Supporting Information

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SI Text

Materials and Methods. ***Analytical ultracentrifugation.*** Sedimentation velocity experiments were carried out using a ProteomeLab XL-I (Beckman Coulter). A protein concentration of 5 μM was used for each sample. Samples were loaded into sector-shaped double channel centerpieces, temperature-equilibrated for 2 h prior to sedimentation in the non-spinning rotor, and spun at 30,000 rpm and 20 °C using an AN50TI rotor. Absorbance scans were collected continuously at a wavelength of 225 nm. Data analysis was with the enhanced van Holde-Weischet analysis module S1, and 2D spectrum analysis (2-DSA) was carried out using the finite element modeling provided with the Ultrascan software [http:/www.ultrascan.uthscsa.edu(S2, S3)]. A particle sedimenting in a gravitational field experiences two opposing forces, the sedimentation force and the buoyant force. Sedimentation is proportional to the mass of the particle and since a sedimenting particle experiences a frictional drag, based on the shape it will experience a frictional force. The 2-DSA analysis method solves a sedimentation profile with respect to both forces and thus provides information about the sedimentation and the frictional coefficient from which conclusions about the molecular mass and shape of a sedimenting particle can be drawn. The sedimentation profiles were analyzed at a grid resolution of 62,500 using 25 grid repetitions. Confidence levels for statistics were derived from 2-DSA data refinement using genetic algorithm followed by 50 Monte Carlo simulations. Calculations to analyze analytical ultracentrifugation data were performed on the UltraScan LIMS cluster at the Bioinformatics Core Facility at the University of Texas Health Science Center at San Antonio, the Lonestar cluster at the Texas Advanced Computing Center (supported by National Science Foundation Teragrid Grant MCB070038 to Borries Demeler), and the National Supercomputer HLRB-II at the Leibnitz-Rechenzentrum, Munich, Germany (supported by Project pr28ci to Johannes Buchner and T.M.F.).

MDH unfolding, refolding and activity assays. MDH (from porcine heart mitochondria, Roche) refolding was monitored by testing for enzymatic activity, essentially as described previously (S4). Samples of 1 μM MDH were held at low pH (in buffer A, pH 2) for 1 h at 37 °C in the absence or presence of 0.5, 1, or 2 μM HdeA, or in the presence of 2 μM HdeB or 2 μM BSA, followed by a 10 min temperature equilibration at 20 °C. Then, the samples were neutralized by addition of 0.133 volumes of 0.5 M sodium phosphate pH 8, and 10 μl aliquots were taken at various time points to determine MDH activity. The final MDH concentration in the assay buffer (50 mM sodium phosphate, 1 mM oxaloacetate, and 150 μM NADH) was 8.8 nM. Activity was then assessed by monitoring the absorbance decrease at 340 nm due to the conversion of β-NADH to β-NAD^+. Activity is reported relative to 8.8 nM native MDH that had been kept at pH 7. Absorbance was monitored by using a Cary100 spectrophotometer equipped with a Peltier temperature control block set to 20 °C.

Alkaline phosphatase unfolding, refolding and activity assays. Alkaline phosphatase (from *E. coli*, Sigma Aldrich) refolding following acid denaturation was monitored by testing for enzymatic activity. Samples of 3 μM alkaline phosphatase were held at low pH (buffer A, pH 2) for 1 h at 37 °C in the absence or presence of HdeA (1.5, 3 or 6 μM), or in the presence of 6 μM BSA or 6 μM HdeB. After the low pH incubation, samples were equilibrated to 20 °C for 10 min. The samples were then neutralized by addition of 0.133 volumes of 0.5 M sodium phosphate pH 8, and 5 μl aliquots were taken at various time points to determine alkaline phosphatase activity, which was measured using a procedure provided by Sigma. This method uses the substrate p-nitrophenyl phosphate and the associated absorbance increase at 405 nm upon the enzymatic cleavage of the phosphate group to yield the products P_i and p-nitrophenol. Activity assays were performed at 20 °C in 100 mM glycine, 1 mM $MgCl_2$ and 1 mM $ZnCl_2$, pH 10.4. Activity is reported relative to an equivalent amount of native alkaline phosphatase.

