Electronic Supplementary Material (ESI) for Physical Chemistry Chemical Physics.

Supplementary Information

Enzyme Aggregation and Fragmentation Induced by Catalysis Relevant Species

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Supplementary Text and Calculations

Calculation of Förster Radius for AlexaFluor 488 and 532:

The Förster radius for the two FRET dyes used in the experiments (AlexaFluor 488 and AlexaFluor 532) is about 6 nm and was calculated using Equations S1-S2 and values below.[1]

$$R_0 = 0.211\sqrt[6]{\kappa^2 n^{-4} Q_D J(\lambda)} \tag{S1}$$

Where R_0 is the Förster radius, κ^2 is the orientation factor, n is the refractive index of the fluid, Q_D is the quantum yield of the donor and $J(\lambda)$ is the overlap integral. The values for Q_D and $J(\lambda)$ were given by https://www.fpbase.org/fret/.

$$R_0 = 0.211\sqrt[6]{0.667(1.33)^{-4}(0.92)(3.44e^{15}\ M^{-1}cm^{-1}nm^4)} \tag{S2}$$

$$R_0 = 62.5\ \dot{A}$$

This indicates that when these two dyes are apart (> 62.5 Å), the emission from AF488 is not enough to excite AF532. However, when these dyes are close together (< 62.5 Å), the emission from AF488 can excite AF532 and a peak at 555 nm appears. This is illustrated in **Figure S10**.

Calculation of FRET Efficiencies:

To describe the FRET efficiency, or the portion of the enzyme population that is within 6 nm of each other, E_{FRET} was calculated using Equation S3,

$$E_{FRET} = \frac{I_{560} - \gamma I_{520}}{I_{520} + (I_{560} - \gamma I_{520})} \tag{S3}$$

Where I_{520} and I_{560} are the fluorescence readings at 510-520 nm and 550-560 nm, respectively, when both dyes are used in the experiment, and γ is the correction factor that corrects for the emission of the donor at 560nm. γ is calculated using Equation S4.

$$\gamma = \frac{I_{560}D}{I_{520}D} \tag{S4}$$

Where $I_{520}D$ and $I_{560}D$ are the fluorescence readings at 510-520 nm and 550-560 nm, respectively, when only the donor dye (AF488) is present in the experiment. An average of three fluorescence readings (fluorescence readings at 510, 515 and 520 nm for I_{520} and fluorescence readings at 550, 555 and 560 nm for I_{560}) was taken for each point to reduce the noise from the instrument. E_{FRET} describes the emission at 560 divided by the total emission and accounts for any overlap of the emission of 488.

Calculation of D-Glucose Concentration Required for Fragmentation:

In order to determine the minimum concentration of D-Glucose required for fragmentation, we examined the experiments where we added invertase and sucrose and saw fragmentation after a time lag of 30-40 minutes.

Calculation of the concentration of D-Glucose present in the solution after 40 minutes was done as follows. For each experiment, we used 760 µL of stock solution. Invertase was added so that its final concentration was 0.1 µM. Thus, number of moles of invertase present in solution.

$$= 0.1 * 10^{-6} \frac{moles}{L} \times 760 \mu L$$

$$= 7.6 * 10^{-11}\, moles$$

The molecular weight of invertase is 270 kDa. Therefore, the amount of invertase in solution

$$= 7.6 * 10^{-11}\, moles \times 270 * 10^{3} \frac{g}{mol}$$

$$= 2.1 * 10^{-5} g$$

Now, according to the Sigma website, the invertase contains ≥ 300 units/ mg solid,

Minimum number of units of invertase in solution

$$= 2.1 * 10^{-5} g \times 300 \frac{units}{mg}$$

$$= 6.3\, units$$

Now, one unit of invertase converts 1.0 µM of sucrose to D-Glucose per min at a pH of 4.5 and temperature of 55°C. Thus 6.3 units will hydrolyze 6.3 µM of sucrose per minute.

In 40 minutes, 6.3 $\frac{units}{min} \times 40\, mins$ = ~ 0.3 millimoles of sucrose is hydrolyzed.

Upon hydrolysis, one mole of sucrose produces 1 mole of D-Glucose. Thus, in solution, ~ 0.3 millimoles of D-Glucose are produced.

