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**Computational Equilibrium Thermodynamic and Kinetic Analysis of K-Ras Dimerization through an Effector Binding Surface Suggests Limited Functional Role**

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**Abstract**

Dimer formation is believed to have a substantial impact on regulating K-Ras function. However, the evidence for dimerization and the molecular details of the process are scant. In this study, we characterize a K-Ras pseudo-*C*2-symmetric dimerization interface involving the effector interacting *β*2-strand. We used structure matching and all-atom molecular dynamics (MD) simulations to predict, refine, and investigate the stability of this interface. Our MD simulation suggested that the *β*2-dimer is potentially stable and remains relatively close to its initial conformation due to the presence of a number of hydrogen bonds, ionic salt bridges, and other favorable interactions. We carried out potential of mean force calculations to determine the relative binding strength of the interface. The results of these calculations indicated that the *β*2 dimerization interface provides a weak binding free energy in solution and a dissociation constant that is close to 1 mM. Analyses of Brownian dynamics simulations suggested an association rate *k*on ≈ 105–106 M−1 s−1. Combining these observations with available literature data, we propose that formation of auto-inhibited *β*2 K-Ras dimers is possible but its fraction in cells is likely very small under normal physiologic conditions.

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**Graphical abstract**



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**INTRODUCTION**

Advances in microscopic and spectroscopic techniques such as electron microscopy (EM), single particle tracking (SPT), and fluorescence energy transfer (FRET)[1](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R1) enabled the study of transient molecular assemblies that underlie many cell signaling processes.[2](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R2) These techniques allowed for relatively detailed characterization of nanoclusters of Ras proteins on the plasma membrane.[3](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R3) The Ras oncoproteins are small GTPases that are ubiquitously expressed in human cells.[4](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R4),[5](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R5) The three most common Ras isoforms in humans, N-, H-, and K-Ras, bind to the inner surface of the plasma via a farnesylated C-terminus plus a proximal palmitoylated cysteine (N- and H-Ras) or polybasic domain (K-Ras). Since in solution the highly conserved catalytic domain (amino acids 1–166) of Ras interacts with effectors as a monomer,[6](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R6),[7](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R7) it was long believed that the functional form of cellular Ras is also monomeric. This view is now changing by the observation that clusters[8](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R8) or dimers of Ras[9](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R9),[10](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R10) are important for function.

Estimation of the average number of Ras cluster sizes through EM analysis of plasma membrane sheets ranges between 5 and 7 proteins per cluster.[3](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R3) However, EM lacks the necessary resolution to quantify the relative distribution of the potentially diverse oligomerization states of Ras that might include dimers. In fact, Ras dimerization has been suggested as early as in 1988 by Santos and co-workers.[11](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R11) In 2000[9](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R9) and more recently,[10](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R10) cell-biological experiments suggested that Ras activates its effector Raf through homodimerization. Moreover, using gel filtration, Dementiev[12](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R12) showed that K-Ras dimerizes upon binding to a synthetic membrane via a meleimide-linked C-terminally farnesylated motif. On the other hand, Werkmüller et al.[13](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R13) found that K-Ras is monomeric in bulk solution at concentrations as high as 2.0 *μ*M based on rotational-correlation times from time-resolved fluorescence anisotropy (TRFA) and lateral diffusion coefficients from fluorescence correlation spectroscopy (FCS) experiments. Another recent report by Nussinov and co-workers[14](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R14) combined microscale thermophoresis and dynamic light scattering experiments with computational modeling to propose that the soluble catalytic domain of GTP-loaded K-Ras forms dimers in solution at concentrations in the tens of micromolar range. An interesting recent work based on nuclear magnetic resonance (NMR) and other spectroscopic techniques probed the potential of C-terminally cross-linked Ras to form dimers.[15](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R15) They concluded that the catalytic domain could not form dimers even at low ionic strength where electrostatic interactions can be strengthened. These examples highlight the controversy about the ability of Ras, and K-Ras in particular, to dimerize via the catalytic domain in an aqueous solvent. However, a degree of consensus is emerging regarding dimerization of K-Ras upon binding to the plasma membrane,[16](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R16) the primary site of its activity in cells.[8](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R8)

Several putative dimerization interfaces may be gleaned from crystal contacts in a number of Ras structures found in the Protein Data Bank (PDB). Most of these involved surface loops, and the most probable state of these structures was predicted to be monomeric by PDBePISA (<http://www.ebi.ac.uk/msd-srv/prot_int/cgi-bin/piserver>), a server for the prediction of probable quaternary structures. However, crystal contacts in some X-ray structures of several members of the Ras superfamily were predicted to be true interfaces of dimers. Intriguing among these were dimers involving the central *β*-strand (*β*2; see [Figure 1](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/figure/F1/)) that form an extended antiparallel *β*-sheet spanning the two monomers. Different variations of this alignment were found in crystal structures of a number of Ras-like proteins, such as Rab9 (PDB id: 1WMS)[17](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R17) and Rac2 (PDB id: 2W2V).[18](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R18) Presumably using these crystal structures as templates, a recent report used structural matching analysis to suggest that K-Ras dimerization, too, involves *β*2.[14](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R14)



