in different stress responses.[1] The tumor suppressor activity of p53 is down-regulated by the human version of the mouse double minute protein 2 (Mdm2) through several mechanisms.[2] Upon the formation of a complex with p53, Mdm2 may either inhibit the transcriptional activity of p53 or may favour its exportation to cytoplasm, promoting its ubiquitin-dependent degradation.[3–5] Therefore, the Mdm2-p53 complex is a preferential target for anticancer drug design to restore normal p53 function in tumor cells by preventing the Mdm2-mediated inactivation of p53.[6] Despite the relevance of this complex, most of the structural information available on the corresponding complex comes from studies on partial domains of both p53 and Mdm2.[7–9] The first study on this complex involving full-length proteins has been done by dynamic force spectroscopy (DFS) on a single couple of interacting partners.[10]

To reach a full comprehension of the molecular interactions at the basis of the functional activity of this system, a detailed knowledge of the energy landscape of the p53-Mdm2 complex, formed by full length proteins, represents a crucial step. Indeed, the energy barrier separating the bound and the unbound state of the complex determines the kinetic and the thermodynamical properties regulating the formation of the complex.[11] The association (k_{on}) and dissociation (k_{off}) rate constants depend on the energy barrier being overcome to form or to dissociate a complex, respectively; whereas the affinity ($K_a = k_{on}/k_{off}$) of a complex is related to the energy barrier between the bound and the unbound state.[12]

Different techniques can be used to evaluate these parameters from samples in the bulk.[13–15] However, the capability to perform measurements at the level of a single couple of interacting partners discloses important effects usually hidden in the ensemble average, such as transient phenomena, crowding effects, population heterogeneity, *etc.*[12,16,17] Moreover, the single molecule approach also offers the possibility to investigate biomolecular systems even when very small quantities of reactants are available. Finally, the knowledge of the free energy for a single couple of interacting biomolecules provides a benchmark for computational experiments whose atomistic resolution is of utmost utility to design drugs, inhibitors, antibodies, *etc.*[18]

Dynamic force spectroscopy is one of the most valuable single molecule techniques for probing inter and intramolecular forces in biomolecular complexes with pico-Newton sensitivity, without labeling and in near-physiological conditions[19,20] In a DFS experiment, an atomic force microscope (AFM) tip is functionalized with one of the biomolecular partners and moved towards a surface on which the other partner has been immobilized, to allow the formation of a complex. Upon retracting the tip from the substrate, a dissociation process between the two partners is induced and the corresponding unbinding force can be measured.

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Since these experiments are performed in non-equilibrium conditions and under the application of an external force at finite velocity, the energy landscape of the complex is modified, and suitable theoretical modelling is generally required to extract information relevant to equilibrium.[21–24] The dissociation rate constant, k_{off}, and the width of the energy barrier, x_β, is usually derived in the framework of the Bell–Evans model which foresees a linear dependence of the unbinding force as a function of the logarithm of the rate, R, at which the pulling force is exerted (loading rate).

Recently, Jarzynski has developed a theoretical procedure which permits the calculation of the equilibrium free energy, ΔG, of a reaction from the mechanical work done along several non-equilibrium (irreversible) unbinding paths. The so-called Jarzynski Identity (JI) given by:[26,27]

$$e^{-\frac{\Delta G}{k_B T}} = \int \rho(W_\lambda) e^{-\frac{W_\lambda}{k_B T}} \mathrm{d}W_\lambda, \qquad (1)$$

connects the equilibrium free energy ΔG of a reaction to the mechanical work $W_\lambda = \int F \mathrm{d}\lambda$, done by the applied force F along a non-equilibrium path, λ, between the initial and final state; $\rho(W_\lambda)$ being the work distribution.