Supplementary Figures

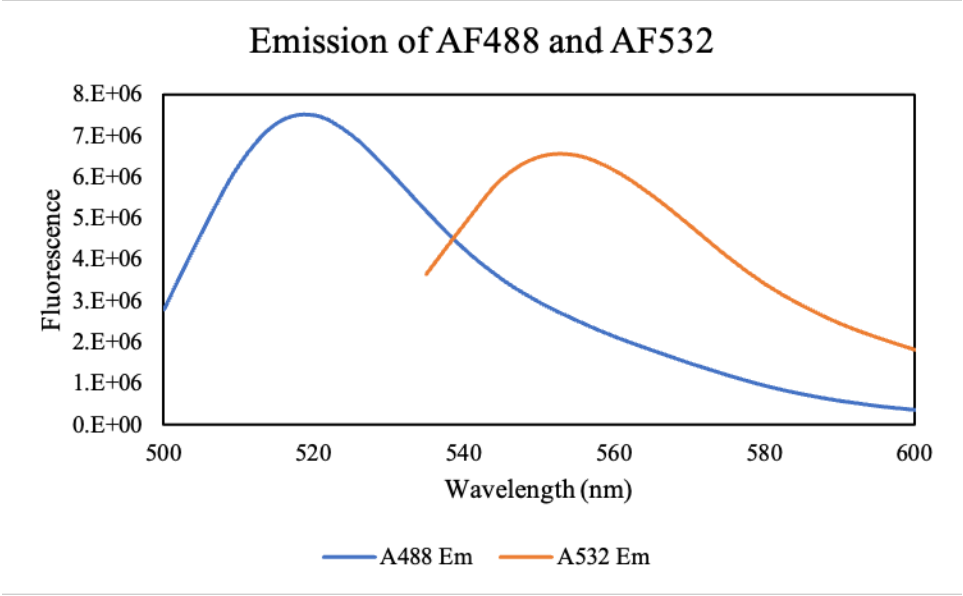

Fig. S1. Emission spectra of AF488 and AF532.
Emission spectra of AF488 (blue line) and emission spectra of AF532 (orange line). Emission is around 520 nm for AF488 and 555 nm for AF532.