Aldolase unfolding, refolding and activity assays. Aldolase (from rabbit muscle, Sigma Aldrich) refolding was monitored by testing for enzymatic activity. Samples of 2.5 μM aldolase were held at low pH (buffer A, pH 2) for 1 h at 37 °C in the absence or presence of 1.25, 2.5 or 5 μM HdeA, followed by a 10 min temperature equilibration at 20 °C. Then, the samples were neutralized by addition of 0.133 volumes of 0.5 M sodium phosphate pH 8, and 20 μl aliquots were taken at various time points to determine aldolase activity, which was determined according to the coupled enzyme procedure provided by Sigma Aldrich. Briefly, aldolase converts its substrate fructose 1,6-diphosphate to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. α-Glycerophosphate dehydrogenase is then used to convert NADH and dihydroxyacetone phosphate to β-NAD^+ and α-glycerophosphate. Thus aldolase activity can be monitored by the absorbance increase at 340 nm associated with conversion of β-NAD^+ to β-NADH. Activity is reported relative to an equivalent amount of native aldolase.

GAPDH unfolding, refolding and activity assays. GAPDH (from baker's yeast, Sigma Aldrich) refolding was monitored by testing for enzymatic activity. A sample of 0.25 μM GAPDH was held at low pH (buffer A, pH 2) for 1 h at 37 °C in the absence or presence of 0.25 μM HdeA. Then, the samples were neutralized by addition of 0.133 volumes of 0.5 M sodium phosphate pH 8, and 10 μl aliquots were taken at various time points to determine GAPDH activity, which was determined by monitoring the absorbance increase associated with the conversion of β-NAD^+ to β-NADH. Assay buffer contained 1 mM β-NAD^+, 4 mM glyceraldehydes-3-phosphate and 10 mM EDTA in 0.1 M potassium phosphate pH 7.4. Activity is reported relative to an equivalent amount of native GAPDH.

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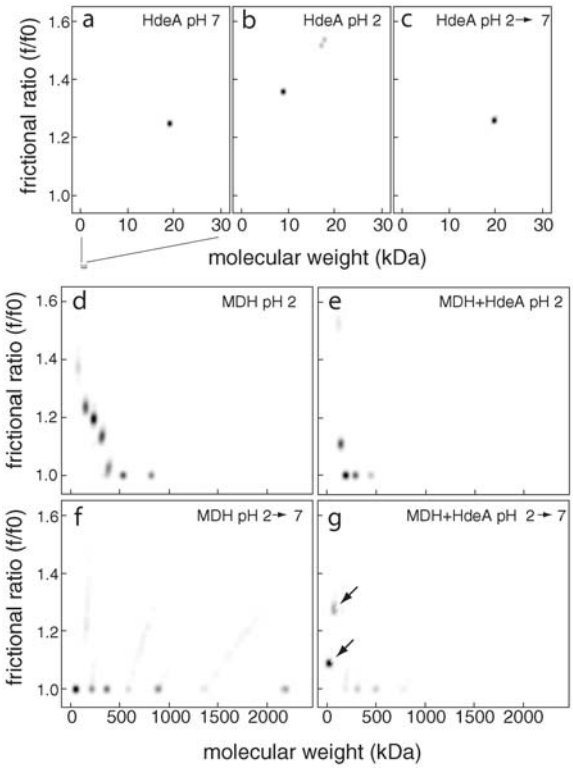

Fig. S1. Analytical ultracentrifugation sedimentation velocity analysis of (*a*) HdeA at pH 7, (*b*) HdeA at pH 2, (*c*) HdeA neutralized from pH 2 → 7. A minor fraction of dimers were still detected at low pH (*b*), which agrees with the previously published K_d of ~50μM for HdeA at pH 2 (7), although the dimers at pH 2 appear to populate different conformations than at pH 7 as indicated by their increased frictional ratio. Sedimentation velocity analysis MDH and HdeA-MDH complexes at low pH and following neutralization are shown in (*d–g*). (*d*) MDH at pH 2, (*e*) MDH + HdeA at pH 2, (*f*) MDH neutralized from pH 2 → 7, and (*g*) MDH + HdeA neutralized from pH 2 → 7. Protein concentrations for both HdeA and MDH were 5 μM. For pH 2 → 7 experiments (*c, f* and *g*), samples were incubated for 1 h at 37 °C at pH 2, then equilibrated at 20 °C and neutralized immediately prior to the analytical ultracentrifugation run. The two major species detected in (*g*) sediment similarly to native HdeA and MDH dimers, and are indicated by arrows.

