APPENDIX

A single mutation in a tunnel to the active site changes the mechanism and kinetics of product release in haloalkane dehalogenase LinB.

Lada Biedermannová, Zbyněk Prokop, Artur Gora, Eva Chovancová, Mihály Kovács Jiří Damborský and Rebecca C. Wade

Multiple turnover analysis. Simple binding kinetics cannot be applied for the second product of 1,2-dibromoethane conversion, since 2-bromoethanol is a substrate and undergoes reaction with both LinB WT and LinB L177W. Multiple turnover analysis was therefore performed for the mixing of 2-bromoethanol with LinB WT and LinB L177W using the stopped-flow fluorescence technique (Figure 7). In this experiment, the observed quench of the fluorescence signal is not associated with the formation of an enzyme-substrate complex (E.R-Br) during the binding step, but it is connected with the second step, nucleophilic attack on the substrate molecule, followed by formation of the alkyl-enzyme intermediate (E-R) and cleavage of the bromide ion (Br^-), which induces the fluorescence quench by interaction with the halide-stabilizing tryptophan (Scheme II) . The transient kinetic data show that only the steady-state fluorescence is observed upon mixing 2-bromoethanol with LinB WT (Figure 7A), indicating that all the steps involved in the formation of the enzyme complex with 2-bromoethanol and the subsequent formation of the alkyl-enzyme intermediate are fast processes, occurring within the dead time of the instrument (0.5-5 ms).

$$\mathrm{E + R\text{-}Br} \underset{}{\overset{K_s}{\rightleftharpoons}} \mathrm{E.R\text{-}Br} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{E\text{-}R.Br^-} \xrightarrow{k_3} \mathrm{E + R\text{-}OH + Br^-}$$

Scheme II

In LinB L177W, the relaxation of the fluorescence signal was significantly slower, showing an exponential kinetic phase upon mixing with 2-bromoethanol (Figure 7B). The observed rate constants for each concentration (k_{obs}) were obtained by fitting the fluorescence traces to single exponentials. The solution for k_{obs} in terms of rate constants (Equation 8) was derived from Scheme II.

$$k_{obs} = \frac{[S] \cdot k_2}{K_S + [S]} + k_{-2} + k_3$$

Equation 8

The dependence of k_{obs} on 2-bromoethanol concentration showed saturation, thus $k_2 = 40 \pm 10\ s^{-1}$ and $K_s = 230 \pm 110$ mM could be determined separately (Figure 7C). The formation of the Michaelis complex appears to be fast process described by a rapid equilibrium with dissociation constant K_s. The dependence of the fluorescence quench amplitudes on 2-bromoethanol concentration indicates that the L177W mutation significantly changes the mechanism of the 2-bromoethanol reaction kinetics (Figure 7D). While a part of sigmoid dependence was observed for LinB WT, indicating cooperative behaviour, a simple hyperbolic dependence indicating classical Michaelis-Menten kinetics with an equilibrium constant K_{eq} = 26 ± 3 mM was observed for LinB L177W.

The multiple turnover data indicate that both the binding and the release of 2-bromoethanol are fast

one-step processes reaching rapid equilibrium and that they cannot be rate determining for the conversion of 1,2-dibromoethane by either LinB WT or LinB L177W. The L177W mutation changed the reaction mechanism and the rate of the second kinetic step during the 2-bromoethanol conversion, but did not affect the kinetics of 2-bromoethanol release after the initial 1,2-dibromoethane dehalogenation.