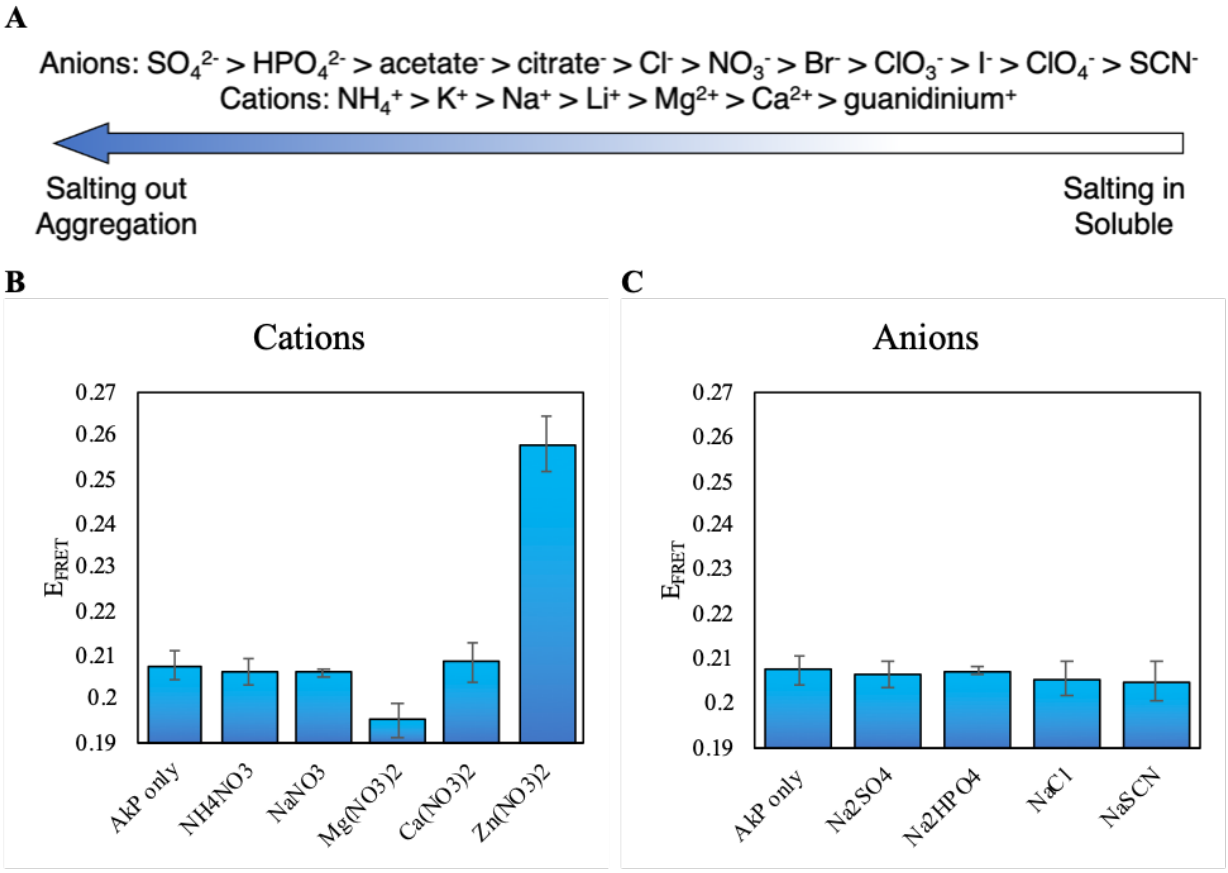

Fig. S2. FRET efficiencies of AkP with salts in the Hofmeister series.
(A) The Hofmeister series. "Salting out" ions will cause aggregation of most proteins and "salting in" salts will cause most proteins to become soluble.[2] FRET efficiency of 0.2 µM alkaline phosphatase with 1 mM **(B)** cations and **(C)** anions from the Hofmeister series. $Zn(NO_3)_2$ is not in the pictured Hofmeister series but is used for comparison. The buffer used to make all experimental solutions was 50 mM HEPES (pH 7). The error bars represent the standard deviation from two trials. The FRET efficiency for 1 mM $Zn(NO_3)_2$ is statistically different from the FRET efficiency with only AkP ($P < 0.05$; see Materials and Methods).

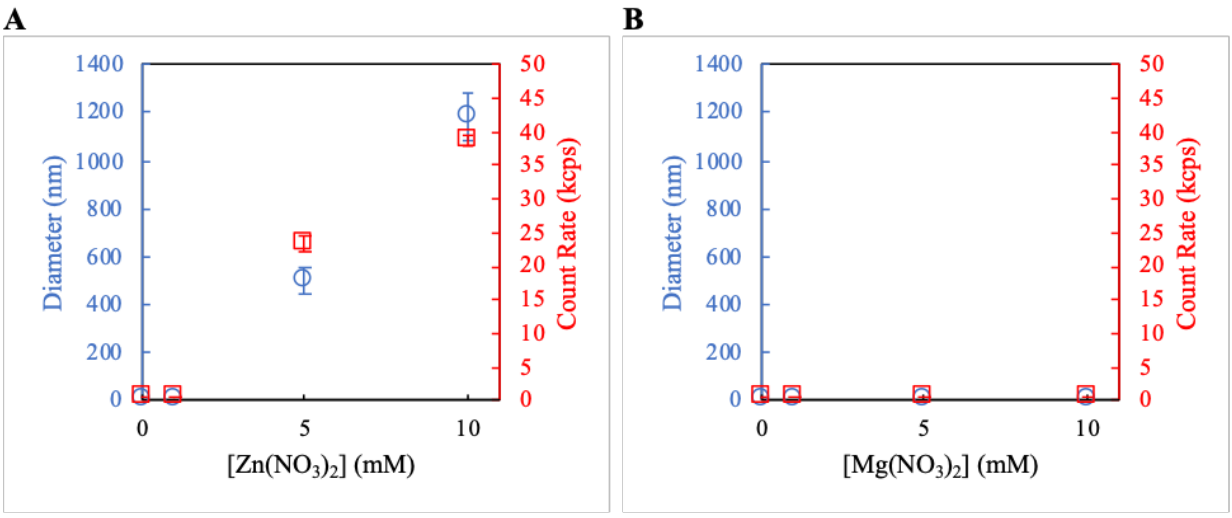

Fig. S3. DLS Data of AkP with increasing concentrations of Zn^{2+} and Mg^{2+}.
(A) Diameter (blue circles) and count rate (red squares) for 3 µM AkP with 0-, 1-, 5-, and 10-mM $Zn(NO_3)_2$. **(B)** Diameter (blue circles) and count rate (red squares) for 3 µM AkP with 0-, 1-, 5-, and 10-mM $Mg(NO_3)_2$. The buffer used to make all experimental solutions was 50 mM HEPES (pH 7). The error bars represent the standard deviation from two trials. The count rates at 1, 5 and 10 mM of $Zn(NO_3)_2$ are statistically different from the count rate with only AkP ($P < 0.05$; see Materials and Methods).