[Figure 1](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/figure/F1/)

Catalytic domain structure of G12D K-Ras (PDB id: 4DSO) is shown in cartoon representation colored in purple (helix), yellow (strand), cyan/white (loop/turn), and blue (switch 1 (residues 25–40) and switch 2 (residues 60–75)). Lobe 1 (residues **...**

These observations suggest that K-Ras dimerization via *β*2 might be viable in solution, but they also raise a number of fundamental questions regarding the potential impact of such a dimer on biological activity. One question is whether dimerization via *β*2 might be autoinhibitory considering that *β*2 is the primary site of effector binding.[19](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R19) Autoinhibition, however, would contradict the reported importance of K-Ras dimerization for MAPK pathway activation.[10](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R10) Another important question is if the thermodynamics and kinetics of K-Ras dimerization via *β*2 are consistent with the well-documented cellular activities of the protein. Tight binding would mean that dimerization might outcompete effector binding while, conversely, weak protein–protein interaction (PPI) would presumably allow for an easier regulation and a reversible response to changes in environmental stimuli.[20](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R20) These questions cannot be fully addressed by biophysical or cell biological techniques alone due to limitations in spatiotemporal resolution. For instance, isothermal titration calorimetry is rather useful for elucidating the overall energetics of dimerization, but it lacks the resolution necessary to identify specific dimerization interfaces.

In this work, we combined template-based dimer prediction, molecular dynamics (MD) simulations, and potential of mean force (PMF) calculations to show that K-Ras dimerization via *β*2 involves a weak PPI, suggesting a small population of such a dimer in solution. In addition, on the basis of structural, thermodynamic, and kinetic arguments, we propose that dimerization via *β*2 is not likely to play a significant role in the biochemical activities of K-Ras.

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**METHODS**

We used the knowledge-based PRotein Interactions by Structural Matching (PRISM) algorithm[21](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R21) to explore possible dimerization interfaces involving *β*2 of K-Ras. We utilized most of the available crystal structures (145 as of 12/2014) of all Ras isoforms in the PDB, taking into account the fact that N-, H-, and K-Ras have a nearly identical catalytic domain.[22](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R22),[23](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R23) [Figure 2](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/figure/F2/) summarizes the overall procedure we adopted for most of the calculations performed in this work.



[Figure 2](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/figure/F2/)

Schematic flowchart showing the overall workflow adopted in this work, including the algorithm used to compute the PMF and the dissociation constant.

**Molecular Dynamics Simulation**

MD simulation was conducted to evaluate the stability of the predicted *β*2 dimer and characterize the physicochemical properties of the interface. The starting structure for the MD simulation was the crystal structure of the oncogenic G12D mutant K-Ras (PDB id 4DSO, residues 1–179). The bound guanosine-diphosphate-monothiophosphate (GSP) was replaced by guanosine triphosphate (GTP), and all other cocrystals were removed. Since the best starting *β*2 dimer model with the lowest energy score (first row in [Table 1](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/table/T1/)) was not predicted for GTP-bound G12D K-Ras, structural alignment was used to reconstruct the model from the 4DSO monomer crystal structure. All crystal waters that have no steric clash with any of the interface residues were kept for reasons described previously.[24](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R24) The resulting dimer model was placed in a cubic box of side 96 Å, containing 31,413 TIP3P water molecules as well as 88 Na+ and 80 Cl− ions, which yielded an ionic strength of 150 mM. A minimum of 14 Å buffer between the edges of the box and protein atoms was used. The solvated system was energy minimized using 50,000 conjugate gradient steps and then gradually heated while keeping the C*α* atoms restrained to their initial positions by a harmonic restraint of force constant *k* = 10 kcal mol−1 Å−2. An equilibration phase followed with *k* progressively reduced to zero by decrements of 2 kcal mol−1 Å−2 every 1 ns.



[Table 1](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/table/T1/)

Summary of the Dominant ***β***2 Dimerization Interfaces Predicted by PRISMa

A 400 ns production run was commenced from the equilibrated system. During the simulation, a 2 fs time step was used with the SHAKE algorithm applied to covalent bonds involving hydrogen atoms. The isothermal–isobaric (NPT) ensemble and periodic boundary conditions were used. Temperature was maintained at a physiologic value of 310 K using Langevin dynamics with a 10 ps−1 damping coefficient. The Nosé–Hoover Langevin piston method was used with a piston period of 200 fs and a decay time interval of 100 fs to maintain constant pressure at 1.0 atm. Short-range van der Waals (vdW) interactions were smoothly switched off between 10 and 12 Å, with a 14 Å cutoff used for nonbonded list updates. Long-range electrostatic interactions were computed using the particle mesh Ewald (PME) method with a grid density of about 1/Å. Simulations were run with the GPU-enabled NAMD2.9[25](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R25) using the CHARMM27[26](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R26) force field and cMAP dihedral correction.

