SUPPORTING INFORMATION

Structure-Reactivity Effects on Intrinsic Primary Kinetic Isotope Effects for Hydride Transfer Catalyzed by Glycerol-3-Phosphate Dehydrogenase.

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EXPERIMENTAL

Materials. DEAE-Sepharose and Sephacryl S-200 were purchased from GE Healthcare. Dowex 50WX4-200R (H^+ form), nicotinamide adenine dinucleotide reduced (NADH, disodium salt), NAD^+ (free acid), dihydroxyacetone phosphate hemimagnesium salt, glycolaldehyde dimer, triethylamine (≥ 99.5%), triethanolamine hydrochloride (≥ 99.5%), ampicillin, isopropyl β-D-thiogalactopyranoside (IPTG) and D,L-dithiothreitol (DTT) were purchased from Sigma-Aldrich. Protease inhibitor tablets (Complete®) and bovine serum albumin, fraction V (BSA) were purchased from Roche. Ammonium sulfate (enzyme grade), Tris base, sodium thiosulfate (anhydrous), sodium hydroxide (1.0 N) and hydrochloric acid (1.0 N) were purchased from Fisher. Sodium phosphite (dibasic, pentahydrate) and sodium phosphate (dibasic, anhydrous) were purchased from Fluka. The water content of sodium phosphite was reduced to $Na_2HPO_3 \bullet 0.4H_2O$ as previously described.[1] Sodium sulfate (anhydrous) was purchased from Mallinckrodt Chemicals. Sodium fluorophosphate (anhydrous) was a generous gift from Dr. Andrew Murkin. Deuterium oxide (99.9% D) and [1-^{2}H]-D-glucose (98%) were purchased from

Cambridge Isotope Laboratories. Water was purified using Milli-Q Academic purification system. All other chemicals were reagent grade or better and were used without further purification.

Solutions and Substrates. Solution pH was determined at 25 °C using an Orion Model 720A pH equipped with a Radiometer pHC4006-9 combination electrode that was standardized at pH 4.00, 7.00 and 10.00 at 25 °C. Triethanolamine and Tris-HCl buffers were prepared by addition of 1M NaOH or 1M HCl and solid NaCl to give the desired pH and final ionic strength. A stock solution of triethylammonium bicarbonate (TEAB) buffer at pH 7.5 was prepared by saturating 1 M triethylamine solution with carbon dioxide at 4 °C for 3-4 hours until the desired pH was reached. The resulting buffered solution was then stored at 4 °C until further use. A stock solution of sodium bicarbonate was prepared by dissolving the salt in water and adjusting the pH to 7.5.

Stock solutions of NADH and *4S*-[4-$^{2}H_{1}$]-NADH (NADD), prepared separately by dissolving the disodium form of the coenzyme in water, were stored at 4 °C. The concentration of NADH and NADD in aqueous solutions were determined from the absorbance at 340 nm using a value of $\varepsilon = 6220\ M^{-1}\ cm^{-1}$. Stock solutions of DHAP were prepared, starting with the hemimagnesium salt, and stirring over Dowex 50WX4-200R (H^{+} form) in water for 5-10 minutes at 25 °C to give the free acid. The Dowex was removed by filtration, and the pH of the resulting solution was adjusted from ~2.0 to 7.5 using 1 M NaOH, and then stored at -20 °C. The concentration of DHAP was determined spectrophotometrically at 340 nm, as the concentration of NADH oxidized upon quantitative conversion to glycerol 3-phosphate catalyzed by GPDH.

Stock solutions of glycolaldehyde dimer (200 mM monomer) were prepared by dissolving the dimer in water and waiting quantitative breakdown of the dimer to the monomer

over a period of three days at room temperature.[1] Stock solutions of sodium salts of oxydianions (phosphite, fluorophosphate, phosphate, sulfate and thiosulfate) were prepared by dissolving the salt in water and adjusting the pH to 7.5 with 1N HCl or 1N NaOH. At pH 7.5, the dianion:monoanion ratio for sodium phosphite and sodium phosphate were 93:7 and 83:17, respectively.