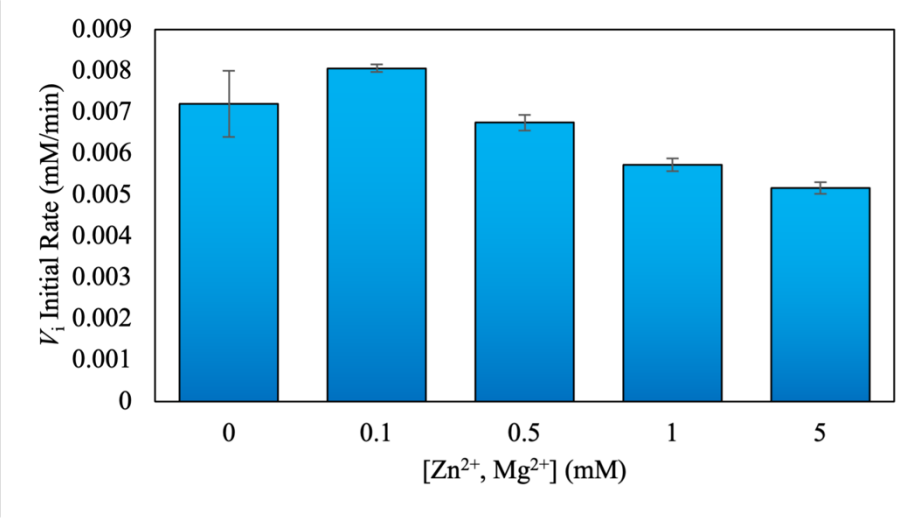

Fig. S4. Activity of AkP with increasing concentrations of Zn^{2+} and Mg^{2+}.
Activity of 0.2 µM tagged alkaline phosphatase from 20-23 min with 0.5 mM p-nitrophenyl phosphate (pNPP) while increasing the concentration of zinc nitrate and magnesium nitrate from 0.1 mM to 5 mM; [Zn^{2+}] = [Mg^{2+}]. See **Fig. S12** for activity over a longer time period. The buffer used to make all experimental solutions was 50 mM HEPES (pH 7). The error bars represent the standard deviation from two trials. None of the activities are statistically different from 0 mM Zn^{2+}, Mg^{2+} ($P > 0.05$; see Materials and Methods).