We also simulated monomeric K-Ras for 140 ns using the same procedure, with the exception that the system size was approximately half of the dimer system because we have only one K-Ras molecule in the simulation box.

**Structural Analysis of Protein–Protein Interaction Interfaces**

Several measures were utilized to characterize the stability and dynamics of the predicted dimer structure. These included intermonomer center-of-mass distance (*r*), relative orientation, root-mean-square deviation (RMSD), and buried solvent accessible surface area at the interface (ΔSASA). Intermonomer salt bridges (SBs), hydrophobic contacts (HCs), and hydrogen bonds (HBs) were also examined. A HB was defined by donor–acceptor distance and donor–hydrogen–acceptor angle cutoffs of 3.0 Å and 20°, respectively. An intermonomer SB was defined by a distance cutoff of 3.2 Å between any side chain oxygen atom of an E or D residue on one monomer and any side chain nitrogen atom of a K or R residue on the other monomer. Similarly, HCs were calculated using a distance cutoff of 3.8 Å between two carbon atoms located across the interface. ΔSASA was defined as the difference in the sum of SASA of the two unbound monomers and that of the dimer: ΔSASA = (SASAm1 + SASAm2) – SASAd, where SASAd, SASAm1, and SASAm2 are the SASA of the dimer and the two unbound monomers. SASA was calculated using a water probe radius of 1.4 Å. Intermonomer separation distance *r* and ΔSASA monitor close contact, while RMSD helps evaluate the stability of these contacts. However, these measures alone cannot uniquely distinguish between different spatial organizations of the monomers. This can be assessed by the angular location and relative orientation of the monomers, which entails measuring the azimuthal and polar angles (*θ*, *ϕ*) of a vector linking the center of mass of one monomer at the origin to the center of mass of another monomer, as well as the three rotation Euler angles (*α*, *β*, *γ*) between the principal axes of the two monomers.

**Potential of Mean Force Calculations**

We used the adaptive biasing force (ABF) method[27](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R27) as implemented in NAMD2.9 to compute the one-dimensional potential of mean force (1D PMF) of K-Ras dimerization along *r*. To increase the efficiency of sampling in a desired range of *r*, the mean force was evaluated by dividing the reaction path into a set of overlapping windows of size 5–12 Å ([Figure 2](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/figure/F2/)). A multiple walker strategy[28](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R28) with six independent walkers was adopted to concurrently sample each window. The duration of the trajectory for each walker was 6–7 ns. A harmonic restraint with force constant *k* = 50 kcal mol−1 Å−2 was applied at the boundary of each window to prevent sampling configurations outside the prescribed window. The instantaneous force in each window was accumulated into bins of width 0.1 Å after discarding the first 10,000 samples where the adaptive biasing force was inactive. For regions of *r* that were still relatively poorly sampled, additional new windows were inserted. The total sampling of the ABF was ~1 *μ*s, which corresponds to an average of ~39 ns/Å.

The mean force evaluated at the *i*th global bin, 〈*F*〉*i*, was combined using a weighted average of the mean forces of all corresponding local window bins:

⟨*F*⟩*i*=∑*Nij*=1*nij*⟨*F*⟩*ij*∑*Nij*=1*nij*

(1)

Here 〈*F*〉*ij* and *nij* are the mean force and the number of samples for the *j*th window at the local bin corresponding to the *i*th global bin, respectively. *Ni* is the number of windows that contain the *i*th global bin, and *j* loops over all such windows. The mean force in each global bin was smoothed out using a weighted running average of neighboring bins within 0.5 Å. This procedure reduced the effects of unphysical sudden changes in the calculated mean forces that might arise from inadequate sampling. The uncertainty in the mean force at the *i*th global bin (*σ*〈*F*〉*i*) was determined using

*σ*⟨*F*⟩*i*−1*Mi*−−−√∑*Mij*=1*mij*(⟨*F*⟩*ji*)2*Ni*−⟨*F*⟩*i*2−−−−−−−−−−−−−−−−−−−−⎷

(2)

where *Mi* is the total number of trajectories that sampled the *i*th global bin. ⟨*F*⟩*ji* and *mij* are the mean force and the number of samples for the *j*th trajectory at the *i*th global bin, respectively.