Although the Jarzynski approach has remarkable potential for the study of irreversible chemical processes, few applications have been registered up to now, especially for biological systems.[28–30] This could be due to some difficulties inherent to its practical implementation, such as the requirement of a large statistical sampling with low experimental errors, or to the necessity to use *ad hoc* experimental architectures.[28]

We present a JI based-analysis of the DFS unbinding data from a complex involving full-length p53 and Mdm2 proteins to extract the unbinding free energy at equilibrium. The value of ΔG, calculated by eqn (1), has been found to be in a good agreement with that obtained from isothermal titration calorimetry measurements for partial domains of both the proteins in the bulk and with the value estimated by computational approaches.[9,31] These results constitute a novel additional insight into the energy landscape of the p53-Mdm2 complex which is of remarkable relevance in the design of new anticancer drugs.

Experimental

The immobilization architecture used to study the p53-Mdm2 interaction is fully described in ref. 10. Briefly, p53 was anchored to a gold substrate (250 nm gold thickness on borosilicate glass (Arrandee, Germany)) previously functionalized with cysteamine–glutaraldehyde linkers able to target the exposed NH_2-groups of the protein. 50 μl of a solution of 1.2 μM p53-GST fusion solution was dropped on the amine-reactive surface of the substrates and incubated overnight at 4 °C. Mdm2 was bound to the silicon nitride tip, previously silanized, by means of a 30 nm-long polyethyleneglycol (PEG) linker (MW 3400) on one end to covalently link, through a maleimide group, the thiol-reactive end of the silane, and on the other to target the exposed NH_2-groups of Mdm2. Functionalized tips were incubated with 50 μl of a 3.2 μM solution of Mdm2 for 4 h at 4 °C, then rinsed with PBS buffer.

Force measurements were performed with a Nanoscope IIIa/Multimode Atomic Force Microscope (AFM; Veeco, Santa Barbara, CA). The used cantilevers, to which the tip is attached, had a nominal spring constant k_{nom} of 0.02 and 0.03 N m^{-1}. Their effective spring constants, determined by the non-destructive thermal noise method,[32] were in the range of 0.017–0.045 N m^{-1}. In all DFS measurements, a relative trigger of 23–35 nm was applied to limit at 0.7 nN the maximum contact force exerted on the protein monolayer by the tip, a ramp size of 150 nm was set and an encounter time (interval between approach and retraction phase) of 100 ms was established. The approach velocity was set, through the software actuating the piezo scan of the AFM apparatus operating in open loop configuration, at a value of 69.8 nm s^{-1}, while the retraction velocity was changed from 50 to 8400 nm s^{-1}. The resulting loading rates were in the range 0.6–72 nN s^{-1}. For each loading rate, a collection of hundreds of force curves were acquired in sequence at the same and at different sites. These curves were scrutinised to discriminate specific and individual events from the non-specific and multiple ones.

Results and discussion

As already mentioned, the application of the JI to determine the unbinding free energy of a complex requires the evaluation of the work done, by the applied force, to induce the unbinding process along iterated non-equilibrium paths related to specific and individual unbinding events. Accordingly, a collection of several force curves are to be acquired and analyzed. With such an aim, we have performed a DFS unbinding experiment on the p53-Mdm2 complex by a tip functionalized with Mdm2 cyclically approached and retracted from a substrate on which p53 has been immobilized (see Fig. 1).

To covalently connect Mdm2 to the AFM tip, we have used a PEG linker which endows the biomolecule with sufficient flexibility to favour the biorecognition of the partner. Moreover, during the tip retraction and before the complex unbinding, PEG undergoes a stretching whose peculiar features can be of valuable help to single out specific and single events (see below).[19,33–36]

A force curve representation of a specific unbinding event is shown in Fig. 2 in which the cantilever deflection, Δz, is plotted *vs.* the piezo displacement, λ, by approaching and retracting the tip towards to the substrate and backwards

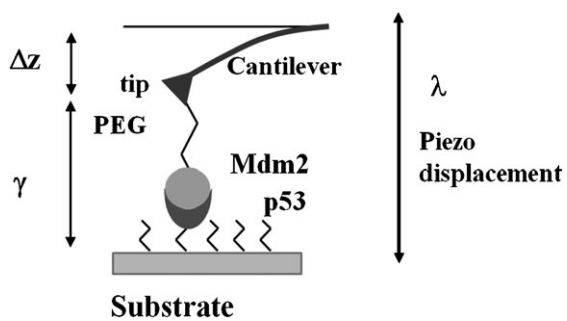

Fig. 1 Sketch of the experimental setup used to investigate the unbinding process of the p53-Mdm2 complex; Δz is the cantilever deflection, γ is the molecular extension and λ is the piezo displacement.