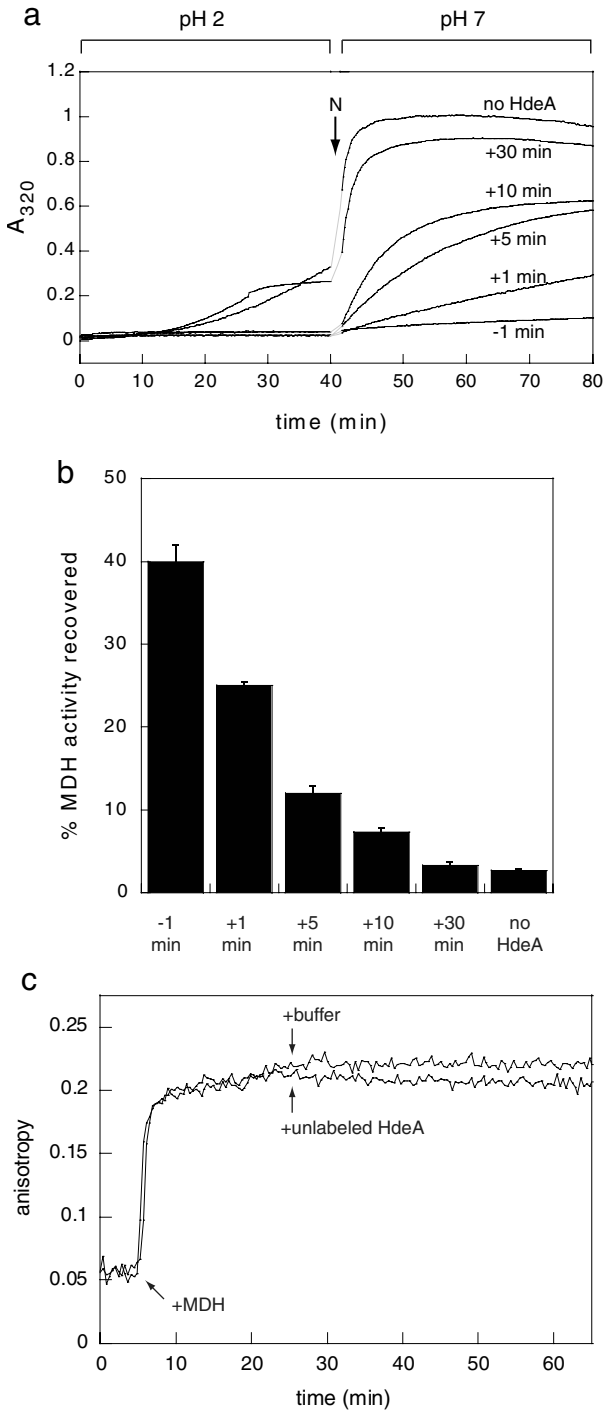

Fig. S2. The effect of HdeA addition time on MDH aggregation and refolding. (*a*) MDH aggregation was initiated by adding MDH (final concentration 8 μM) to buffer A, pH 2 at 37 °C. The time of MDH addition was set to 0; HdeA (final concentration 16 μM) addition time was relative to this point. HdeA was added at time points −1 min, +1 min, +5 min, +10 min , +30 min, or not at all (i.e., MDH alone). After 40 min incubation at pH 2, the pH was neutralized (final pH equals 7) by addition of 0.133 volumes 0.5 M phosphate pH 8, as indicated by the arrow (N). Aggregation was monitored by apparent changes in absorbance due to light scattering at 320 nm in a Varian Cary 100 spectrophotometer equipped with Peltier temperature control. The gaps in the data after pH neutralization are due to the time required to mix the samples and resume data collection; traces are connected with gray lines only to guide the eye. (*b*) The effect of HdeA addition time on MDH reactivation. 8 μM MDH was acid denatured in buffer A, pH 2 for 40 min at 37 °C. The time of MDH addition was set to 0; HdeA (final concentration 16 μM) addition time was relative to this point. HdeA was added at time points −1 min, +1 min, +5 min, +10 min , +30 min, or not at all (i.e., MDH alone). After 40 min at pH 2, each sample was diluted 20-fold into 50 mM sodium phosphate, 150 mM NaCl, pH 7 to initiate refolding. Samples were allowed to refold at 20 °C for 3 h, after which time 10 μl aliquots were taken in triplicate and assayed for MDH activity. Activity is expressed relative to native non-acid denatured MDH of the same concentration (4 nM final concentration in the each activity assay). (*c*) Stability of HdeA-MDH complexes at pH 2 as monitored by fluorescence anisotropy. Duplicate samples of 0.5 μM HdeA(S27C)-bimane were prepared at pH 2 and equilibrated to 37 ° C. After 5 min, MDH was added to both samples to a final concentration of 0.5 μM. Both samples were allowed to equilibrate for 20 min at which point a 10-fold excess of unlabeled HdeA (or buffer) was added and fluorescence anisotropy was further monitored.