The PMF at a given reaction coordinate value, *W*(*rk*), was estimated by integrating the negative of the mean force between monomers over *r* using the midpoint rule numerical integration method along with subintervals of length Δ*r* = 0.1 Å, as follows

*W*(*rk*)=−∫*rkr*min⟨*F*⟩*rdr*≈−Δ*r*∑*i*=1*k*⟨*F*⟩*k*

(3)

The uncertainty in the potential of mean force at the *k*th global bin was approximated using

*σW*(*rk*)=Δ*r*∑*ij*=1*kρijσ*⟨*F*⟩*iσ*⟨*F*⟩*j*−−−−−−−−−−−−⎷

(4)

where *ρij* is the correlation coefficient between each trajectory mean force value in the *i*th and *j*th global bin.

**Calculation of Equilibrium Dissociation Constant and Dimerization Rate Constant**

The equilibrium dissociation constant (*K*d) of two relatively rigid and anisotropic molecules can be rigorously estimated from a six-dimensional PMF,[29](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R29) *W*(*r*, Ω), using

*K*d=8*π*2*C*0∫boundd*r*dΩe−*W*(*r*,Ω)/*RT*

(5)

Here *C*0 is the 1 M standard concentration ( ~11660molecule/Å3), *R* is the universal gas constant, and *T* is the temperature in Kelvin. Given a Cartesian coordinate system whose origin and axes correspond to the center of mass and three principal moment of inertia axes of the first monomer, respectively, *r* can be defined as the intermonomer separation distance and Ω as the relative orientation of the second monomer; Ω encompasses the angular location (*θ*, *ϕ*) and relative orientation (*α*, *β*, *γ*) of the second monomer. In practice, however, sampling limitations require that calculations are routinely restricted to the separation distance, angular locations, and relative orientations close to that of the bound state.[30](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R30) In this case, *K*d can be evaluated from the 1D PMF profile, *u*(*r*), utilizing the approximation

*K*d≈8*π*2*C*0*δω*∫bound*r*2d*r*e−*u*(*r*)/*RT*

(6)

where *δω* reflects the orientational freedom of the bound monomer and can be estimated from the ranges and averages of angular locations and relative orientations extracted from MD trajectories of the dimer via

*δω* ≈ Δ*θ*Δ*ϕ*Δ*α*Δ*β*Δ*γ*〈sin*θ*sin*β*〉

(7)

where the angle brackets denote averaging over time and Δ in this context describes the orientational freedom in each angular dimension (e.g., Δ*θ* = *θ*max − *θ*min).

We used Brownian dynamics (BD) simulations[31](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R31) conducted with the program SDA7[32](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R32) to investigate the kinetics of K-Ras dimerization via *β*2. Specifically, we ran 1,000,000 independent BD trajectories to calculate the rate constant of dimerization (*k*on). For each run, one monomer (the mobile monomer) was placed on a sphere of radius 143 Å centered at the geometric center of the other (stationary) monomer. The initial angular location and relative orientation of the mobile monomer varied among the different BD runs. Simulations were terminated (i.e., the reaction was deemed unsuccessful) when the intermonomer distance reached 286 Å. The electrostatic potential in these calculations was obtained by solving the linear Poisson–Boltzmann (PB) equation at a salt concentration of 150 mM. An adaptive time-stepping scheme with time-steps of 1–20 ps was used to integrate the BD equations of motion. A monomer translational and rotational diffusion coefficient of 0.011 Å2/ps and 2.2 × 10−5 rad/ps were used, respectively. *k*on was calculated using the Northrup–Allison–McCammon method,[31](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R31) where an encounter complex was counted when a prescribed number of native dimer contacts were sampled.

**Electrostatic Surface Potential Calculations**

We used the Adaptive Poisson–Boltzmann Solver (APBS)[33](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R33) to calculate the electrostatic potential around the catalytic domain of K-Ras by solving the nonlinear PB equation. We used the PDB 2PQR Web server[34](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R34) to assign charges and atomic radii based on the CHARMM27 force field. A fine grid spacing of 0.25 Å was used. Solvent effects were accounted for using a 0.15 M 1:1 electrolyte and dielectric constants of 2.0 and 78.54 for the protein interior and the solvent water, respectively.

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**RESULTS AND DISCUSSION**

**Prediction of the *β*2 Dimer Interface through Structural Matching**

Our initial search for bona fide homodimers of small GTPases in published reports and the PDB database yielded an intriguing result. Namely, some members of the Ras superfamily, such as GDP-bound Rab9 (PDB id: 1WMS),[17](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R17) dimerize via a central *β* strand in such a way that an antiparallel alignment of the strand leads to an extended *β* sheet spanning the two protomers. We wondered if K-Ras might form a dimer through a comparable interface but did not observe such an interface during protein–protein docking with Rosetta[35](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R35) (Rosetta yielded other interfaces that will be discussed elsewhere). We therefore turned to knowledge-based PPI prediction techniques, such as the structural matching strategy of PRISM.[21](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R21) To improve the statistics of the data and to test if predictions are affected by the initial structure, we used over 145 X-ray structures as input and obtained a large number of predicted dimer models. Among the many interfaces identified, different variations of the *β*2 dimerization mode appeared to be prevalent and energetically favored ([Table 1](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/table/T1/)). PRISM essentially uses transitive relations to extrapolate known protein–protein interaction interfaces available in the PDB to other protein pairs that share similar interfaces. Therefore, the multiplicity of the *β*2 dimerization mode predicted by PRISM reflects the diversity of Ras-related template structures that have intermonomer contacts involving *β*2.