Stock solutions of *hl*GPDH (7 or 14 mg/mL) were dialyzed exhaustively against 20 mM triethanolamine at 4 °C. Dilutions of the stock solutions were then prepared in 20mM triethanolamine buffer (pH 7.5) that contains 5-10mM DTT and 0.1 mg/mL BSA. The enzyme concentration was determined by absorbance at 280 nm using the extinction coefficient of 18 450 $M^{-1}cm^{-1}$ and a subunit molecular mass of 37 500 Da.[2]

***4S*-[4-^{2}H$_1$]-NADH.** The disodium salt *4S*-[4-$^2H_1$]-NADH (NADD) was prepared by the following literature procedure, with minor modifications.[3] A 12 mL solution containing 50 mM $NaHCO_3$, 30 mM [1-^{2}H]-D-glucose and 15 mM (120 mg) NAD^+ was adjusted to pH 7.5. The reaction was initiated by the addition of 1 mg of glucose dehydrogenase (≥200 U/mg) to the buffered NAD^+ solution, and monitored at 260 and 340 nm, and judged to be complete after 5 hours, when the value of A_{340}/A_{260} reached a maximum. The solution was diluted to 15 mL and loaded onto a DEAE-Sephadex A25 column, previously equilibrated with 0.01 M triethylammonium bicarbonate. The column was eluted with a linear 1.0 L gradient of 0.01 – 0.50 M triethylammonium bicarbonate (10-500 mM, 1 L). Fractions with a ration A_{260}/A_{340} = 2.27 ± 0.10 were pooled, concentrated to 5 mL by rotary evaporation, and lyophilized to dryness. The triethylammonium salt of NADD was passed over a Dowex 50WX4-200R (Na^+ form) column at 4 °C to give the disodium salt solution, which was lyophilized to give a 67% yield of

NADD. A comparison of the NMR-spectra of NADD and known hydrogen labeled standard showed that the deuterium enrichment at the C-4 position was ≥99%.

Human Liver Glycerol-3-Phosphate Dehydrogenase (*hl*GPDH). The plasmid pDNR-dual donor vector containing the gene for wild-type human liver GPDH (*hl*GPDH) gene insert was purchased from the Harvard plasmid repository. The insert gene was subcloned into a bacterial expression vector pET-15b from Novagen and used for transformations of cells from *E. coli* strain *Bl 21*(DE3). These cells were then grown, and GPDH purified to homogeneity by following a published procedure.[4] The protein concentration was determined from the UV absorbance at 280 nm, using the extinction coefficient of 18 450 M^{-1} cm^{-1} calculated for a subunit molecular weight of 37500 Da using the ProtParam tool available on the ExPASy server.[5,6]

Enzyme Assays. All enzyme assays were conducted at 25 °C and an *I* of 0.12 (NaCl) in a final volume of 1 mL. Glycerol-3-phosphate dehydrogenase was assayed by monitoring the oxidation of NADH by DHAP. Initial velocities of the oxidation of NADH (≤ 5% reaction) either by the whole substrate DHAP or the truncated substrate glycolaldehyde (GA) were calculated from the change in absorbance at 340 nm using an ε of 6220 $M^{-1}cm^{-1}$. The standard assay mixture for GPDH activity contained 100 mM TEA (pH 7.5), 1 mM DHAP, 0.20 mM NADH, 0.1 mg/mL BSA, 50 μM DTT, and ca. 0.67 nM GPDH at an *I* of 0.12 (NaCl).

BSA was used in assays to determine the activity of GPDH activity for catalysis of reduction of DHAP, in order to control for absorption of enzyme onto the glass cuvettes. BSA was omitted from assays to determine GPDH activity for catalysis of reduction of GA, because absorption does not cause a significant change in the larger enzyme concentrations used for these assays.

***hl*GPDH-Catalyzed Reaction of DHAP.** Assay mixtures for the *hl*GPDH-catalyzed reduction of DHAP contained 20 mM TEA (pH 7.5), 0.1 mg/mL BSA, 50 μM DTT, 0.02–1 mM DHAP, up to 50 μM NADH and ca. 0.67 nM GPDH at an *I* of 0.12 (NaCl). Initial velocities for each assay were determined within 5–10 minutes. Isotope effects on the initial velocities for the *hl*GPDH-catalyzed reduction of DHAP obtained by running parallel kinetic assays using solutions that contained NADD instead of NADH. The kinetic parameters, including the primary deuterium kinetic isotope effects (1° DKIEs) were determined from the global fits of kinetic data to the appropriate kinetic scheme, using the fitting software provided by the Prism graphing program (GraphPad) and SigmaPlot 12.5 (Systat). The value of K_m for the reactive carbonyl form of DHAP was calculated from the observed K_m and a value of $f_{car} = 0.55$ for the fraction of DHAP present in the free carbonyl form.[7]