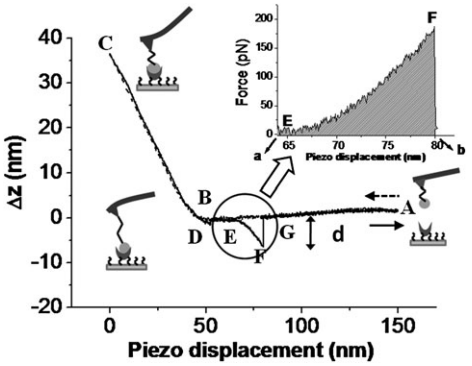

Fig. 2 A representative experimental force curve (approach and retraction) for a specific unbinding event of the p53-Mdm2 complex. Inset: zoom of the region of the retraction curve used to calculate the work by eqn (3); the force values have been obtained from the cantilever deflection Δz through $F = -k\Delta z$, where k is the effective cantilever spring constant. The dashed region represents the area underlying the force curve providing the work value.

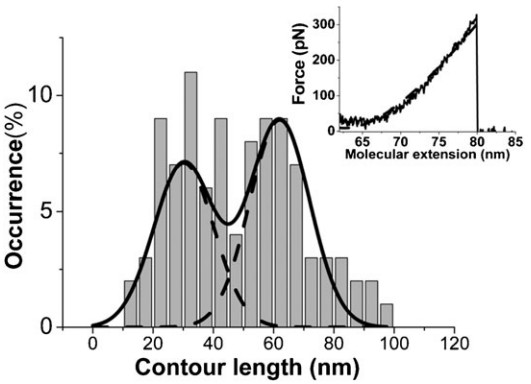

Fig. 3 Histogram of the contour length L, extracted from a fit by eqn (2) of the non-linear portion of the retraction curve by the WLC model. The data arises from a collection of a hundred force curves recorded at an effective loading rate of 4.5 nN s^{-1}. Inset: the dashed line represents a fit of a force curve as a function of the molecular extension with eqn (2).

from it. From the contact point (B) between the tip and the substrate at zero deflection, the two partners may undergo a biorecognition process, then form a specific complex; further approach results in an upwards cantilever deflection due to molecular repulsion. Upon reaching a preset maximum value of contact force (point C), commonly kept below 1 nN to avoid damage of the sample, the approach is stopped and the tip is retracted (path CD). During the retraction, and after the contact point B, adhesion forces, and/or bonds formed in the contact phase, cause the tip to adhere to the sample up to a distance beyond the initial contact (path DE) with a downwards cantilever deflection. As the retraction process continues, a nonlinear course of the deflection curve is observed (EF path); such a course being related to the stretching of the linker.[37] When the applied force overcomes the interaction forces, the tip pulls off sharply (jump-off), going to a non-contact position (FG path). The measure of the cantilever deflection, d, in correspondence with such a jump provides an estimation of the unbinding force between the biomolecular partners, through the expression $F_{unb} = -kd$, where k is the cantilever spring constant.

As due to the stochastic character of the unbinding process in the single molecule regime, the force curves collected in sequence, even at the same tip-substrate location, can significantly differ among them and from that in Fig. 2. Indeed, a variety of shapes related to nonspecific, multiple events, or even to no event at all can be registered (for some examples see ref. 20). Accordingly, a careful analysis of these curves is necessary to single out those corresponding to specific and single events. We have analyzed the trend of the non-linear portion of the retraction force curve after the baseline deflection and before the jump-off (EF path in Fig. 2). This is expected to reflect the stretching of the PEG according to well-defined features.[38,39] In particular, we have accepted curves whose non-linear course could be fitted by the worm-like chain (WLC) model:[40]