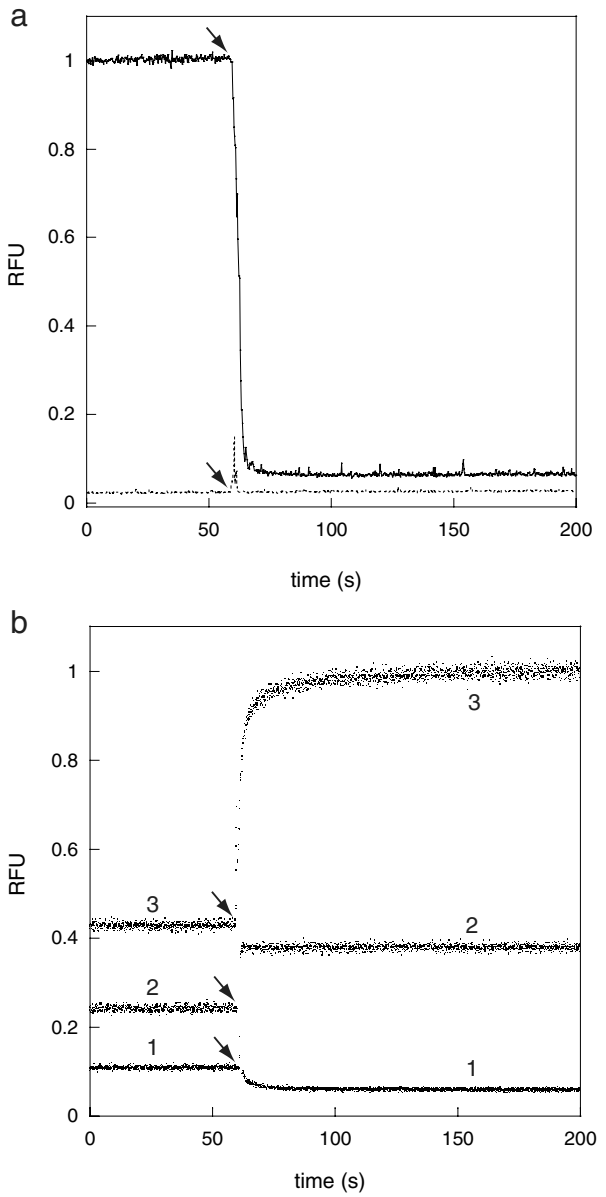

Fig. S3. Refolding and dimerization of substrate-free HdeA following pH neutralization. (*a*) HdeA refolding by bis-ANS fluorescence. The dye bis-ANS is essentially non-fluorescent in aqueous (i.e., polar) solutions unless bound to exposed hydrophobic regions of a protein (5), thereby allowing assessment of the refolding of HdeA following pH neutralization. bis-ANS (10 μM) was incubated in buffer A, pH 2, at 20 °C in the absence (dotted line) or presence (solid line) of 2 μM HdeA. As expected, bis-ANS alone exhibited a very low fluorescence. However, in the presence of HdeA at pH 2, the fluorescence signal increased approximately 40-fold. Upon pH neutralization (indicated by the arrows), the signal rapidly declined, which is indicative of the refolding of HdeA and the release of bound bis-ANS. (*b*) HdeA dimerization as monitored by FRET. The protein HdeA(S27C) was labeled with either Alexafluor532 (AF532) or bimane as described previously (6). 0.5 μM HdeA(S27C)-bimane (trace 1), 0.5 μM HdeA(S27C)-AF532 (trace 2), or 0.5 μM of each bimane- and AF532-labeled protein (trace 3) were incubated in buffer A, pH 2 at 20 °C. The pH was then neutralized (at the time indicated by the arrows) by addition of 0.133 volumes 0.5 M phosphate, pH 8. Fluorescence was monitored by preferentially exciting the bimane donor at 390 nm (5 nm bandpass) and measuring AF532 (acceptor) fluorescence at 550 nm (5 nm bandpass).

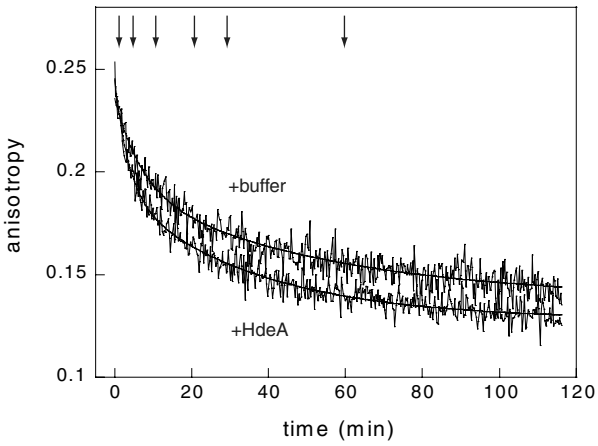

Fig. S4. HdeA does not rebind released MDH after pH-induced dissociation. Duplicate samples of 0.25 μM HdeA(S27C)-bimane and 0.25 μM MDH were incubated in buffer A (pH 2) for 1 h at 37 °C. The temperature was then adjusted to 20 °C and after 10 minutes the pH was neutralized by addition of 0.133 volumes 0.5 M sodium phosphate pH 8. Complex dissociation was monitored by fluorescence anisotropy. To one of the samples, aliquots of excess unlabeled active HdeA (i.e., from a concentrated stock solution at pH 2) were added at the points indicated by arrows. Each arrow represents addition of a 2-fold excess of unlabeled HdeA. Traces were then fit to double exponential functions; triplicate experiments yielded averages of $k_1 = 0.32 \pm 0.1$ and $k_2 = 0.022 \pm 0.005$ in the absence of additional unlabeled HdeA (plus buffer) and $k_1 = 0.37 \pm 0.1$ and $k_2 = 0.027 \pm 0.002$ in the presence of additional unlabeled HdeA (plus HdeA).