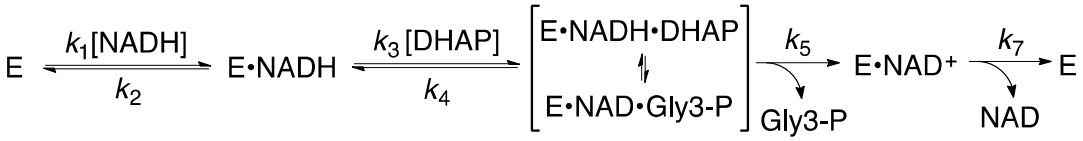
Unactivated and Dianion-Activated hlGPDH-Catalyzed Reactions of Glycolaldehyde. Assay mixtures for the *Hs*GPDH-catalyzed reduction of glycolaldehyde (GA) in the absence of phosphite contained 10 mM TEA (pH 7.5), 20–60 mM GA, 0.20 mM NADH, 45 μM *Hs*GPDH at *I* = 0.12 (NaCl). Assay mixtures for the *Hs*GPDH-catalyzed reduction of GA in the presence of dianions contained 10 mM TEA (pH 7.5), 2–60 mM GA, 0.20 mM NADH, a measured concentration of the dianion at *I* = 0.12 (NaCl) and the following enzyme concentrations: 0.4 μM *Hs*GPDH, HPO_3^{2-}; 0.2 μM *Hs*GPDH, FPO_3^{2-}; 4 μM *Hs*GPDH, SO_4^{2-}; 4 μM *Hs*GPDH, $HOPO_3^{2-}$; 8 μM *Hs*GPDH, $S_2O_3^{2-}$. The initial velocity of enzyme-catalyzed reduction of GA by NADH was determined by monitoring the decrease in absorbance at 340 nm over a period of 20 minutes for the dianion activated hydride transfer reactions, and a period of 120 minutes for the unactivated hydride transfer reactions. Isotope effects on the initial velocities for the *hl*GPDH-catalyzed reduction of GA in both the absence and presence of oxydianion

activators were obtained by running parallel kinetic assays using solutions that contained NADD instead of NADH. The kinetic parameters, including the primary deuterium kinetic isotope effects (1° DKIEs) were determined from the global fits of kinetic data to the appropriate kinetic scheme, using the fitting software provided by the Prism graphing program (GraphPad) and SigmaPlot 12.5 (Systat).

RESULTS.

Figure S1 in the Supporting Information shows Michaelis-Menten plots of v_{obs} against [NADH] or [NADD] for *hl*GPDH-catalyzed reduction of DHAP at 25 °C, pH 7.5 (100 mM TEA) and different fixed concentrations of DHAP. This Figure shows the nonlinear least squares fit of data to eq 2, derived for an ordered reaction mechanism, where NADH binds first followed by DHAP (Scheme 1).[8,9] The following equations connect these empirical parameters to the rate constants for the ordered steady-state mechanism shown in Scheme S1: $k_{cat} = k_5k_7/(k_5 + k_7)$, $K_a = k_5k_7/k_1(k_5 + k_7)$, $K_b = k_7(k_4 + k_5)/ k_3(k_5 + k_7)$, $K_{ia} = k_2/k_1$.[10] These data were fit to eq 1 to give the kinetic parameters reported in Table S1. These fits also gave values for E_X (1°DKIE - 1) that were used to calculate the primary deuterium isotope effects (1°DKIE) reported in Table S1.[8,9]

Scheme S1



$$\left(\frac{v_{obs}}{[E]_t}\right) = \left(\frac{k_{cat}[A][B]}{K_{ia}K_b(1+F_iE_{K_{ia}}) + K_b[A](1+F_iE_{V/Kb}) + K_a[B](1 + F_iE_{V/Ka}) + [A][B](1+F_iE_V)}\right) \quad (1)$$

Figures S2 – S5 show the dependence of $(v_{obs} - v_{un})/[E]$ (s^{-1}) on $[FPO_3^{2-}]$, $[SO_4^{2-}]$, $[HOPO_3^{2-}]$ and $[SSO_3^{2-}]$, respectively for dianion activated reduction of GA by saturating 0.2 mM NADH (top graph) or by 0.2 mm NADD (bottom graph) catalyzed by *hl*GPDH at pH 7.5 (10 mM TEA), 25 ˚C and $I = 0.12$ (NaCl). These Figures show the nonlinear least squares fit of the experimental data to eq 4, derived for Scheme 2 for the main text.[8,9] Table 1 from the main text reports the kinetic parameters and 1°DKIE obtained from the fit of these kinetic data.

Table S1. Kinetic Parameters and KIEs for the Wild-type *hl*GPDH-Catalyzed Reduction of DHAP.[a]

Kinetic Parameter (Scheme S1)[b,c]	
k_{cat} /s^{-1}	240 ± 10
K_a/μM	6.7 ± 0.3
(K_b)/μM	52 ± 3 [d]
K_{ia}/μM	7.0 ± 0.9 [d]
$k_{cat}/K_a/(M^{-1}s^{-1})$	(3.6 ± 0.2) x 10^7
$k_{cat}/K_b/(M^{-1}s^{-1})$	(4.6 ± 0.3) x 10^6
$^{D}k_{cat} = (k_{cat})_H/(k_{cat})_D$	1.5 ± 0.1
$^{D}K_{ia} = (K_{ia})_H/(K_{ia})_D$	1.0 ± 0.2
$^{D}(k_{cat}/K_a) = (k_{cat}/K_a)_H/(k_{cat}/K_a)_D$	1.2 ± 0.1
$^{D}(k_{cat}/K_b) = (k_{cat}/K_b)_H/(k_{cat}/K_b)_D$	1.5 ± 0.1