$$F(\gamma) = \frac{k_B T}{l_p}\left[\frac{1}{4}\left(1-\frac{\gamma}{L}\right)^{-2} + \frac{\gamma}{L} - \frac{1}{4}\right] \qquad (2)$$

where F is the applied force, γ is the molecular extension, l_p is the persistence length and L is contour length of the polymer, *i.e.* the maximum distance between the ends of the linear polymer chain;[36] the molecular extension can be obtained from $\gamma = \lambda - \Delta z$, where $\Delta z = F/k$ is the deflection of the cantilever and λ is the piezo displacement, (see Fig. 1). From a fit of the force curves, we have extracted both the persistence length and the molecular extension; an example of the fit being shown in the inset of Fig. 3 (for more details see the corresponding legend). The persistence length value, l_p, of 0.37 ± 0.04 nm has been found to be in a good correspondence with that expected for our PEG polymer.[41]

Fig. 3 shows the histogram of the contour length obtained from the analysis of a collection of a hundred force curves at the effective loading rate of 4.5 nN s^{-1}. The histogram can be satisfactorily described by two Gaussian distributions, one centered at 30.2 nm and the other one at 61.5 nm. Since the used PEG linker is expected to have a contour length of (30 ± 5) nm under stretching,[41] our results, are consistent with the occurrence of single and double unbinding events

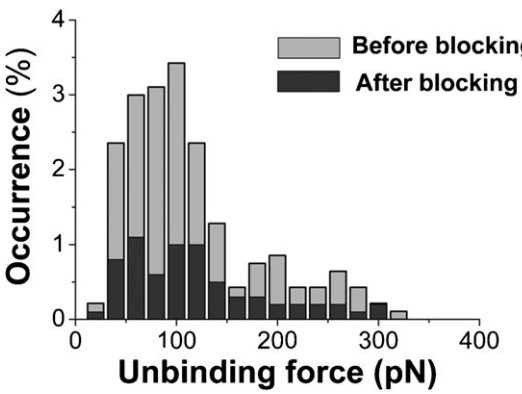

Fig. 4 Histogram of the unbinding force as determined from the jump-off events of force curves attributed to specific events, before and after blocking. The data have been extracted from a collection of a hundred force curves recorded at the effective loading rate of 4.5 nN s^{-1}.

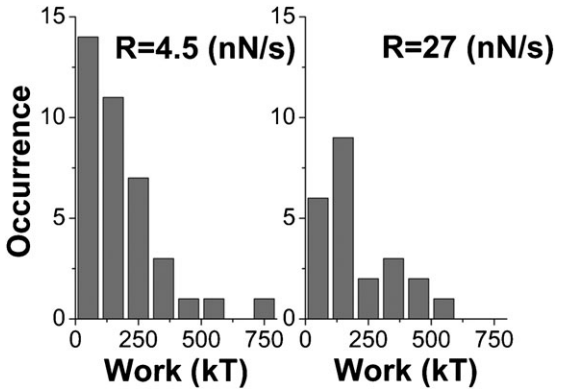

Fig. 5 Histograms of the work done by the applied force at the two different loading rates. The work has been calculated by a numerical integration of the force curves through eqn (3), over the piezo displacement, by the Newton–Simpson algorithm.

occurring in sequence.[35] Accordingly, we have retained those curves whose L values fall in the (30 ± 5) nm interval and discarded those with longer L values.

