SI Appendix

Liu *et al.*

Chart 1A

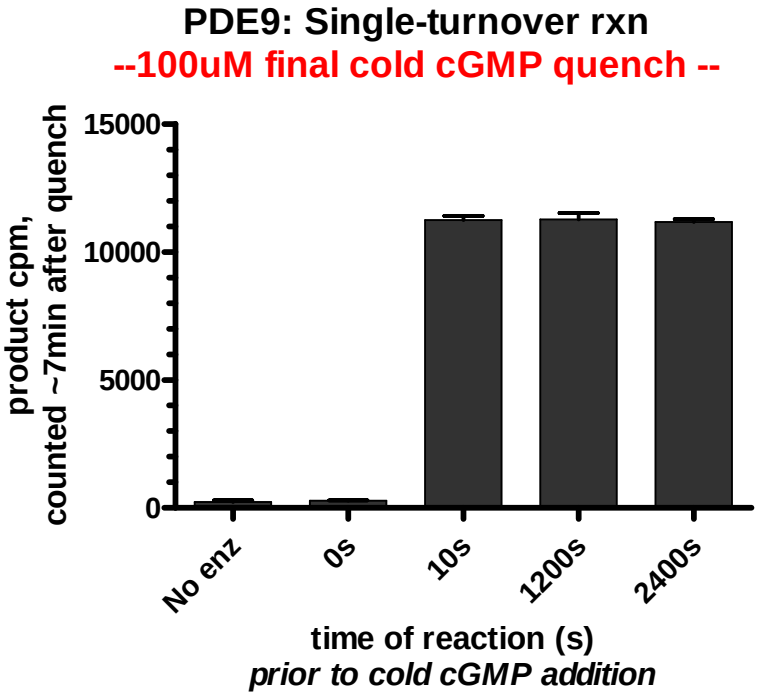


Chart 1B

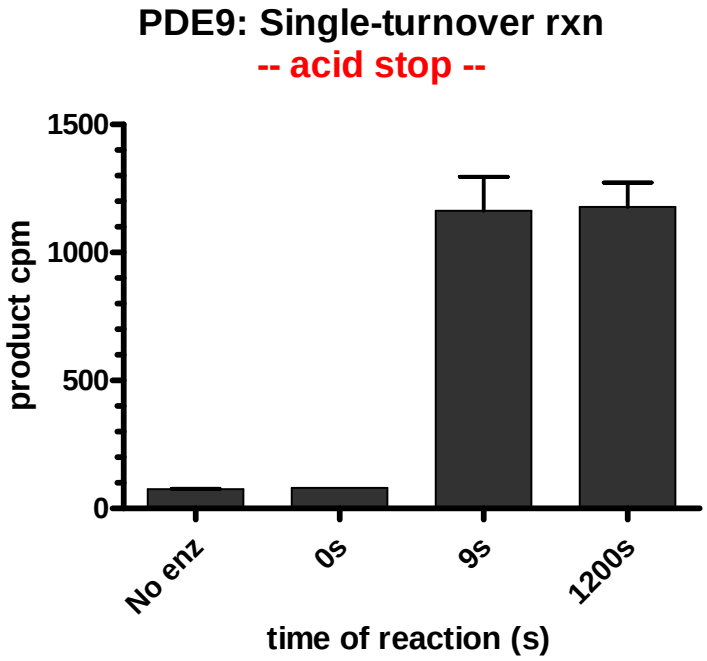
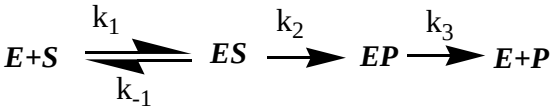