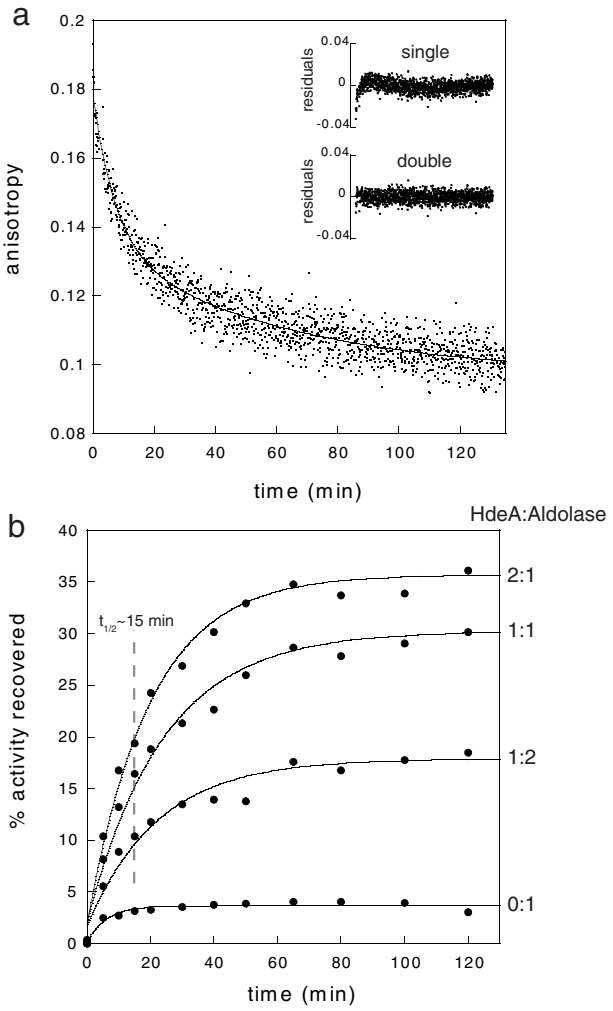

Fig. S5. Comparison of the rate of aldolase release from HdeA with the rate of aldolase refolding. (*A*) Aldolase release from HdeA following pH neutralization. 0.25 μM bimane-labeled HdeA(S27C) +0.25 μM aldolase was incubated in buffer A (pH 2) at 37 °C for 1 h, at which point the temperature was re-equilibrated to 20 °C for 10 min. After equilibration, the pH was neutralized by addition of 0.133 volumes 0.5 M sodium phosphate pH 8. Data acquisition began immediately upon neutralization. The data were fit with a double exponential function (smooth line, curve fit parameters $A_1 = 0.04$, $k_1 = 0.14\text{min}^{-1}$, $A_2 = 0.04$, $k_2 = 0.016\text{min}^{-1}$). Residuals for single and double exponential fits are shown in the inset. (*B*) Aldolase refolding following release from HdeA as monitored by the recovery of enzymatic activity. Samples were prepared as in (*A*) except that protein concentrations were 2.5 μM aldolase and 0, 1.25, 2.5 or 5 μM HdeA. Aliquots were taken and assayed for aldolase activity at various time points after neutralization. The refolding of aldolase alone, which was treated in the same way except in the absence of HdeA, was tested as a control for spontaneous refolding.