For each selected curve, we have also determined the unbinding force by measuring the jump-off d and multiplying it for the effective cantilever spring constant; a representative unbinding force histogram being shown in Fig. 4. The distribution exhibits a main peak at about 88 pN; this most probable force value is consistent with the experimental results from other biomolecular complexes at the same loading rate (4.5 nN ps_{-1}).[39,42,43]

The binding specificity of the complex has been assessed by performing a blocking experiment. First, we have determined the unbinding frequency, given by the ratio between successful events (*i.e.* events corresponding to specific unbinding processes) over the total recorded events. Then, we have repeated the experiment after incubation of the p53 substrate with a solution of free Mdm2 to saturate the Mdm2 binding sites of p53 on the substrate. At the same loading rate, we have observed a reduction of the unbinding frequency from 20 down to 6% which is indicative of the specificity of the p53-Mdm2 complex formation, in a very good agreement with previous results.[10] At the same time, we noted that the unbinding force distribution, before and after blocking, shows a good overlap, which indicates the same nature of the corresponding interactions (Fig. 4).[38]

For each selected curve, we have finally evaluated the work done by the applied force during the unbinding process, by calculating the integral from the beginning of the nonlinear course in the retraction curve (point a in the inset of Fig. 2) up to the end of the jump-off event (point b):

$$W = \int_a^b F \mathrm{d}x \qquad (3)$$

where the integration variable, x, is given by the piezo displacement λ, according to recent theoretical suggestions.[44] This work is then given by the area underlying the force curve as indicated by the dashed region in the inset of Fig. 2 and corresponds to the work expended on the whole system, including the instrument (this work being called accumulated work).[44] Previous studies applying the JI to molecular systems have estimated the work by using the molecular extension γ as the integration variable obtaining, then, only the work transferred to the molecular system;[26,45] this may be susceptible to introducing significant errors as discussed in ref. 44.

From the work values, we can determine the total complex free energy, ΔG_{bond}, through the JI, given by the following expression, suitably adapted from eqn (1) according to ref. 30:

$$e^{-\frac{\Delta G_{\mathrm{bond}}}{k_{\mathrm{B}}T}} = \sum_{i=1}^{N} \frac{1}{N} e^{-\frac{W_i}{k_{\mathrm{B}}T}} \qquad (4)$$

where N is the number of independent iterations of the unbinding process and the W_i is the work along the ith unbinding path done under the application of the external force. In this respect, we mention that the rate at which the force is applied generally affects the modulation of the energy landscape which, in turn, may lead the system to cross different regimes, for instance from the thermally-activated to diffusive or drift regimes.[46] Indeed, the Bell–Evans model, widely used in the analysis of DFS experiments, allows the extraction of the dissociation rate constant, k_{off}, and the width of the energy barrier, x_β, from the trend of the unbinding force as a function of the logarithm of the loading rate. This can be done under the assumption that the applied force yields a small perturbation of the energy landscape barrier with a concomitant decrease of the lifetime of the system.[21,25] It is therefore crucial to know the response of the system with the loading rate R. On such a basis, we have separately evaluated through eqn (3) the work done by the applied force during the unbinding process, for a collection of force curves recorded at seven different loading rates. In particular, for each loading rate, we have analyzed a hundred force curves which were preliminarily attributed to specific unbinding events. The obtained histograms of the work values at two loading rate values, are shown in Fig. 5. In both the cases, a higher occurrence is observed at low work values; a similar spread of the work values having been observed for the other loading rates.

The bond free energy, ΔG_{bond}, as plotted as a function of $1/R$, is shown in Fig. 6. We note that it rapidly decreases with $1/R$, reaching a constant value.

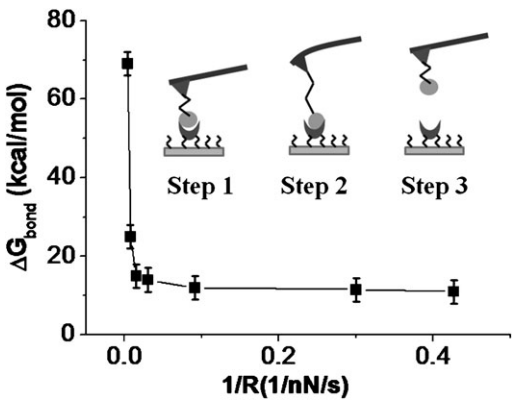

Fig. 6 The free energy difference, ΔG_{bond}, plotted as a function of $1/R$. Error bars indicate the standard deviations. Inset: sketch of the main steps occurring during the retraction of the tip. Step 1: PEG collapsed and complex formed; step 2: PEG stretched and complex still formed; Step 3: complex broken and PEG collapsed.