Chart 1. Time courses of product generation of single turn over reactions. The reactions were quenched at different reaction times by adding unlabelled cGMP (final concentration 100 μM, 1A), or 4N HCl (1B). The final concentration of [^{3}H]cGMP used was 20nM, and the final concentration of PDE9 was 120 nM or 240 nM for unlabelled cGMP or 4N HCl quenched reactions respectively. Time 0 readings, obtained from reactions with enzyme and quenching agents added at the same time, are 282±5 cpm (measured at 7 minutes after quenching) and 80±2 cpm for unlabelled cGMP and 4N HCl quenched reactions respectively. For reactions quenched by unlabelled cGMP, time 0 readings slowly drifted upward at the rate of a normal steady state reaction, but only reached 2088±18 cpm overnight due to enzyme inactivation by Zn^{2+} after 1 hour (the

SPA beads contain 18 mM Zn^{2+}). To minimize the continuous hydrolysis of cGMP in time 0 reactions quenched by unlabelled cGMP, it is necessary to conduct experiments under minimum light condition and to centrifuge the readout plates briefly at low speed to achieve readout within 10 minutes. Within experimental errors, all the non-time 0 readings (from 8 seconds up to 40 minutes) are essentially the same (12,236±167 cpm, 1A; 1,074±116 cpm 1B). Practically, the product readings at 20 or 40 minutes can be considered as the maximum readings when all 20nM substrates are hydrolyzed. The background readings without enzyme are 227 ±50 cpm and 78±2 cpm for reactions quenched by unlabelled cGMP and 4N HCl respectively.



Scheme 1. PDE9 hydrolytic reaction. E, S, ES, EP stand for enzyme, substrate, enzyme*substrate complex and enzyme*product respectively. [*E*], [*S*], [*ES*] and [*EP*] stand for their concentrations. k_1 and k_{-1} are rates of *ES* formation and dissociation, k_2 is the rate of substrate hydrolysis, and K_3 is the dissociation rate of *EP*, respectively. Because the large hydrolysis energy of the cyclic phosphate bond (7-11 Kcal/mol) and the observed lack of product inhibition, *ES->EP* and *EP->E+P* are treated irreversible.

Scheme 1 is used for kinetic analysis of PDE9 hydrolytic reaction. Applying the steady-state approximations (28), equations for [*ES*] and [*EP*] at the kinetic steady state can be obtained:

$$[ES] = \frac{\frac{k_3}{k_2 + k_3} * [E]_0 * [S]}{\frac{(k_{-1} + k_2) * k_3}{k_1 * (k_2 + k_3)} + [S]} \qquad (1)$$

$$[EP] = \frac{k_2}{k_3} * [ES] \qquad (2)$$

Under steady-state, $[S] >> [E]_0$, where $[E]_0$ stands for the overall enzyme concentration. Applying equations (1) and (2), equations for steady-state reaction kinetics are obtained.

$$v = k_3 * [EP] = k_2 * [ES]$$

$$\frac{\frac{k_2 * k_3}{k_2 + k_3} * [E]_0 * [S]}{\frac{(k_{-1} + k_2) * k_3}{k_1 * (k_2 + k_3)} + [S]} \qquad (3)$$

$$= \frac{kcat * [E]_0 * [S]}{[S] + k_m}$$

$$km \equiv \frac{(k_{-1} + k_2) * k_3}{k_1 * (k_2 + k_3)} \qquad (4)$$

$$kcat \equiv \frac{k_2 * k_3}{k_2 + k_3} \qquad (5)$$

$$\frac{kcat}{km} \equiv \frac{k_1 * k_2}{k_{-1} + k_2} \qquad (6)$$

Equations (4), (5) and (6) link the microscopic kinetic rate constants to the apparent steady-state reaction parameters. *kcat*, *km* and *kcat/km* are experimentally determined to be 0.02 S^{-1}, 80 nM and 2.5×10^5 $M^{-1}s^{-1}$ respectively.