[a]Determined at pH 7.5 (20 mM TEA buffer), 25 °C, and I = 0.12 (NaCl). [b] These kinetic parameters were determined from the non linear least squares fit of data to eq 1 for a sequential bisubstrate reaction. The following equations connect these empirical parameters to the rate constants for the ordered steady-state mechanism (Scheme S1): $k_{cat} = k_5k_7/(k_5 + k_7)$, $K_a = k_5k_7/k_1(k_5 + k_7)$, $K_b = k_7(k_4 + k_5)/ k_3(k_5 + k_7)$, $K_{ia} = k_2/k_1$.[10] [c] The quoted uncertainties are standard deviations provided by the fitting program. [d] Reported for the free carbonyl form of DHAP which is 55% of the total substrate.

FIGURE LEGENDS

Figure S1. Dependence of v_{obs}/[E] on [NADH] or [NADD] for *hl*GPDH-catalyzed reduction of DHAP at 25 °C, pH 7.5 (20 mM TEA) and different fixed [DHAP], which are shown on the right-hand side of the top Figure. The solid lines show the non linear least squares fit of these data to eq 2 in the main text, which was derived for an ordered reaction mechanism where NADH binds first followed by DHAP.[8,9]

Figure S2. Dependence of ($v_{obs} - v_{un}$)/[E] (s^{-1}) on [GA] for FPO_3^{2-}-activated *hl*GPDH-catalyzed reduction of GA by 0.2 mM NADH or NADD at pH 7.5 (10 mM TEA), 25 °C and I = 0.12 (NaCl). The individual curves compare dianion activation of reactions of NADH or NADD at the fixed [GA] shown on the right-hand side of the top Figure. The solid lines show the nonlinear least squares fit of the experimental data to eq 4, derived for Scheme 2 in the main text.[8,9]

Figure S3. Dependence of ($v_{obs} - v_{un}$)/[E] (s^{-1}) on [GA] for SO_4^{2-}-activated *hl*GPDH-catalyzed reduction of GA by 0.2 mM NADH or NADD at pH 7.5 (10 mM TEA), 25 °C and I = 0.12 (NaCl). The individual curves compare dianion activation of reactions of NADH or NADD at the fixed [GA] shown on the right-hand side of the top Figure. The solid lines show the nonlinear least squares fit of the experimental data to eq 4, derived for Scheme 2 in the main text.[8,9]

Figure S4. Dependence of ($v_{obs} - v_{un}$)/[E] (s^{-1}) on [GA] for $HOPO_3^{2-}$-activated *hl*GPDH-catalyzed reduction of GA by 0.2 mM NADH or NADD at pH 7.5 (10 mM TEA), 25 °C and I = 0.12 (NaCl). The individual curves compare dianion activation of reactions of NADH or NADD at the fixed [GA] shown on the right-hand side of the top Figure. The solid lines show the

nonlinear least squares fit of the experimental data to eq 4, derived for Scheme 2 in the main text.[8,9]

Figure S5. Dependence of $(v_{obs} - v_{un})/[E]$ (s^{-1}) on [GA] for $S_2O_3^{2-}$-activated *hl*GPDH-catalyzed reduction of GA by 0.2 mM NADH or NADD at pH 7.5 (10 mM TEA), 25 °C and I = 0.12 (NaCl). The individual curves compare dianion activation of reactions of NADH or NADD at the fixed [GA] shown on the right-hand side of the top Figure. The solid lines show the nonlinear least squares fit of the experimental data to eq 4, derived for Scheme 2 in the main text.[8,9]

