Dissociation kinetics for RBD and CRD

According to the single-molecule imaging kinetics formulated by Xie (2001), the reaction rate equation for a simple stochastic dissociation is:

\[
\frac{dA}{dt} = -k_d A(t).
\]

(S1)

Here, A is the probability that a molecule is in the association state (R or C in this study) and \(k_d\) is the reaction rate constant for dissociation. Because in single-fluorescent-molecule imaging experiments, the fluorophore is photobleached in parallel with the dissociation, the rate equation S1 should be modified to:

\[
\frac{dA}{dt} = -(k_d + k_b) A(t)
\]

(S2)

for visible molecules. Here, photobleaching is assumed to occur with a rate constant \(k_b\), independently of the dissociation. Now, A is the probability that a molecule is in the observable (fluorescent) association state. In the experiments, all the fluorescent molecules were in the A state at \(t = 0\), when the molecule appeared on the membrane. Solving (S2) with this initial condition gives:

\[
A(t) = \exp\left\{- (k_d + k_b)t\right\}.
\]

(S3)

The on-time distributions for RBD and CRD (Fig. 4A and B in the text) reflect the probability density functions for the disappearance of the observable association states, which include the true dissociation and photobleaching. Then, the function \(F(t)\), which describes the on-time distribution, is:

\[
F(t) = (k_d + k_b) A(t) = (k_d + k_b) \exp\left\{- (k_d + k_b)t\right\}.
\]

(S4)

The value for \(k_d + k_b\) can be estimated by least-squares fitting the on-time distribution to \(F(t)\). The value for \(k_b\) was determined on the plasma membrane of cells fixed with cold methanol to prevent the dissociation of the GFP-tagged molecules from the membrane (Hibino et al., 2003). \(k_b = 0.09 \text{ s}^{-1}\) under our experimental conditions. The best-fit values were \(k_d = 3.70 \text{ s}^{-1}\) \((R^2 = 0.997)\) for GFP–RBD and 2.29 \text{ s}^{-1}\ \((R^2 = 0.986)\) for CRD–GFP. These results are almost the same as the results for global fitting in the
Dissociation kinetics for RBDCRD

The coupled differential equations for the reaction Scheme C (in text) are:

\[
\frac{dRC}{dt} = -(k_1 + k_b)RC(t) + k_{1r}C(t),
\]

\[
\frac{dC}{dt} = k_1RC(t) - (k_{1r} + k_2 + k_b)C(t).
\]  (S5)

Here, RC is the probability that a molecule is in the association state with RAS using both RBD and CRD. \(k_{1r}\) is the first-order association rate constant of RBD from the association state C. The initial condition is \(RC(0) = 1\) and \(C(0) = R(0) = 0\) (Hibino et al., 2009). The function that describes the on-time distribution of RBDCRD–GFP (Fig. 4C in text) is:

\[
G(t) = k_2C(t) + k_b\{RC(t) + C(t)\}.
\]  (S6)

The reaction rate constant \(k_{1r}\) was determined by fitting the function S6 to the on-time distribution of RBDCRD (Fig. 4C in text), obtained by solving S5 numerically using Ode45 solver in MATLAB (MathWorks). In this fitting, the values for \(k_1\) (3.70 s\(^{-1}\)) and \(k_2\) (2.29 s\(^{-1}\)) were fixed to those derived from fittings to the on-time distributions of RBD and CRD, respectively. \(k_b\) was 0.22 s\(^{-1}\) under the experimental condition for RBDCRD. The best-fit value for \(k_{1r}\) was 0.91 s\(^{-1}\) (\(R^2 = 0.972\)), similar to the result for the global fitting described in the next section.