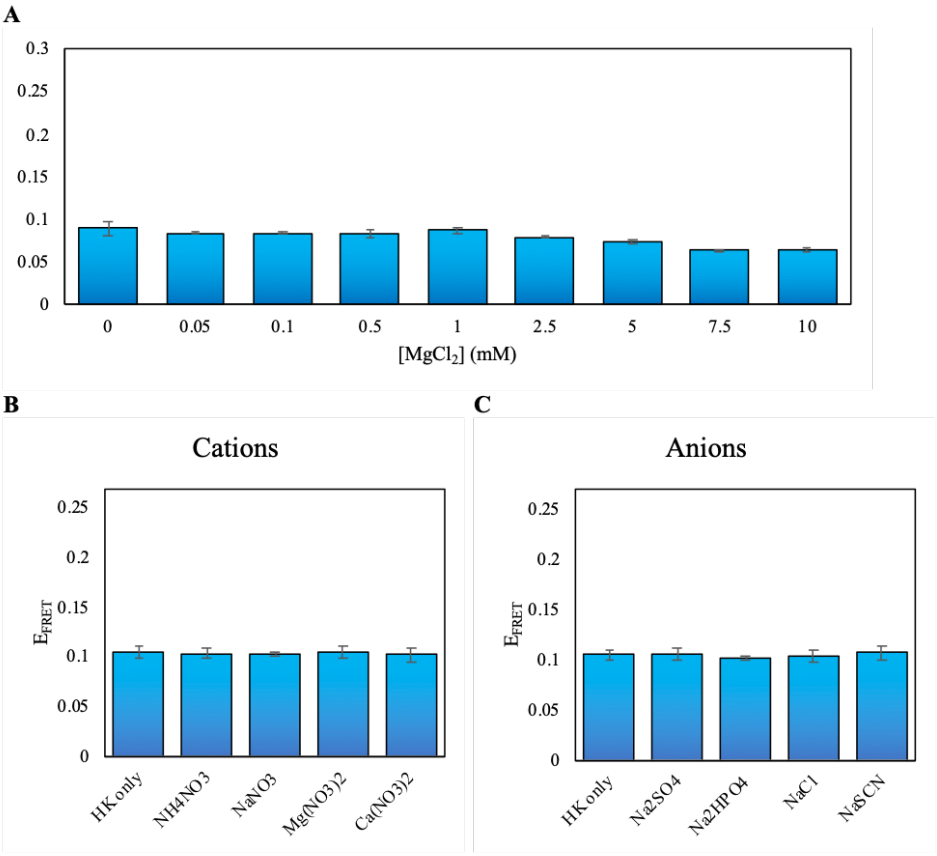

Fig. S5. FRET efficiencies of HK with $MgCl_2$ and with salts in the Hofmeister series. **(A)** FRET efficiency of 0.2 µM hexokinase with increasing mM concentrations of magnesium chloride (blue bars). FRET efficiency of 0.2 µM hexokinase with 1 mM **(B)** cations and **(C)** anions in the Hofmeister series. The buffer used to make all experimental solutions was 50 mM HEPES (pH 7). The error bars represent the standard deviation from two trials. The FRET efficiencies for 5 – 10 mM $Mg(NO_3)_2$ are statistically different from the FRET efficiency with only HK ($P < 0.05$; see Materials and Methods).

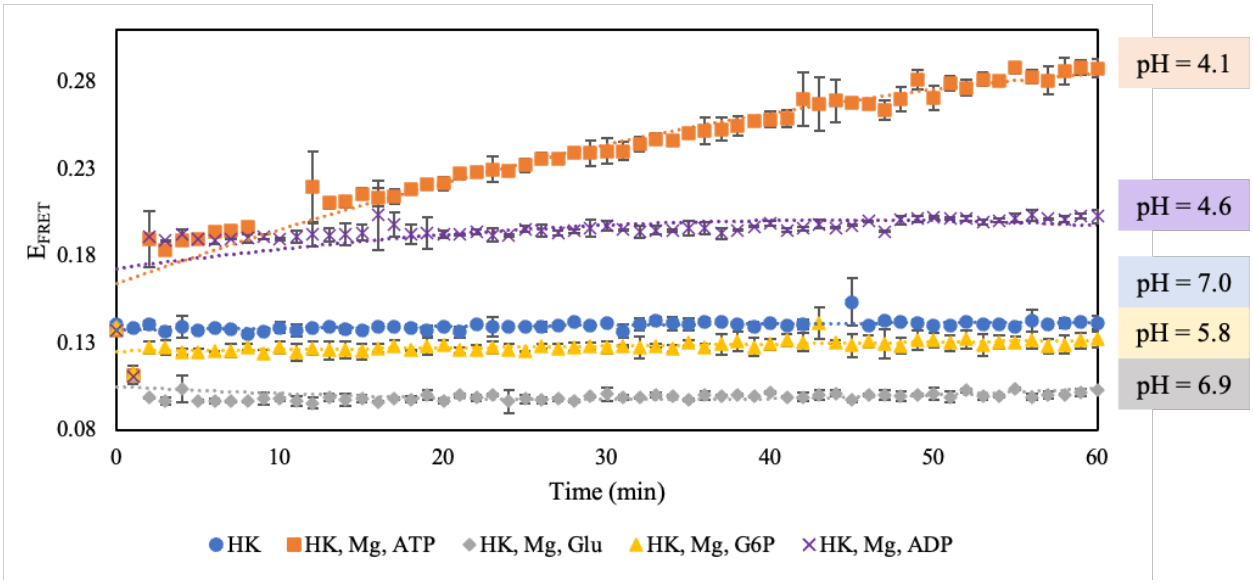

Fig. S6. FRET efficiencies of HK with species involved in catalysis.
FRET efficiency of 0.2 µM HK (pH 7.0; blue circles); 0.2 µM HK, 40 mM $MgCl_2$, 20 mM ATP (pH 4.1; orange squares); 0.2 µM HK, 40 mM $MgCl_2$, 20 mM D-Glu (pH 6.9; gray diamonds); 0.2 µM HK, 40 mM $MgCl_2$, 20 mM G6P (pH 5.8; yellow triangles); 0.2 µM HK, 40 mM $MgCl_2$, 20 mM ADP (pH 4.6; purple crosses). The buffer used to make all experimental solutions was 50 mM HEPES (pH 7). The dashed lines are to guide the eye. The error bars represent the standard deviation from two trials.