Figure S1
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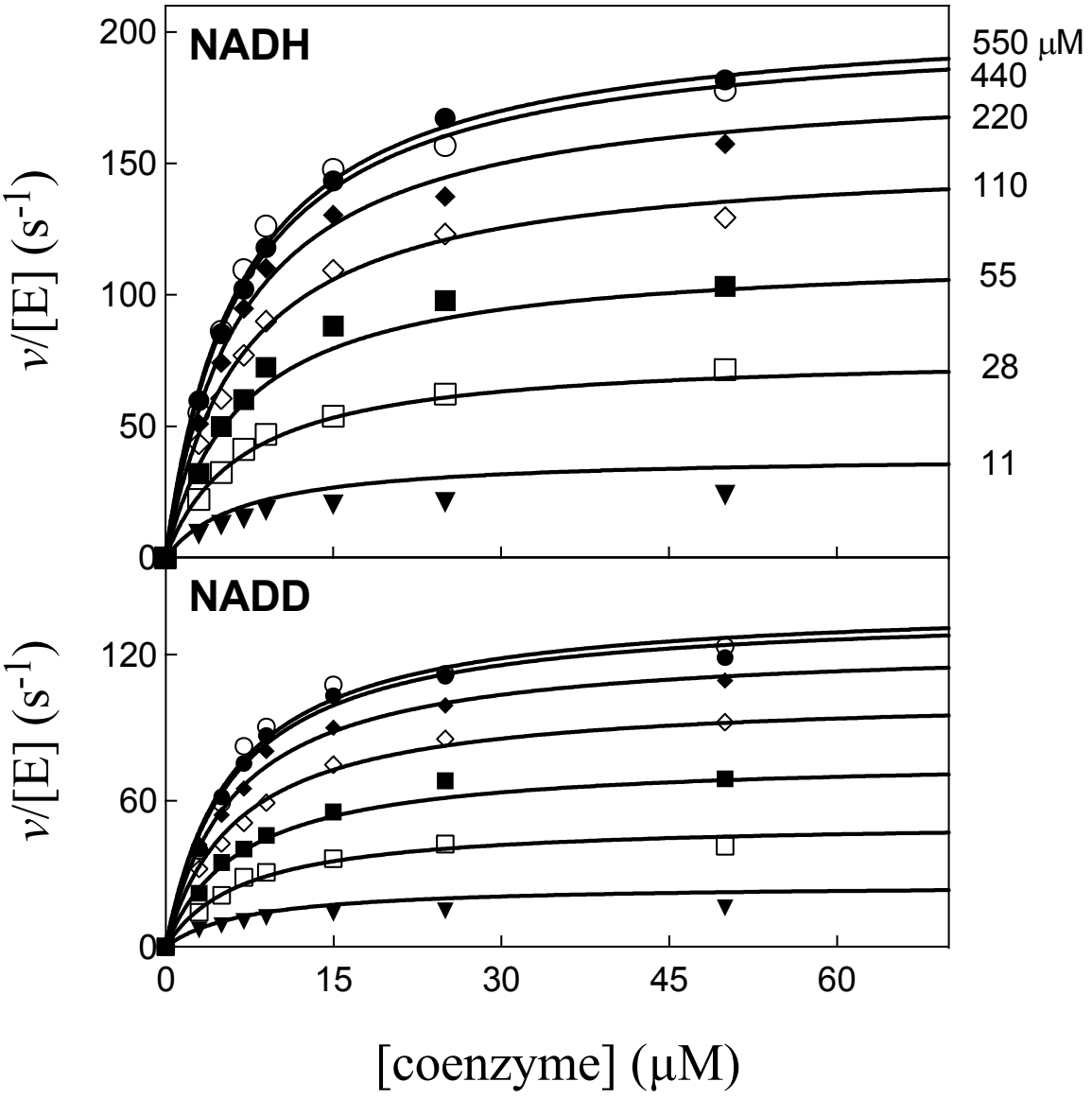