A more general model for the dissociation of RBDCRD includes both R and C states as dissociation intermediates (Supplement Fig. 1A). Fitting to the on-time distribution of RBDCRD using this scheme, with \(k_1 = 3.70\) s\(^{-1}\) and \(k_2 = 2.29\) s\(^{-1}\), returned the best-fit values of \(k_{1r} = 1.74\) s\(^{-1}\) and \(k_{2r} = 630\) s\(^{-1}\) (\(R^2 = 0.962\)). The large association rate constant (\(k_{2r}\)) for the reassociation of CRD suggests that the R state is negligible as a dissociation intermediate.

Global fitting of the on-times of RBD, CRD, and RBDCRD

On-time distributions for RBD, CRD, and RBDCRD (Fig. 4 in text) were simultaneously analyzed by fitting the functions S4 and S6 to determine the best-fit values for the common rate constants \(k_1\), \(k_{1r}\), and \(k_2\) by solving equations S2 and S5 numerically using Ode45 solver in MATLAB. The nonlinear least-squares fitting was performed with the lsqcurvefit function in MATLAB. The best-fit values were \(k_1 = 3.57\) s\(^{-1}\), \(k_{1r} = 1.04\) s\(^{-1}\), and \(k_2 = 2.15\) s\(^{-1}\). \(R^2\) for the results of fitting were 0.997, 0.985, and 0.968 for RBD, CRD, and RBDCRD, respectively. The fitting curves are shown in Fig. 4 in the text. The best-fit values from the global fitting were used as the reaction parameters in the text and for the following analysis.
Dissociation kinetics of RAF

The coupled differential equations for the reaction scheme D (Fig. 5 in text) are:

\[
\frac{dRC}{dt} = -(k_1 + k_{RC} + k_b)RC(t) + k_{rl}C(t) + k_3RCX(t),
\]

\[
\frac{dC}{dt} = k_1RC(t) - (k_{rl} + k_2 + k_c + k_b)C(t) + k_3CX(t),
\]

\[
\frac{dRCX}{dt} = k_{RC}RC(t) - (k_1 + k_3 + k_4 + k_b)RCX(t) + k_{rl}CX(t),
\]

\[
\frac{dCX}{dt} = k_{RC}C(t) + k_1RCX(t) - (k_{rl} + k_2 + k_3 + k_4 + k_b)CX(t) + k_{XC}X(t),
\]

\[
\frac{dX}{dt} = k_2CX(t) - (k_{XC} + k_3 + k_4 + k_b)X(t),
\]

\[
\frac{dRC_p}{dt} = k_4RCX(t) - (k_1 + k_b)RC_p(t) + k_{rl}C_p(t),
\]

\[
\frac{dC_p}{dt} = k_4CX(t) + k_1RC_p(t) - (k_{rl} + k_2 + k_b)C_p(t).
\]

(S7)

Here, RCX and CX are the probabilities that a RAF molecule is simultaneously associated with RAS and its kinase using both RBD and CRD, or only CRD for association with RAS, respectively. X is the state in which the complex between RAF and the kinase on the plasma membrane is dissociated from RAS. RC_p and C_p are the phosphorylated forms of the RC and C states, respectively. k_{RC} and k_c are the first-order association rate constants between CAD and the kinase from the RC state and C state, respectively. k_3 is the dissociation rate constant of CAD from the kinase. k_4 is the catalytic rate constant of the kinase. k_{XC} is the first-order association rate constant between CRD and RAS from the X state.

The on-time distribution of RAF (Fig. 4D in text) is:

\[
H(t) = k_2C(t) + (k_3 + k_4)X(t) + k_2C_p(t)
\]

\[
+ k_b \{RC(t) + C(t) + RCX(t) + CX(t) + X(t) + RC_p(t) + C_p(t)\}.
\]

(S8)

The initial condition is 1 for RC(0) and 0 for all the other states (Hibino et al., 2009).