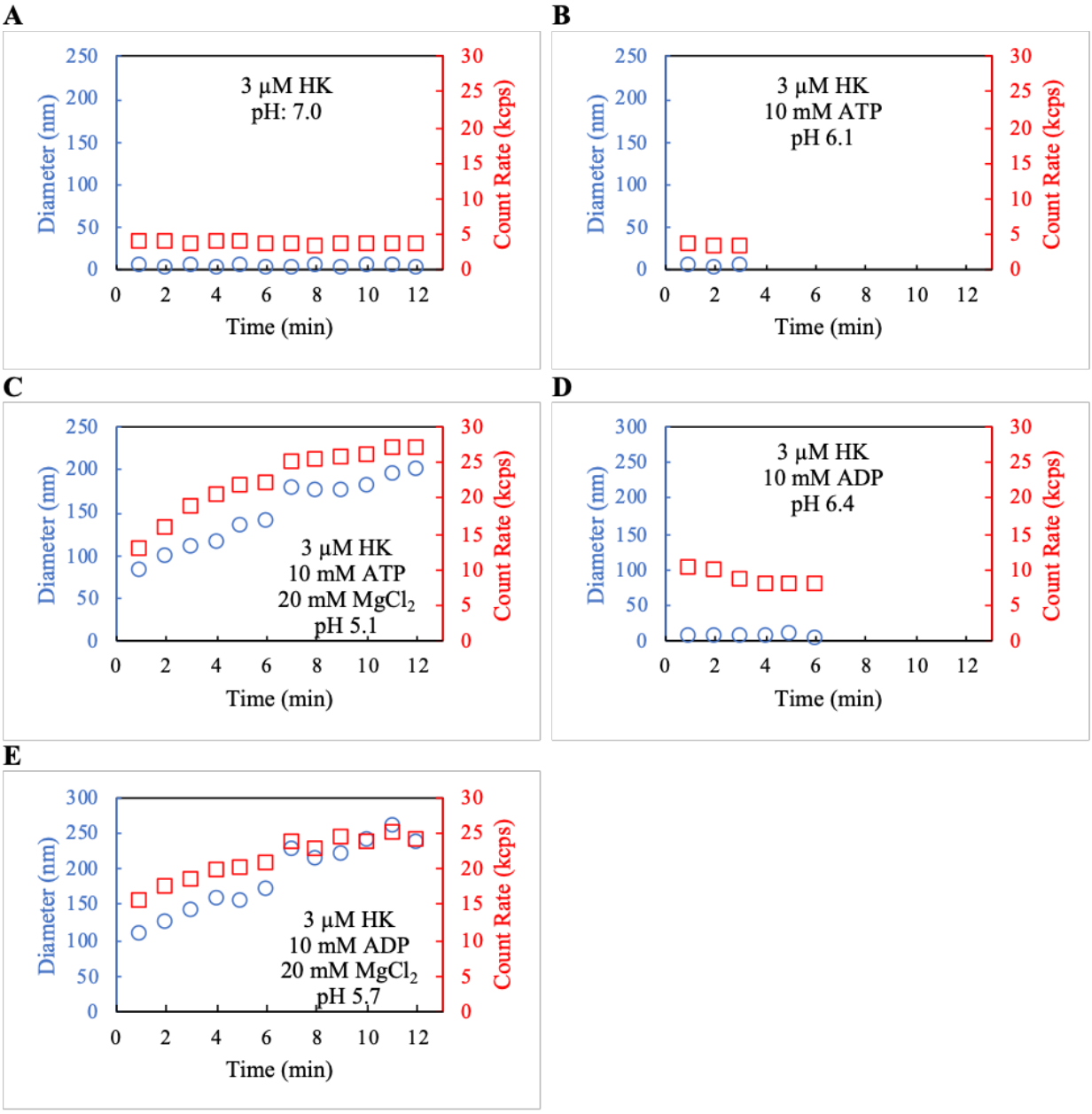

Fig. S7. DLS Data of HK with Mg^{2+} and ATP or ADP.