Figure S2
Reyes et. al

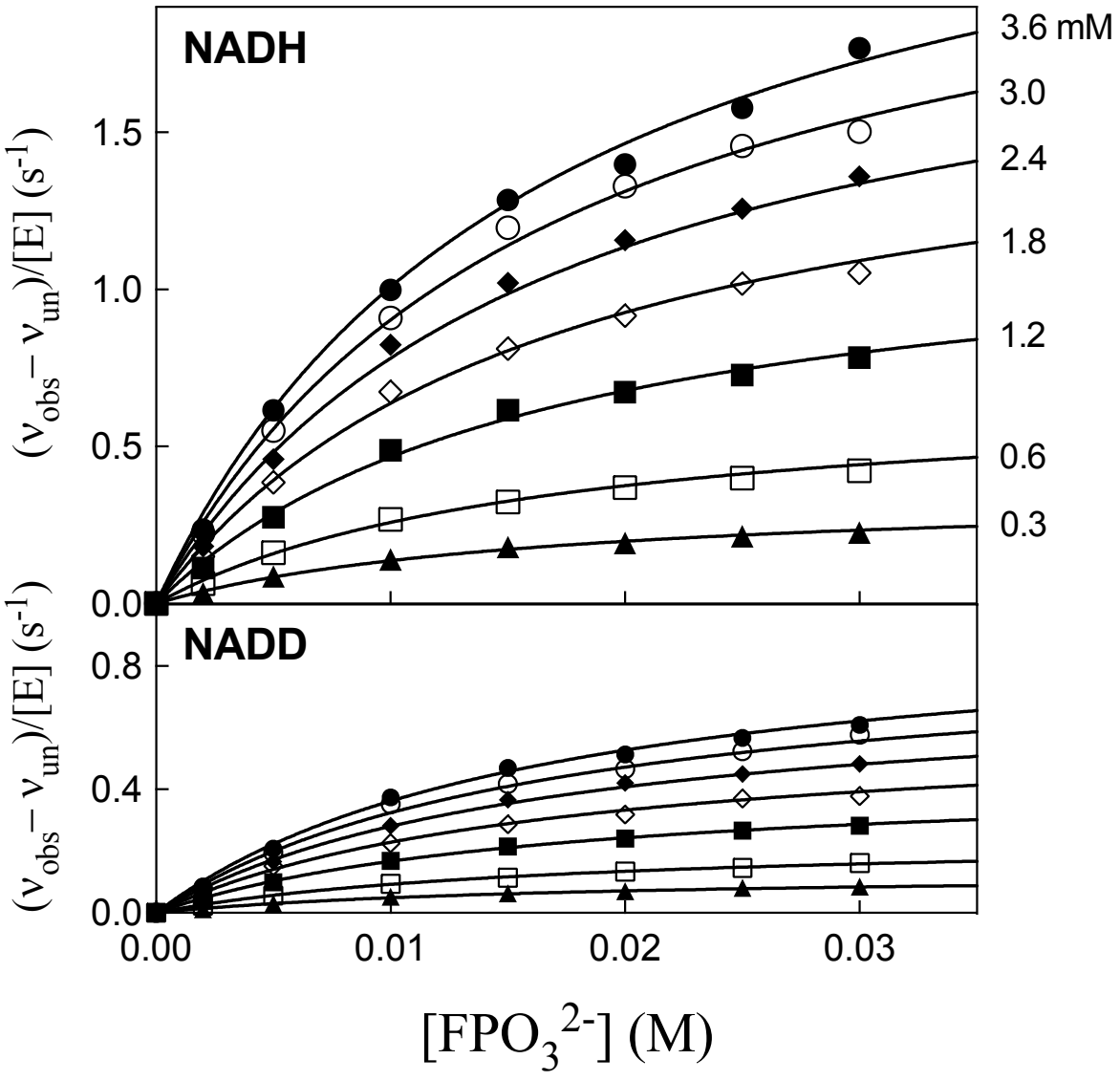

Figure S3
Reyes et. al

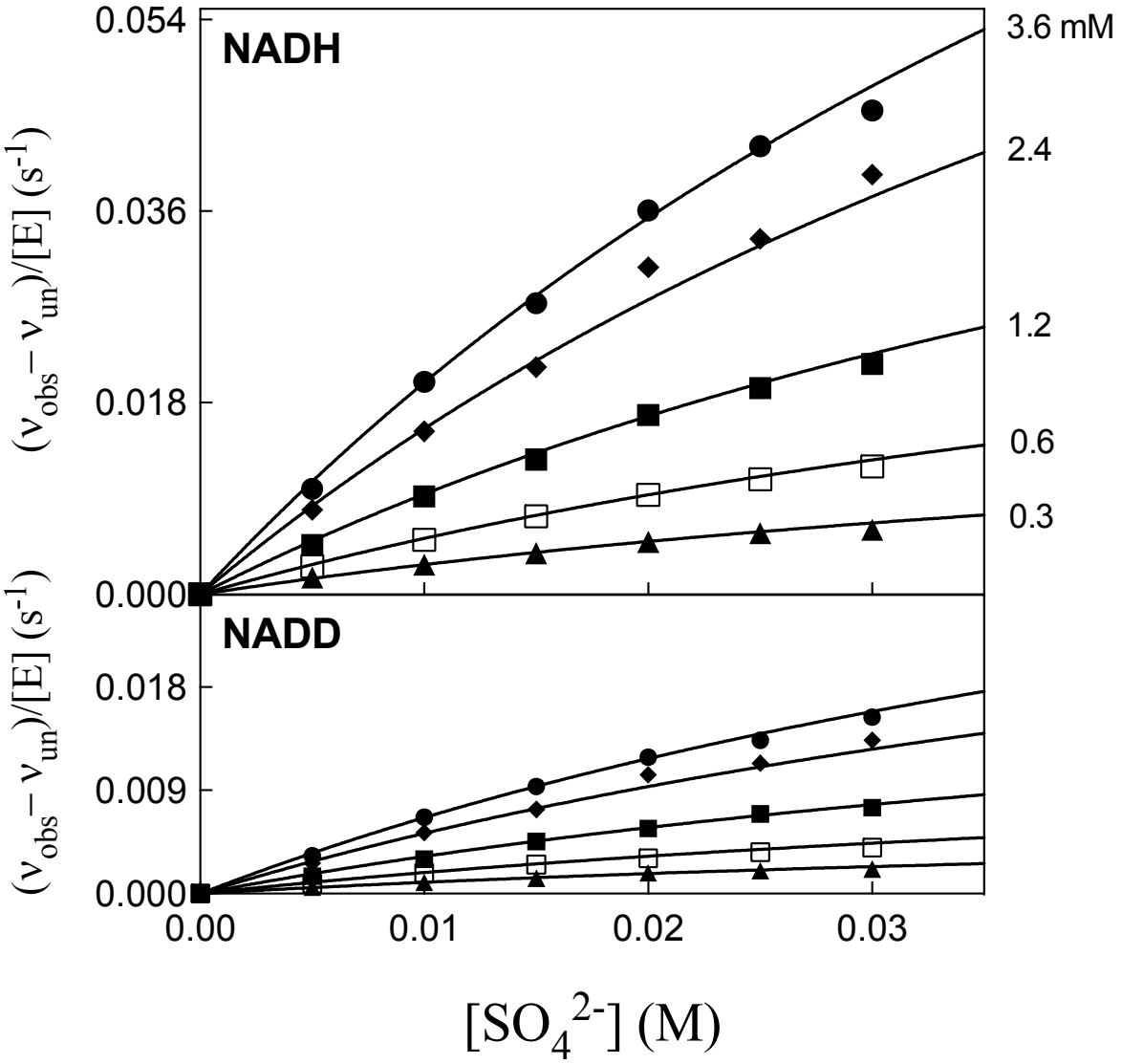