Dimerization via *β*2 ([Figure 3A](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/figure/F3/)) involves only lobe 1 (residues 1–86 of the catalytic domain residues 1–166[23](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R23)); lobe 2 (residues 87–166) and the hyper-variable region (HVR, residues 167–185) do not participate. Addition of the HVR to the truncated monomers within a preassembled *β*2 dimer did not prevent the full-length K-Ras from membrane binding (not shown), suggesting that dimerization via *β*2 may be viable in cells. This prompted us to conduct a comprehensive characterization of the interface in terms of its structural integrity and thermodynamic stability. As already noted, PRISM used different templates to predict several variations of what can be broadly referred to as the *β*2 interface (see [Table 1](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/table/T1/)). We used statistics and the binding energy score to select a representative model for further investigation using MD simulation, Brownian dynamics (BD), and PMF calculation. The model based on the PDB structure 2ERX was dominant in terms of both binding energy score and frequency of occurrence; both of these quantities were about twice larger (in absolute value) than those for the next best model ([Table 1](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/table/T1/)). Therefore, this model was selected as a representative of *β*2 dimers.



[Figure 3](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/figure/F3/)

(A) Snapshot from a MD simulation of the *β*2 dimer model showing an extended antiparallel *β*2-sheet spanning the two monomers. (B–F) Time evolution of distances between selected residue pairs representing key intermonomer interactions **...**

**Structural Stability and Dynamics of the *β*2 Dimer Interface**

Networks of hydrogen bonds and salt bridges at PPI interfaces are very important contributors to stability.[36](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R36) In our *β*2 dimer model, a number of specific interfacial interactions were found to stabilize the complex ([Figure 3B–H](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/figure/F3/)). In fact, the interface spans a well-defined network of residues, such as D33 and S39 ([Figure 3G](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/figure/F3/)), that are involved in interactions with the Ras binding domain (RBD) of effectors PI3K, Ral GDS, and Raf.[37](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R37) In particular, S39 forms two backbone hydrogen bonds that helped create a continuous *β*-sheet across the interface. Two additional backbone hydrogen bonds were formed between R41 and E37 ([Figure 3B](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/figure/F3/)). The resulting 12-stranded *β*-sheet remained stable during a 400 ns MD simulation. To test the role of inter-monomer backbone hydrogen bonding in K-Ras dimerization, we examined the impact of disrupting the *β*2 strand on K-Ras clustering because clustering is likely mediated by dimer formation. This was achieved by introducing proline residues at *β*2 and an EM analysis technique described in previous work (e.g., see ref [3](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R3)). Briefly, plasma membrane (pm) sheets from BHK (baby hamster kidney) cells expressing monomeric green fluorescent protein (mGFP)-tagged wild type and E37P/D38P double mutant K-Ras were labeled with anti-GFP conjugated gold and visualized by EM. Analysis of the spatial pattern of the gold particles suggested that clustering is significantly (>30%) reduced in the mutant relative to the wild-type control. This shows that dimerization of K-Ras via *β*2 is possible in cells, and that backbone hydrogen bonds are critical for its formation. Binding is further enhanced by a salt bridge between the side chains of D33 and K42 ([Figure 3C](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/figure/F3/)). Similarly, R41 and E37 are engaged in a network of dynamic interactions alternating between salt bridges and hydrogen bonds ([Figure 3D](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/figure/F3/)). Y40 and H27 also stabilized the interface via T-shaped aromatic ring interactions ([Figure 3E and F](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/figure/F3/)). Additional packing interactions involving I21, F28, and V29 provide significant nonspecific hydrophobic contacts at the interface. The involvement of many of these residues in at least two stabilizing interactions suggests that their mutation will have a significant impact on *β*2 dimerization kinetics and thermodynamic stability.