Dissociation kinetics of CRDCAD

The coupled differential equations for the reaction scheme E (Fig. 5 in text) are:

\[
\frac{dC}{dt} = -(k_2 + k_{RC} + k_b)C(t) + k_3CX(t),
\]

\[
\frac{dCX}{dt} = k_{RC}C(t) - (k_2 + k_3 + k_4 + k_b)CX(t) + k_{XC}X(t),
\]
The function that describes the distribution is:

\[
\frac{dX}{dt} = k_2 CX(t) - (k_{XC} + k_3 + k_4 + k_b)X(t),
\]

\[
\frac{dC_p}{dt} = k_4 CX(t) - (k_2 + k_b)C_p(t).
\]

(S9)

The initial condition \(C(0) = 1\) and \(CX(0) = X(0) = C_p(0) = 0\) was adopted for fitting because the association rate of CAD–GFP in the cytoplasm with the plasma membrane was as low as the rate of the nonspecific association of GFP, suggesting that most of the initial association state of CRDCAD was not the X state but the C state.

**Global fitting of the on-time distributions of RAF and CRDCAD**

The on-time distributions for RAF and CRDCAD (Fig. 4D and E in text) were simultaneously analyzed by fitting the functions S8 and S10, respectively, to determine the best-fit values for the common rate constants \(k_{RC}, k_C, k_3, k_4\), and \(k_{XC}\) by numerically solving equations S7 and S9, respectively, using Ode45 solver in MATLAB. In this fitting, \(k_1, k_{1r},\) and \(k_2\) were fixed as the values determined from the kinetics of RBD, CRD, and RBDCRD in the previous sections. Nonlinear least-squares fitting was performed with the lsqcurvefit function in MATLAB. The best-fit values were \(k_{RC} = 46.80\) s\(^{-1}\), \(k_C = 0.56\) s\(^{-1}\), \(k_3 < 10^{-4}\) s\(^{-1}\), \(k_4 = 0.49\) s\(^{-1}\), and \(k_{XC} = 5.30\) s\(^{-1}\). R\(^2\) for the results of fitting were 0.952 and 0.972 for RAF and CRDCAD, respectively. The fitting curves are shown in Fig. 4 in the text.

**Parameter dependence**

We examined how changes in the parameter values affected the on-time distributions and phosphorylation efficiencies of RAF and CRDCAD (Supplement Figure 2). The on-time distributions \((\tau_{1/2})\) were sensitive to the parameters for the interaction between RAS and RAF \((k_1, k_{1r},\) and \(k_2\)). The dissociation rate constant between RAF and the kinase \((k_3)\) must be 0.1 s\(^{-1}\) or smaller to reproduce the experimentally determined on-time distributions. The first-order association rate constant between the RC state and the kinase \((k_{RC})\) must be of the same order of magnitude as or larger than the best-fit value. The on-time distributions were also sensitive to the values of \(k_4\) and \(k_C\), but relatively insensitive to the value of \(k_{XC}\).

Based on the reaction scheme using the best-fit values of the parameters (Fig. 6A in text), the probability of each association state of RAF on the membrane as a function of time after its association with RASGTP (Fig. 6B in text) was simulated. The simulation suggested a high phosphorylation efficiency (95%) for the RAF molecules once they have associated with RASGTP. This suggestion seems robust because there was a clear reduction in the phosphorylation efficiency only with parameter values
that changed the on-time distribution significantly from those observed in the experiments.

**Steady-state flow of the RAF activation network**

Consider the reaction network of the RCX, CX, and X states (Fig. 6A in text). Because the reaction path C → CX can be neglected (Supplement Fig. 4), practically all RAF molecules flow into the network from the RCX state and flow out through one of the three states. Therefore, the changes in the probabilities of each state per unit time are:

\[
\begin{align*}
\Delta \text{RCX} &= F_{\text{in}} - (k_1 + k_4) \text{RCX} + k_{1r} \text{CX}, \\
\Delta \text{CX} &= k_1 \text{RCX} - (k_{1r} + k_2 + k_4) \text{CX} + k_{XC} \text{X}, \\
\Delta \text{X} &= k_2 \text{CX} - (k_4 + k_{XC}) \text{X}, \\
F_{\text{out}} &= k_4 (\text{RCX} + \text{CX} + \text{X}).
\end{align*}
\]  