Figure S4
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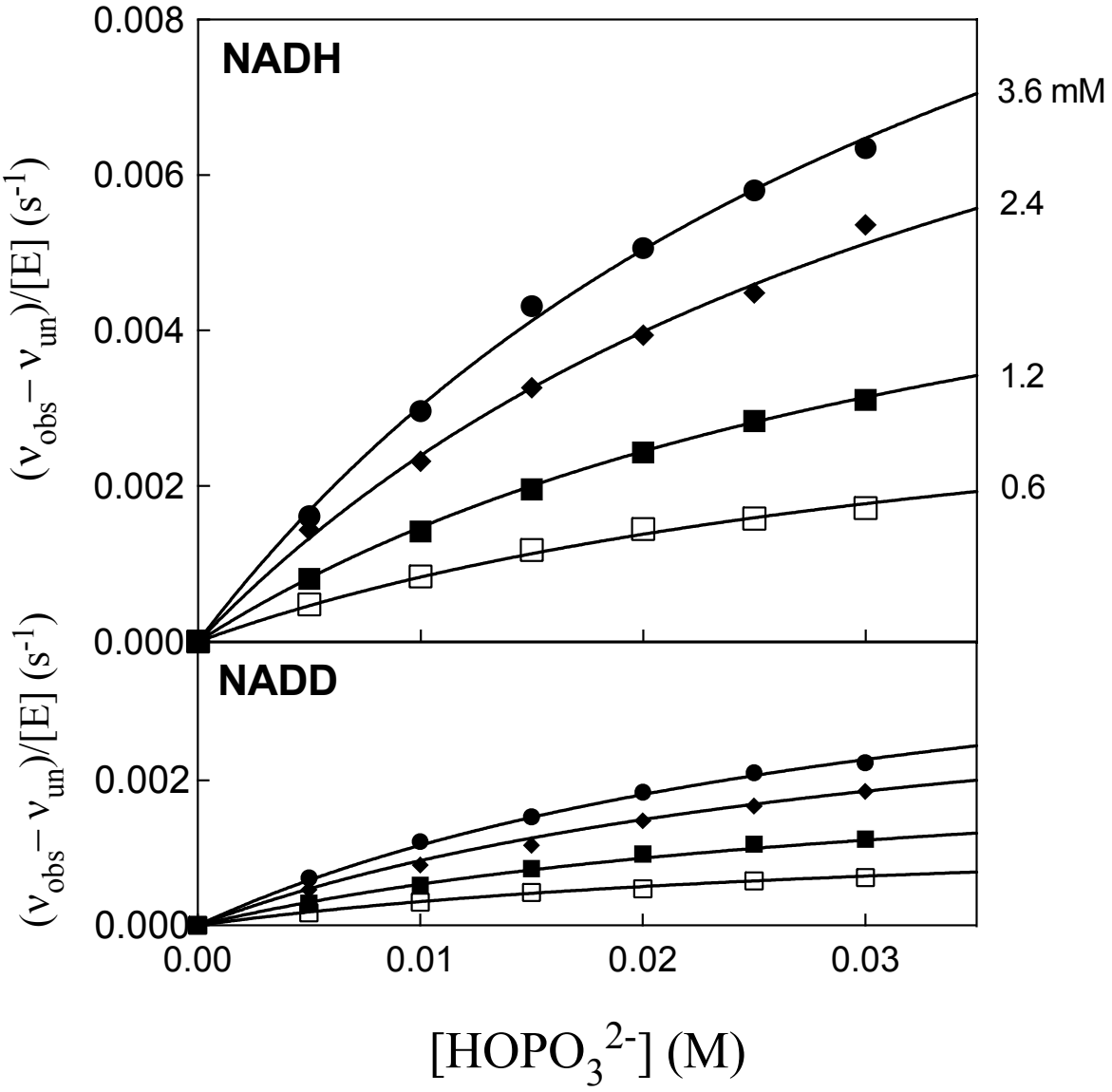