In principle, the JI is expected to be independent of the rate at which the external force is applied, however the effective lifetime of the system depends on the loading rate value and this can alter, in practice, the response of the system. In fact, at high loading rates, the drastic decrease of the lifetime of the complex can result into a significant deviation from the adiabatic regime, with the introduction of large statistical fluctuations affecting the extracted ΔG_{bond} values. We note on passing that a high number of repetitions N are needed in eqn (4), to lower the statistical errors.[28] On the other hand, at low loading rates, the smaller deviations from the equilibrium lifetime favour approaching the adiabatic regime with concomitant low fluctuations in the ΔG_{bond} values.[25,30] Notably, a behaviour similar to that shown in Fig. 6 has been observed and for the free unbinding energy of the syntaxin–synaptobrevin complex.[30]

We have estimated the total complex free energy from the asymptote of the plot of ΔG_{bond}, obtaining a value of -10.2 ± 0.5 kcal mol^{-1}. It should be noted that this free energy ΔG_{bond}, appearing in eqn (4), takes into account the contributions from both the stretching of the PEG linker and the unbinding process of the complex. The different steps of the unbinding process, as a function of time, have been sketched in the inset of Fig. 6. Step 1 represents the starting configuration in which the PEG is collapsed and the complex is formed. Step 2 indicates the tip retraction stage at which the PEG is stretched but the complex is still formed. Finally, Step 3 represents the stage after the breaking of the complex and at which the PEG snaps to a collapsed state. Since the bond extension is negligible until rupture of the complex, all the extension prior to rupture is attributed to the PEG polymer. Accordingly, ΔG_{unb} related merely to the unbinding process of the p53-Mdm2 complex can be calculated from the expression:

$$\Delta G_{unb} = \Delta G_{bond} - \Delta G_{PEG} \quad (5)$$

where ΔG_{PEG} is the free energy related to the stretching of the PEG linker.

The free energy, ΔG_{PEG}, related to the stretching of a 30 nm long PEG linker has been experimentally evaluated to be -1.78 kcal mol^{-1},[36] therefore an unbinding free energy, ΔG_{unb}, of $-(8.4 \pm 0.5)$ kcal mol^{-1} can be derived for the p53-Mdm2 complex. Such a value is in a good agreement with those obtained in bulk for partial domains of both p53 and Mdm2, by isothermal titration calorimetry measurements ranging from -8.8 kcal mol^{-1} to -6.6 kcal mol^{-1}.[9] Such an agreement is surprising since the two systems, and even the experimental conditions, are quite different. Indeed, in our single molecule experiment, the biomolecules are anchored to the tip *via* a stretchable linker and to the substrate, while they are free in solution bulk experiments. Nevertheless, our results are also in a good agreement with the ΔG values obtained from the computational method (-7.4 kcal mol^{-1}) on single partial domains of our interacting proteins.[18] This suggests that the interaction features of p53 and Mdm2 are essentially regulated by the partial domains directly involved in the molecular interaction without any substantial interference from the other portions of the biomolecules.

Conclusions

The Jarzynski approach has allowed us to determine the unbinding free energy from the evaluation of the mechanical work performed along irreversible unbinding paths for the p53-Mdm2 complex involving full length individual proteins. This result provides an important piece of information on the molecular properties of this complex which could be at the basis of further investigations aimed at disclosing possible effects of ligands or drugs on the p53-Mdm2 interaction. This could be a remarkable help to design new drugs for innovative anticancer strategies. Furthermore, we would like to remark that the evaluation of the unbinding free energy from the unbinding data collected by the same experimental setup commonly used in DFS experiments allows us to extend the potential of DFS in the investigation of biorecognition processes. Indeed, the possibility to have access, from the same experimental data, to the k_{off} rate constant and to the energy barrier width, together with the unbinding free energy, could be of high relevance in the characterization of biomolecular complexes at single molecular level.

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