(S11)

Here, \(F_{\text{in}}\) and \(F_{\text{out}}\) are the inlet and outlet of RAF molecules into this network, respectively. At the steady state, \(\Delta \text{RCX} = \Delta \text{CX} = \Delta \text{X} = 0\) and \(F_{\text{in}} = F_{\text{out}}\). Solving S11 under these conditions with the best fit values for the rate constants, the probabilities of the three states were calculated to be \(\text{RCX}:\text{CX}:\text{X} = 0.26:0.54:0.20\). The flows between the states and to the outlet can be calculated from these probabilities and the reaction rates. Because the fitting of on-times was relatively insensitive to the \(k_{XC}\) value (Supplement Fig. 2), similar calculations were performed using a \(k_{XC}\) value 10 times smaller (0.53 s\(^{-1}\)) or larger (53 s\(^{-1}\)) than the best-fit value (5.3 s\(^{-1}\)). The steady-state probabilities were \(\text{RCX}:\text{CX}:\text{X} = 0.19:0.26:0.55\) or \(0.29:0.68:0.03\), respectively.

**References in the Supplement**


**Supplement Figure 1: General model for the sequential dissociation of RBDCRD**

(A) A general scheme for the sequential dissociation of RBD and CRD in the RBDCRD fragment. The numbers indicate the best-fit values of the reaction rate constants. (B) The results of fitting to the on-time distribution.

**Supplement Figure 2: Parameter dependency**

The half-lives of the on-time (\(\tau_{1/2}\)) and the phosphorylation efficiency (\(\phi_p\)) were examined by changing the value of each kinetic parameter in simulation. The differences from the standard values for \(\tau_{1/2}\) and \(\phi_p\) under the best-fit parameter values were plotted. All parameters, except \(k_3\), were made smaller (white bars) or larger (gray bars) than the best-fit values by a factor of three. White dotted bars for \(k_{RC}\) show the results when a value for \(k_{RC}\) (4.7 s\(^{-1}\)) that was 10 times smaller was used. For \(k_3\), the results of the
calculation when $k_3 = 0.1$ or $1.0$ s$^{-1}$ are shown.

**Supplement Figure 3: Simulations of dissociation time course of RAF**

(A) Accumulation of the dissociation state ($\phi$) with time after association with RASGTP. Simulations were performed for RBD (blue) and RBDCRD (magenta). CRD has an effect to elongate the on-time. (B) Dissociation time course between RASGTP and RAF on the plasma membrane in the simulations with (blue) and without (magenta) the X state. RASGTP dissociates from RAF faster in the simulation with the X state.

**Supplement Figure 4: Effects of the reduction of minor reaction paths and states**

The on-time distributions (A) and phosphorylation time courses (B) were simulated using reaction networks in which the minor reaction paths and/or kinetic states were reduced (C). Lines 1–6 in (A) and (B) indicate the results of simulations using reaction schemes 1–6 shown in C, respectively. Reaction scheme 1 is the original. Reduction of the reaction path from the C state to the RC or the CX state (Scheme 2, 3) does not effectively affect to the kinetics. The effects of the additional reduction of the X state (Scheme 4) on the kinetics of RAF dissociation from the plasma membrane and its phosphorylation were not large, although it significantly changed the interaction between RAS and RAF (Supplement Fig. 3B).
A

RC \xrightarrow{k_1 = 3.7} C \xleftarrow{k_{tr} = 1.7}

\begin{align*}
k_2 &= 2.3 \\
k_2 &= 630 \\
k_1 &= 3.7 \\
k_2 &= 2.3
\end{align*}

R \rightarrow \phi

B

Supplement Figure 1. Hibino et al.
Supplement Figure 2. Hibino et al.
Supplement Figure 3. Hibino et al.
Supplement Figure 4. Hibino et al.