In a single turn over reaction (Chart 1) quenched with unlabelled cGMP at reaction time t, the amount of 5'-[^{3}H]GMP detected is the result of cGMP hydrolysis prior to qhenching plus the amount of cGMP bound in the Es complex and subsequently hydrolyzed. The huge excess of the unlabelled cGMP used in quenching changes the reaction from single turnover ([E] > [S]) to multiple cycle ([S] >> [E]) by diluting the

residual unbound [^{3}H]cGMP or [^{3}H]cGMP dissociating from the [ES] complex available for hydrolysis by more than 5000 folds. Thus, the amounts of 5'-[^{3}H]GMP generated from any unbound as well as dissociated [^{3}H]cGMP after quenching are insignificant. Thus, only the continuous hydrolysis of pre-formed *ES* complex contributes to the labeled product after quenching. The following calculations, based on the single turn over reactions quenched by unlabelled cGMP and the steady state kinetics parameters described above, convincingly indicate that substrate binding, dissociate and hydrolysis are relative fast, and the rate limiting step of the reaction is the product release.

Under single turn over reaction condition ($[E]_0 > [S]_0$, $[S]_0$ stands for substrate concentration at time 0), the lower limit of the free substrate concentration, $[S]_t$, at time t can is given by equation (7):

$$[S]_t=[S]_0*e^{(-k_1*[E]_0*t)} \qquad (7)$$

Equation (7) omits *ES* dissociation and the decrease in the concentration of the free enzyme available for substrate binding. In other word, for any given reaction time t, to reach the same concentration of free substrate concentration $[S]_t$ requires k_1 larger than the value given by equation (7). Similarly, the upper limit of the concentration of labeled product (both free and in the form of *EP*), $[P]_t$, generated at time *t* before quenching is given by equation (8):

$$[P]_t=[S]_0*[1-e^{(-k_2*t)}] \qquad (8)$$

Equation (8) omits the dissociation of *ES* and assumes that the association of *ES* is infinitely fast. For the same t and $[P]_t$, k_2 should be larger than the value given by this equation. After quenching at time t, the existing *ES* complexes dissociate at rate of k_{-1} and hydrolyze at the rate of k_2. Again, assuming all the remaining substrate exist in the form of *ES* complex, the upper limit of the labeled product generated by continuous hydrolysis of *ES* complex after quenching is given by equation (9):

$$[P]_{t,\,after} = \frac{k_2}{k_{-1} + k_2} * ([S]_0 - [P]_t) \quad (9)$$

From (9), the upper limit of total concentration of labeled product when the reaction is quenched at time t, $[P]_{t,total}$ is given by (10):

$$[P]_{t,\,total} = [P]_t + [P]_{t,\,after}$$
$$= \frac{[S]_0 * k_2}{k_{-1} + k_2} + \frac{k_{-1} * [P]_t}{k_{-1} + k_2} \quad (10)$$

From our single turn reaction experiment, the amount of the radioactive product generated by 120 nM enzyme quenched at 9 seconds is more than 98% of $[S]_0$. From calculations based on our conservative equations (7), that requires $[S]_{9s}/[S]_0<0.02$, or $k_1*[E]_0>0.46$, or $k_1>3.6\text{x}10^6\ M^{-1}s^{-1}$. Based on equation (6), $k_{-1}>14.5*k_2$. Based on equation (10), for $[P]_{9s,total}>98\%[S]_0$, $[P]_{9s}>97.8\%[S]_0$. From equation (8), $k_2>0.43\ s^{-1}$. Based on equation (5), $k_2/k_3>21.3$, or $k_3<0.021\ s^{-1}$. These calculations show that cGMP

hydrolysis rate is relative fast, and the product dissociation is the rate limiting step of the reaction, determining the overall reaction rate at steady-state.

The calculations of acid quenched reactions are simpler. Since 4N HCl can immediately denature PDE9 and release all the bound guanine nucleotides (chart 1B), based on equation 8, the lower limit of k_2 can be deduced to be $>0.48\ s^{-1}$ from the time course of labeled product generation.