To characterize the monomer–monomer relative motion, we first looked at the RMSD of the C*α* atoms after aligning the catalytic domain of one monomer to the initial structure while excluding the flexible switches 1 (residues 25–40) and 2 (residues 60–75). As shown in [Figure 4A](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/figure/F4/), no major reorganization of the dimer or conformational change of the monomers occurred during the simulation. Similarly, the intermonomer separation distance (*r*) remained stable during the entire simulation ([Figure 4B](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/figure/F4/)), with its average over the last 350 ns being 31.8 ± 0.3 Å. Notice, however, that *r* increased slightly to an average of 32.2 ± 0.3 Å in the last 50 ns. [Figure 4D](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/figure/F4/) also shows small changes in the relative angular location and orientation of the monomers, suggesting tight binding and limited relative orientational freedom of the monomers. Another quantity that yields an insight into the strength of the dimer is the buried SASA ([Figure 4C](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/figure/F4/)). The average Δ*S*ASA of our dimer (14.8 ± 0.9 nm2) is somewhat smaller than the ~19 ± 8 nm2 observed in known biological PPI interfaces,[38](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R38) but it still translates to an estimated 8.3 ± 1.3 kcal/mol favorable contribution to the binding free energy based on the formula Δ*G*nonpolar = 0.5ΔSASA + 0.86 kcal/mol.[39](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R39) This estimate encompasses contributions of hydrophobic interactions and an entropic gain due to the release of water molecules upon dimerization. For instance, the total number of HBs between solvent and the catalytic domain of the two proteins was 304 ± 14 in the dimer, while the number of HBs between solvent and the catalytic domain of the free monomer was found to be 158 ± 10, suggesting that about a dozen water molecules were released from the interface.



[Figure 4](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/figure/F4/)

Time evolution of different quantities used to characterize the dimer structural stability. (A) C*α* root-mean-square deviation (RMSD) from the initial structure. (B) Intermonomer center of mass distance. (C) Buried solvent accessible surface area **...**

Parts A and B of [Figure 5](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/figure/F5/) show the number of intermonomer HBs and HCs. Despite substantial fluctuations, there was no drift in either of these quantities, suggesting a stable set of contacts at the interface. The average number of intermonomer HBs was 4.0 ± 1.0, which includes buried backbone HBs that can potentially have large contributions to binding free energy. The average number of intermonomer HCs, 11 ± 3, is also substantial considering the relatively conservative cutoff used and the overall polarity of the interface. These values are generally consistent with the expected average numbers of interfacial HBs, HCs, and SBs in known protein complexes.[36](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R36)



[Figure 5](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/figure/F5/)

Time evolution of the number of intermonomer hydrogen bonds (A) and hydrophobic contacts (B). Black represents data sampled every 100 ps, and thick solid lines are 50 ns running averages. (C) Root-mean-square fluctuations (RMSF) of subunits 1 and 2 in **...**

Comparison of C*α* atom root-mean-square fluctuations (RMSFs) in the dimer and the monomer indicated similar fluctuations overall except at switches 1 and 2 ([Figure 5C](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/figure/F5/)). As expected, the RMSF of the interfacial switch 1 residues is dampened by dimer formation, but quite unexpectedly, the fluctuation of switch 2 residues is enhanced upon dimerization. Increased flexibility upon complex formation often compensates for entropic loss associated with the reduced translational and rotational freedom of the reactants.[40](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R40) To further examine the impact of complex formation on the global dynamics of K-Ras, we used principal component analysis (PCA) of each protomer in the dimer and the free monomer, following a previously described protocol.[23](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R23) The fluctuations captured by the first two principal components (PC) displayed in [Figure 5D](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/figure/F5/) show that K-Ras samples a wide region of configurational space both in its free and complexed forms. However, the phase spaces sampled by the protein in the dimer and monomer are somewhat different. Whereas the free monomer sampled a region close to that populated by a cluster of GTP-bound Ras X-ray structures,[23](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R23) each subunit in the dimer sampled a region closer to GDP-bound G12V H-Ras structures. This difference is partly due to the differential dynamics at switch 2 and partly due to minor reorganizations in intramonomer hydrogen bonds induced by dimer formation.

**Potential of Mean Force and Equilibrium Dissociation Constant Calculations Suggest Modest Stability**

The fact that no dissociation occurred within the limited time scale of the MD simulation suggests that the dimer is potentially stable. However, this says little about the actual equilibrium thermodynamics of the complex. The free energy of the complex relative to an appropriate reference state, typically the unbound state, is the most reliable and general means of determining thermodynamic stability. Binding free energy change can be approximated from a PMF along an appropriate reaction coordinate that distinguishes between the unbound and bound states. We calculated the PMF of K-Ras dimerization along *r*, and the result is shown in [Figure 6](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/figure/F6/). Accurate calculation of PMF requires that the total sampling time for each window is exhaustive to ensure visiting an ensemble of uncorrelated configurations.[41](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R41) In the case of Ras, a multi microsecond scale of simulation may be needed to fully sample the dimer configurational space due to, for example, the slow rotational diffusion of the monomer (see [Figure 6D](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/figure/F6/)). This is computationally expensive. However, we were able to extend our sampling long enough to obtain a reliable estimate of the binding energetics, as can be seen from the relatively small estimated errors for both the PMF ([Figure 6A](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/figure/F6/)) and the mean forces ([Figure 6B](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/figure/F6/)). Moreover, the number of samples used to evaluate the mean force at any given *r*, plotted in [Figure 6C](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/figure/F6/), shows that most of the 0.1 Å-wide bins were sampled at least 106 times, which is equivalent to 2 ns/bin.