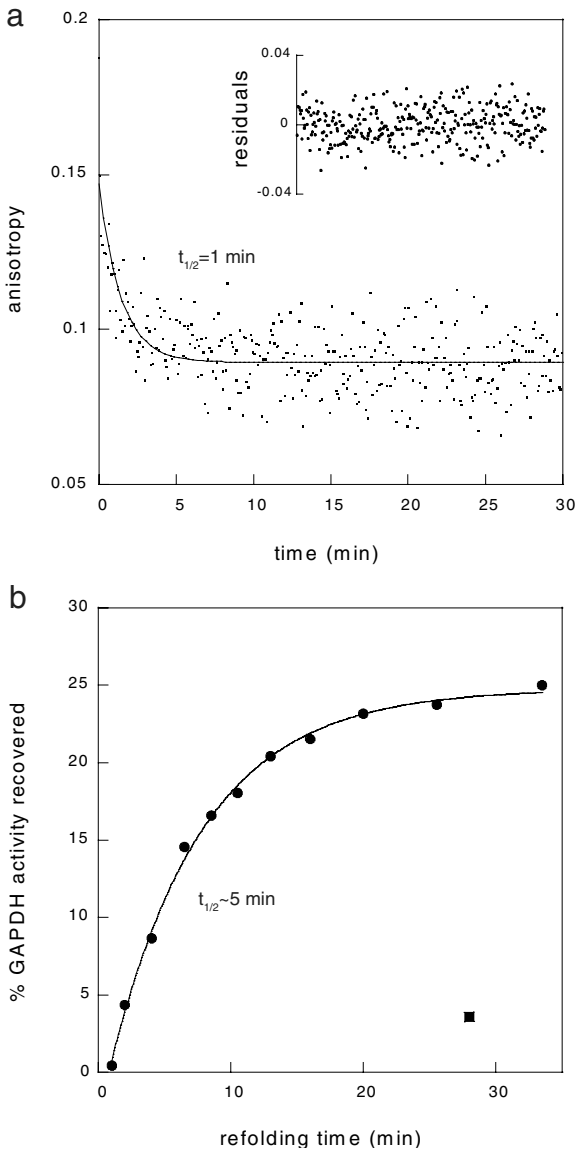

Fig. S6. Comparison of the rate of GAPDH release from HdeA with the rate of GAPDH refolding. (*A*) GAPDH release from HdeA following pH neutralization. 0.25 μM bimane-labeled HdeA(S27C) +0.25 μM GAPDH was incubated in buffer A, pH 2.0 at 37 °C for 1 h, at which point the pH was neutralized by addition of 0.133 volumes 0.5 M sodium phosphate, pH 8. Data acquisition began immediately upon neutralization. The data were fit with a single exponential function (curve fit parameters $A = 0.12$, $k = 0.67\text{min}^{-1}$). Residuals for the fit are shown in the inset. (*B*) GAPDH refolding following release from HdeA as monitored by the recovery of enzymatic activity. Aliquots were taken directly from the cuvette in which anisotropy was being measured and assayed for GAPDH activity at various time points after neutralization (•). The refolding of GAPDH alone, which was treated in the same way except in the absence of HdeA, was tested as a control for spontaneous refolding (■).

