Supporting Information

for

Experimental Evidence for Hydrogen Tunneling when the Isotopic Arrhenius Prefactor (A_H/A_D) is Unity

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Contents

Mutagenesis, Expression and Purification of the SLO-1 double mutant

The SLO-1 double mutant Leu^{546}Ala/Ile^{553}Ala plasmid was prepared following the Stratagene QuickChange II protocol[1] starting from the WT SLO-1 plasmid with mutation at position 546 followed by mutation at position 553. Site-directed mutagenesis at position 546 was performed using the forward primer 5′–CGC ACT TGC CAG GCA ATC TGC AAT TAA TGC TAA TGG C–3′ and the reverse primer 5′–GCC ATT AGC ATT AAT TGC AGA TTG CCT GGC AAG TGC G–3′. The mutant Leu^{546}Ala plasmid was isolated and further subjected to mutation at position 553 utilizing the forward primer 5′– CTA ATG GCA TAG CAG AGA CAA CCT TTT TGC–3 and the reverse primer 5′–GCA AAA AGG TTG TCT CTG CTA TGC CAT TAG C–3. The Leu^{546}Ala single mutation and Leu^{546}Ala/Ile^{553}Ala double mutation were confirmed by sequencing the mutant plasmids utilizing three different primers targeting different regions of the gene; the beginning of the gene, a 500-bp region of the gene containing the mutation site(s) and a 500-bp region covering up to the end of the gene.[2]

The SLO-1 double mutant was expressed using the pT7-7 plasmid in *E. coli* and purified as described previously[3,4] with some minor modifications. The starting culture was diluted 300 fold and incubated at 37 ℃ with shaking until OD_{600} reached ~0.7. The temperature was then rapidly lowered to 15 ℃ and incubated with shaking for an additional 96 h. The cells were harvested and stored at -80 ℃ until purification.

Cell paste from 4.5 L (~20 g) was resuspended in ~ 80 mL lysis buffer [25 mM Tris-base (pH 7.5), 0.1 mM EDTA, 1X BugBuster (Novagen), 1250 units of benzonase (Novagen), ~0.5 mM aminoethylbenzenesulfonyl fluoride (AEBSF, Sigma), ~0.2 mg/mL of lysozyme (Sigma)]. The lysis reaction was allowed to proceed for ~40 minutes at RT followed by cooling at 4 ℃ for 20 minutes. The lysis mixture was centrifuged at ~ 20k rpm for 20 minutes to pellet the insoluble cellular debris.

Kinetics Measurements

Steady-state kinetics were performed on a Cary 50 spectrophotometer in the single wavelength mode. The reaction progress was monitored by following the generation of the product, 13-(*S*)-HPOD (ε_{234} = 23,600 M^{-1} cm^{-1}).[3] All assays were performed in 100 mM borate (pH 9.00) under ambient atmosphere in a constant temperature regulated by a water-jacketed cuvette holder as described previously with minor adjustments.[5] Kinetic assays were performed at 0.50-80 μM linoleic acid (LA). The UV kinetic profile shows a lag phase, which is longer for the 11,11-$^2H_2$-LA at lower concentration and at lower temperatures ($T \leq 30$ ℃). The lag phase gradually decreased on either increasing the substrate concentration or increasing the assay temperature and subsequently became much smaller for reactions performed at higher temperatures ($T \geq 45$ ℃) with higher concentrations of the substrate (above 20 μM LA). The lag phase is followed by a linear rate, which in turn, is followed by a steady decrease in the reaction rate as the substrate concentration is depleted. The initial rates were fitted to the Michaelis-Menten equation to obtain the kinetic parameters k_{cat} and k_{cat}/K_M.[6] The errors associated with each k_{cat} measurements were used to weight the exponential Arrhenius fit. The rate constants were corrected for the iron content of the mutant enzyme as determined by ICP (Perkin Elmer Optima 3000 DV), using standardized iron solutions.

All data presented herein were obtained under ambient atmosphere (20.8% O_2) and corrected to O_2 saturation as needed. As SLO-1 is a bisubstrate enzyme, the k_{cat} refers to the condition under which both LA and O_2 are saturating. A correction was applied for the Leu^{546}Ala/Ile^{553}Ala SLO double mutant to account for a small degree of subsaturating O_2 using the protio-substrate. As discussed in earlier studies [cf. (5)], the greatly reduced K_m for O_2 with the dideutero-substrate ensured saturating conditions at all temperatures investigated. The impact of subsaturating O_2 correction was assessed by comparing the apparent k_{cat} obtained under ambient atmosphere (approx 20.8% O_2) to that obtained under 100% O_2, and then correcting the ambient rates. The correction

factor at each temperature is given in Table S1. The apparent k_{cat} values under both ambient and 100% O_2 were obtained by monitoring the consumption of O_2 with a Clark-type electrode. The reactions were stirred and thermostated at respective temperatures. LA (80 μM) in borate buffer (pH 9.00) was used in kinetic measurements. The substrate was allowed to equilibrate for ca. 5 minutes at either ambient or 100% O_2, and the reactions were initiated by the addition of 2-4 μL of concentrated enzyme via a gastight syringe.

Table S1. Correction of empirical rate constants.

Temperature ℃	Correction Factor
15	1.025
20	1.055
25	1.195
30	1.116
35	1.053
40	1.088
45	1.089
50	1.022

Table S2. Empirical rate constants and isotope effects for the Leu^{546}Ala/Ile^{553}Ala mutant of SLO-1

T, ℃	k_{cat}(H), s^{-1}	k_{cat}(D), s^{-1}	K_M(H), μM	K_M(D), μM	$^{D}k_{cat}$	$^{D}(k_{cat}/K_M)$
15	1.39 (0.04)	0.0101 (0.0003)	34.7 (3.3)	13.1 (1.6)	137 (1)	51 (2)
20	1.62 (0.04)	0.0111 (0.0005)	39.4 (4.4)	5.8 (1.3)	146 (2)	22 (2)
25	2.15 (0.04)	0.0150 (0.0005)	36.5 (2.4)	3.9 (0.7)	143 (1)	15 (1)
30	2.21 (0.09)	0.0172 (0.0003)	21.6 (3.8)	4.3 (0.3)	128 (3)	25 (3)
35	2.16 (0.05)	0.0187 (0.0003)	12.2 (1.5)	2.4 (0.2)	115 (1)	23 (1)
40	2.67 (0.05)	0.0261 (0.0006)	16.9 (1.6)	4.4 (0.4)	102 (1)	27 (1)
45	2.73 (0.07)	0.0314 (0.0010)	10.1 (1.3)	4.2 (0.6)	87 (1)	35 (1)
50	2.95 (0.04)	0.0321 (0.0009)	9.8 (0.7)	5.8 (0.8)	91 (1)	54 (3)

References

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(6) *KaleidaGraph*; Synergy Software, Reading, PA.