[Figure 6](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/figure/F6/)

(A) Potential of mean force (PMF) profile characterizing the reversible dissociation of the *β*2 dimer along the intermonomer distance. The PMF is shown in a thick black line, while the uncertainty in it is in gray shading. (B) Mean force profile **...**

The profile of the mean force along *r*, calculated by averaging over all other degrees of freedom, exhibits a maximum of 2.8 kcal/Å at *r* = 33.6 Å. The inflection point immediately after this distance likely coincides with the rapture of intermonomer backbone hydrogen bonds and the start of the rise of the PMF from its global minimum. The PMF, whose numerical value was set to 0 at *r* = 50 Å, remains negligibly small for *r* between 45 and 50 Å, suggesting that the monomers were far apart and not interacting with each other. For *r* < 45 Å and going to the left, the PMF rises slightly until a small maximum is reached at about 43 Å (see the bump in [Figure 6A](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/figure/F6/)). This reflects the initial encounter of the monomers via unfavorable nonspecific contacts resulting in a small barrier of height <0.5 kcal/mol. The impact of this minor barrier on the kinetics of the reaction is probably small, although underestimation is possible due to insufficient sampling. The profile begins to decline at a separation distance of ~40.0 Å and falls smoothly until it reaches an energy well of depth of −8.6 ± 0.9 kcal/mol at 32.5 Å (almost identical to the average *r* in the last 50 ns of the free MD run), before rising sharply at the onset of atomic collisions. Overall, the PMF profile suggests that the *β*2 dimer of K-Ras is thermodynamically stable but it is substantially weaker than some of the well-known strong PPIs such as barnase-barstar which has a binding free energy of −19 kcal/mol.[30](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R30)

Quantifying *K*d, a quantity most relevant in experimental studies, can provide additional insights into the population of K-Ras dimers present in the system at given conditions. We note that, during the calculation of *K*d via [eq 6](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#FD6), the exact definition of the bound state along *r* is not critical as the integral is dominated by the negative values of the PMF at the energy well. This means that limiting the integral upper bound to any *r* value between 42 and 50 Å would yield roughly the same *K*d. Utilizing the time course and statistics of a monomer’s angular location and orientation relative to the other monomer (see [Figure 4D](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/figure/F4/)), we calculated Δ*θ*, Δ*ϕ*, Δ*α*, Δ*β*, Δ*γ*, and 〈sin*θ* sin*β*〉 to be 0.668, 0.972, 0.345, 0.702, 0.685, and 0.738, respectively. Plugging these values into [eq 7](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#FD7) yielded *δω* = 0.08 rad[5](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R5), which measures the orientational freedom of the monomer. Using this value and integrating the PMF profile shown in [Figure 6A](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/figure/F6/) over the entire range of *r*, we obtained *K*d = 870 *μ*M. This indicates a weak PPI[42](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R42) (e.g., a dimer fraction of 2% in a 10 *μ*M solution), and is consistent with the failure of various experimental studies to detect K-Ras dimers in solution[13](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R13) except at a relatively high concentration or upon membrane binding.[12](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R12)

We estimated *k*on of K-Ras dimerization via the *β*2 interface using BD simulations (see Methods). As expected the estimate of *k*on varies with the reaction criteria, including the cutoff and the number of native contacts used to define the formation of an encounter complex ([Figure 7](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/figure/F7/)). For example, if a reaction was defined to occur when at least three independent native contacts are formed using a cutoff of 3.5 Å, *k*on ≈ 1 × 106 M−1 s−1. This value is in the same range of very tight complexes such as the BPTI–trypsin complex[43](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R43) and likely represents an upper bound considering that K-Ras dimers are not ubiquitously observed in experiments. We obtained *k*on ≈ 1 × 105 M−1 s−1 if a reaction was defined to occur when at least four independent native contacts have formed. This value is more consistent with a less stable and transient complex, as we expect is the case for K-Ras dimerization. The actual *k*on may be even slower, since it will be influenced by the relaxation time for the transition from an encounter complex to the final stereospecific dimer. Nonetheless, the two examples provide a reasonable estimate for the approximate range of the actual *k*on. Ultimately, both the association and dissociation rates would be needed to determine the overall kinetics of dimer formation. Combining our *k*on estimates with the *K*d value obtained from the PMF calculations, we obtained a dissociation rate constant (*k*off) of 87–870 s−1 or a half-life of 0.8–8 ms (calculated as *t*1/2 = ln 2/*k*off). This further supports our conclusion that the *β*2 dimer is transient and short-lived in solution when compared to, for example, the BPTI–trypsin complex which has a *k*off value of 6.6 × 10−8 s−1 and a half-life of 17 weeks.[43](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R43)



[Figure 7](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/figure/F7/)