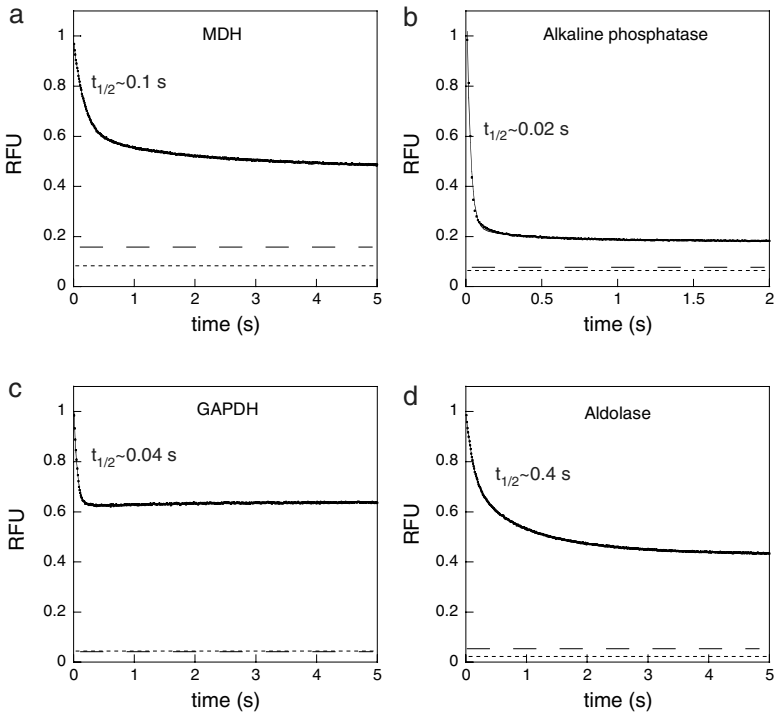

Fig. S7. Decrease in fluorescence of substrate-bound bis-ANS following pH neutralization indicates fast folding to a less hydrophobic intermediate state. 10 uM bis-ANS was pre-incubated with 100 nM MDH (*A*), Alkaline phosphatase (*B*) GAPDH (*C*), or aldolase (*D*) at pH 2, and was loaded into syringe A of a Kintek SF2004 stopped flow system. After a 5-min incubation at 20 °C, the pH was neutralized by rapid mixing with an equal volume of 0.125 M sodium phosphate containing 10 uM bis-ANS, pH 8.0 for a final pH of ~7. Upon neutralization, the decrease in bis-ANS fluorescence was monitored (excitation equals 390 nm, emission equals 450 nm longpass filter). The fluorescence decrease is consistent with each substrate folding to an intermediate state (or ensemble of intermediates) that is less competent to bind bis-ANS. The short dashed lines in each panel show the fluorescence of bis-ANS alone in the absence of substrate; the long dashed lines mark the fluorescence of bis-ANS in the presence of native substrate protein (100 nM) at pH 7. The fluorescence in each panel was normalized to the maximum signal; i.e., that of bis-ANS in the presence of the acid-denatured protein. Each curve was fit to a double exponential function, with the following curve fit parameters: (*A*) $A_1 = 0.39$, $k_1 = 5.4\ s^{-1}$, $A_2 = 0.12$, $k_2 = 0.43\ s^{-1}$; (*B*) $A_1 = 1.2$, $k_1 = 41 s^{-1}$, $A_2 = 0.05$, $k_2 = 2.1 s^{-1}$; (*C*) $A_1 = 0.45$, $k_1 = 19 s^{-1}$, $A_2 = -0.02$, $k_2 = 0.60 