Figure S5
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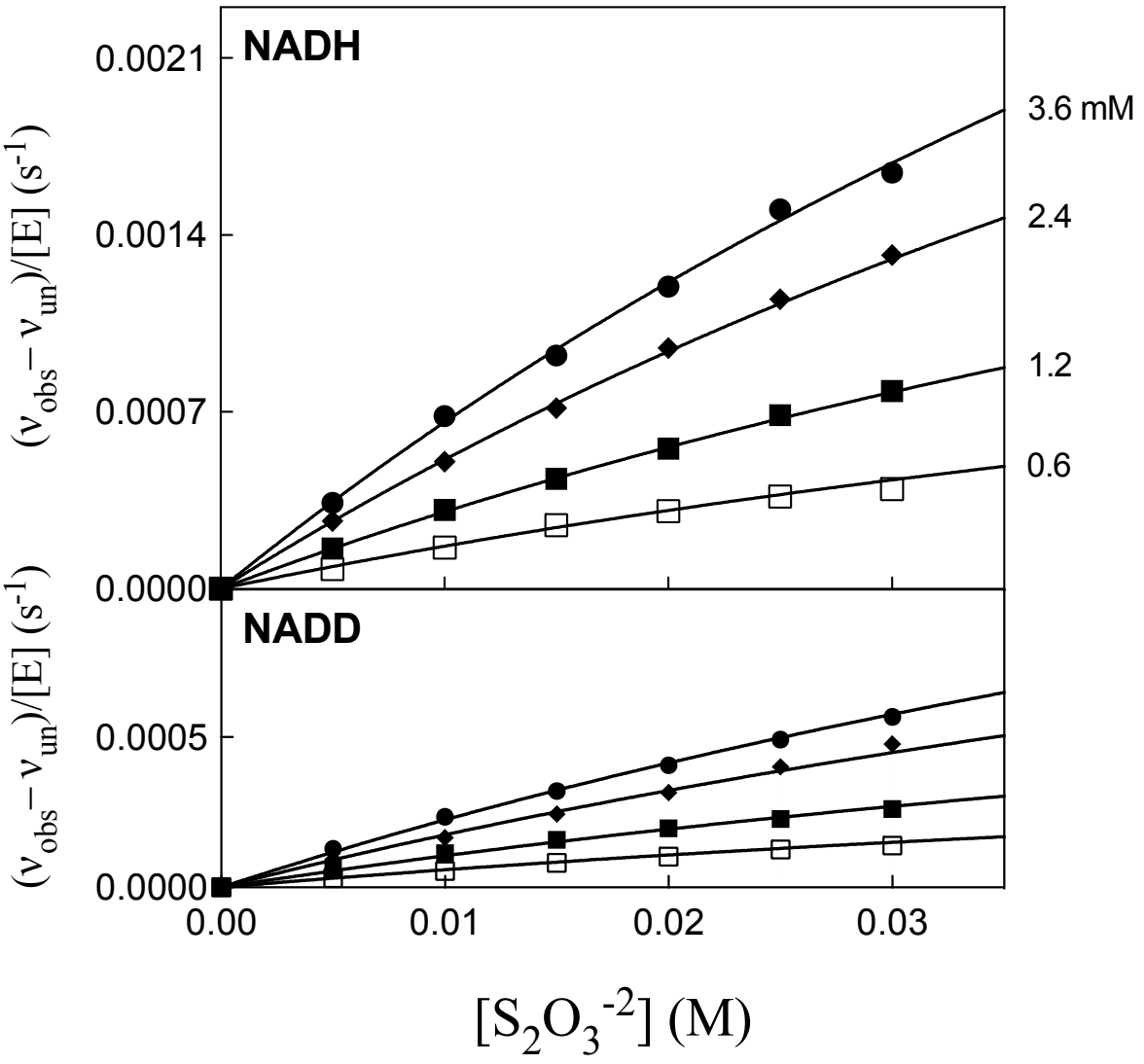

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