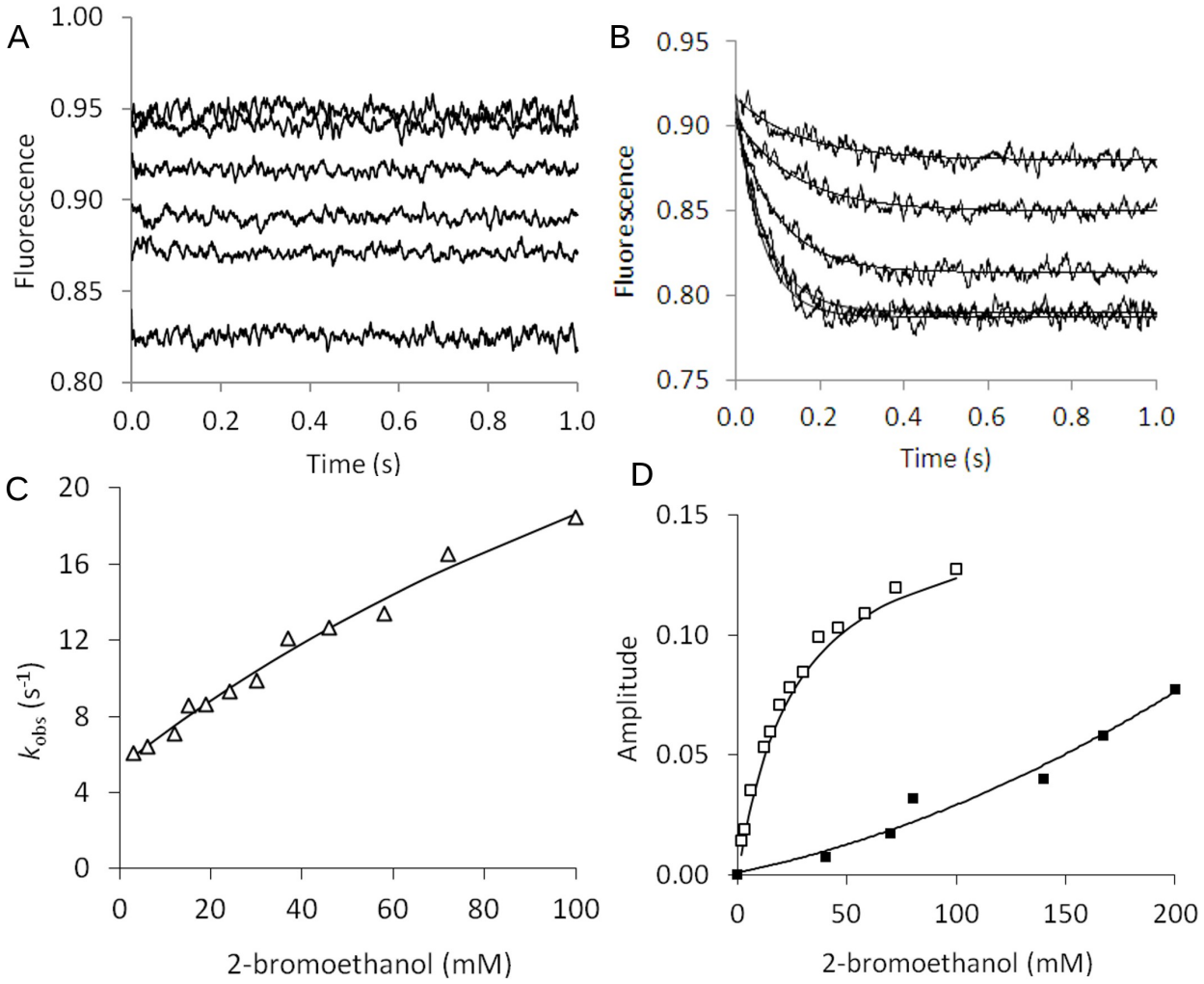

FIGURE 7. Stopped-flow analysis of the mixing of 2-bromoethanol with LinB WT and LinB L177W. Fluorescence traces obtained upon mixing 15 μM LinB WT with 2-bromoethanol to a final concentration of 0 - 200 mM (**A**). Fluorescence traces obtained upon mixing 30 μM LinB L177W with 2-bromoethanol to a final concentration of 0 - 100 mM (**B**), solid lines represent the best fit to the data by using a single exponential equation. The dependence of the observed rate constants on the concentration of 2-bromoethanol (**C**), the solid line represents the best fits obtained by using Equation 8. The dependence of the fluorescence quench amplitude on the concentration of 2-bromoethanol (**D**) in reaction with LinB WT (black squares) and LinB L177W (open squares). Solid lines represent the best fit by using: (i) the hyperbolic equation $A = A_{lim} . [S] / K_{eq} + [S]$ for the LinB L177W data, in which A is the amplitude of fluorescence quench, A_{lim} is the limiting (maximum) amplitude, K_{eq} is the equilibrium constant and S is the concentration of 2-bromoethanol, and (ii) the Hill equation $A = A_{lim} . [S]^n / K_{eq}^n + [S]^n$ for LinB WT in which n is the Hill coefficient. A unique solution for the individual parameters of the Hill equation cannot be obtained.