s^{-1}$; (*D*) $A_1 = 0.32$, $k_1 = 7.1 s^{-1}$, $A_2 = 0.25$, $k_2 = 0.91 s^{-1}$. The half-times given in *A*–*C* are for the fast decay phase, which comprised the majority of the overall fluorescence decrease; the half-time in *D* is an average of the two observed exponential phases, as the amplitudes for the two observed exponential phases were approximately equal.

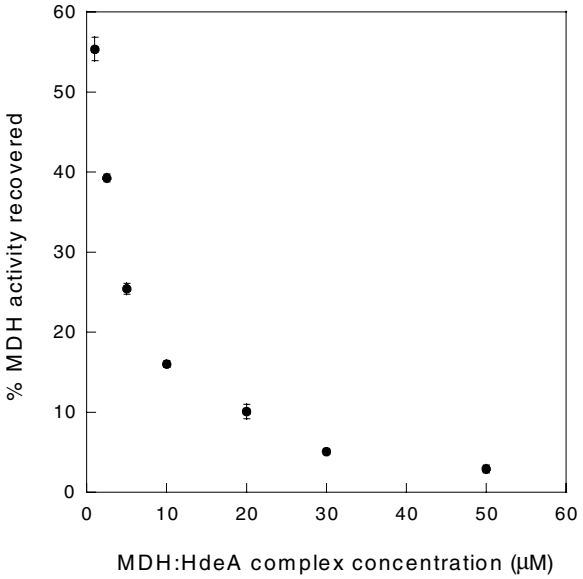

Fig. S8. Increasing HdeA:MDH complex concentration leads to decreasing MDH refolding yields. HdeA was incubated in buffer A, pH 2 at a concentration of 1, 2.5, 5, 10, 20, 30, or 50 μM. To each sample, and equimolar amount of MDH was added, and the complexes were incubated at low pH for 1 h at 37 °C. The temperature was then re-equilibrated to 20 °C for 10 min. After equilibration, the pH was neutralized by addition of 0.133 volumes 0.5 M sodium phosphate pH 8. Refolding was allowed to proceed for 3 h, after which the MDH activity of each sample was measured in triplicate. Error bars represent 1 s.d. Activity is reported relative to an equivalent amount of native MDH.

Table S1. Summary of analytical ultracentrifugation data

Sample	pH	Mw (kDa)	Sw20 (1e-13 s)	Sed Coefficient (1e-13 s)	f/f0
HdeA	2	19.5 – 1	2.09	2.09 – 0.04	1.26 – 0.02
HdeA	7	9.1 – 1	1.19	1.19 – 0.09	1.33 –0.04
HdeA	2 → 7	18.8 – 1	2.07	2.07 – 0.02	1.24 – 0.01
MDH	7	59.8 – 2	4.33	4.33 – 0.05	1.18 – 0.02
MDH	2	150–800	13.34	ND	ND
MDH	2 → 7	170–2200	21.40	ND	ND
HdeA+MDH	2	140–400	10.75	ND	ND
HdeA+MDH	2 → 7	18/69 – 2	8.36	2.17/4.43 – 0.05	1.15/1.27 – 0.02