Association rate constant for encounter complexes defined by the presence of three and four “native dimer” contacts at different reaction cutoffs shown in black and red, respectively. The green shaded region highlights the cutoff range **...**

**Proposed Biochemical Context and Relevance of K-Ras Dimerization through the Effector Binding Surface**

Ras signals from the plasma membrane[8](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R8) and membrane binding facilitates Ras dimerization.[12](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R12) The latter is presumably due to the increased effective local concentration and the reduced dimensionality of the search space for intermolecular encounter. In addition, intracellular salt and crowding conditions[44](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R44) likely have a significant effect on dimerization. Therefore, it is possible that the marginal solution stability of the *β*2 dimer suggested by our calculations represents a lower limit of the actual affinity in the cell. However, the fact that the interface overlaps with the effector-binding surface and yet there is no published report of direct competition between dimerization and effector binding suggests that the tendency to dimerize is probably small also in cells. If this is true, then the fraction of autoinhibited *β*2 K-Ras dimers is minor under endogenous expression levels (approximately 3.6 × 106 molecules[45](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R45)). Moreover, the nearly perfect electrostatic complementarity between Ras and the RBD of effectors[46](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R46) and the fact that the measured *K*d of Ras–effector interaction is well below 100 nM[47](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R47) seem to further suggest that activated K-Ras would predominantly exist in its effector bound form. However, this does not rule out the existence and possible physiologic role of auto-inhibited K-Ras dimers, particularly in cells harboring specific mutations at the canonical switch regions that enhance surface complementarity. The *β*2 surface of the catalytic domain of unbound, monomeric K-Ras is dominated by a negative electrostatic potential ([Figure 8](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/figure/F8/)). This means that dimerization requires overcoming a long-range repulsive net force, which should impede dimerization. Even if this repulsion is attenuated by environmental factors such a high salt concentration, the lack of long-range steering forces that facilitate the formation of an encounter complex[48](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R48) will reduce the association rate.[49](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/#R49)



[Figure 8](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/figure/F8/)

(A) The catalytic domain of K-Ras in cartoon representation with surface accessible area rendering of the key residues involved in the dimerization shown in green. (B) Electrostatic potential displayed using the same view as in part A with red and blue **...**

A closer look at the electrostatic surfaces of K-Ras in the complex, i.e., taking into account the specific antiparallel orientation of the interacting surfaces, yielded an intriguing insight. Namely, there exists local electrostatic complementarity despite the long-range ant-complementarity at the global level. In other words, electrostatic complementarity emerges once the monomers are aligned in the right orientation ([Figure 9](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/figure/F9/)). Combining these observations, we propose that repulsive forces and rotational diffusion affect the dimerization reaction negatively until a perfect antiparallel alignment is achieved. After that, the reaction shifts to a downhill process, driven by electrostatic forces, leading to the tight intermonomer hydrophilic and hydrophobic interactions discussed in previous sections. The interplay between all of these forces results in dimeric species that are unlikely to be highly populated in cells. We thus propose that Ras–effector interaction would be the dominant process for cases in which the relative concentration (or expression levels) of Ras and effector molecules is comparable. At a dramatically higher relative concentration of Ras, however, formation of *β*2 dimers may lead to a nonlinear relationship between Ras and Ras–effector complexes.



[Figure 9](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5048749/figure/F9/)

Induced electrostatic complementarity demonstrated by the electrostatic potential at the surface of the two bound monomers with a top view on their *β*2 dimerization interface.

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**CONCLUSIONS**

A major goal of this work was to evaluate the thermodynamic and kinetic bases for the proposed dimerization of K-Ras via its central *β*-strand that normally serves as part of the effector binding surface. Starting with a survey of putative dimerization interfaces in the Ras superfamily of proteins followed by predictions using structure matching algorithms, we obtained several variations of a K-Ras dimerization interface involving the central *β*-strand and a few other surrounding residues. MD simulation of the statistically most significant and lowest-energy predicted dimer model enabled us to characterize the intermonomer interactions in great detail, and found that the interface exhibits physicochemical features that compare well with bona fide protein dimers. The simulation also allowed us to identify key residue–residue interactions including backbone and side chain hydrogen bonds as well as hydrophobic contacts that stabilize the interface. Despite these stabilizing interactions, however, an extensive (~1 *μ*s long) PMF calculation suggested that the dimer is thermodynamically only marginally stable with a dissociation constant close to 1 mM. Moreover, analysis of Brownian dynamics simulations of the diffusional association yielded an association rate *k*on ≈ 105–106 M−1 s−1, which is significantly slower than that of known strong PPIs, presumably due to the lack of long-range electrostatic complementarity. Taken together with our initial experiments with EM, these observations suggest that auto-inhibitory K-Ras dimerization via the effecter binding surface is possible but its concentration in the cellular milieu is likely very small at normal expression levels.

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**Footnotes**

The authors declare no competing financial interest.

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