(A) Diameter (blue circles) and count rate (red squares) for 3 µM hexokinase (pH 7.0) over a period of 12 minutes. Diameter (blue circles) and count rate (red squares) for 3 µM hexokinase with **(B)** 10 mM ATP (pH 6.1), **(C)** 10 mM ATP and 20 mM $MgCl_2$ (pH 5.1), **(D)** 10 mM ADP (pH 6.4), **(E)** 10 mM ADP and 20 mM $MgCl_2$ (pH 5.7) over a period of 12 minutes. The buffer used to make all experimental solutions was 50 mM HEPES (pH 7).

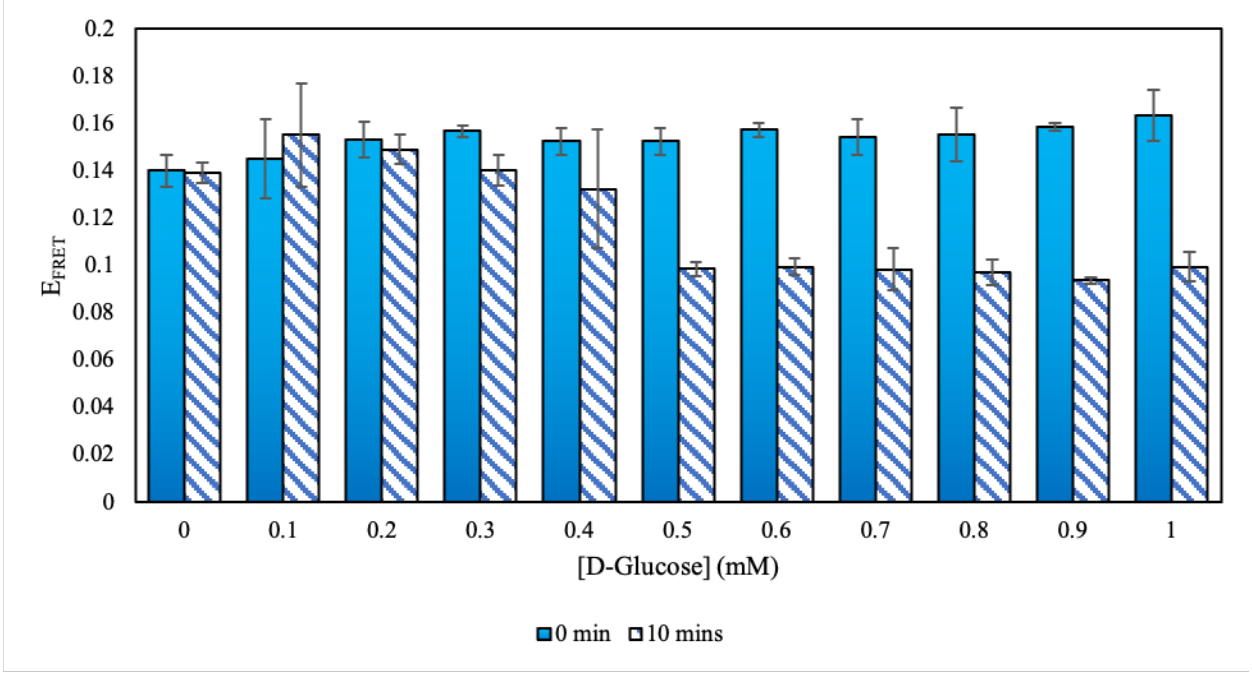

Fig. S8. FRET efficiencies of GOx with increasing concentrations of D-Glu.
FRET efficiency for fragmentation using 0.2 μM GOx and varying quantities of added D-Glucose. Blue bars represent the FRET efficiency at 0 min; striped bars represent the FRET efficiency 10 minutes after the addition of D-Glucose. The buffer used to make all experimental solutions was 50 mM MES (pH 6). The error bars represent the standard deviation from three trials of the experiments. The FRET efficiencies for 0.3 and 0.5 – 1 mM D-Glu at 10 mins are statistically different from the FRET efficiencies at 0 min ($P < 0.05$; see Materials and Methods).

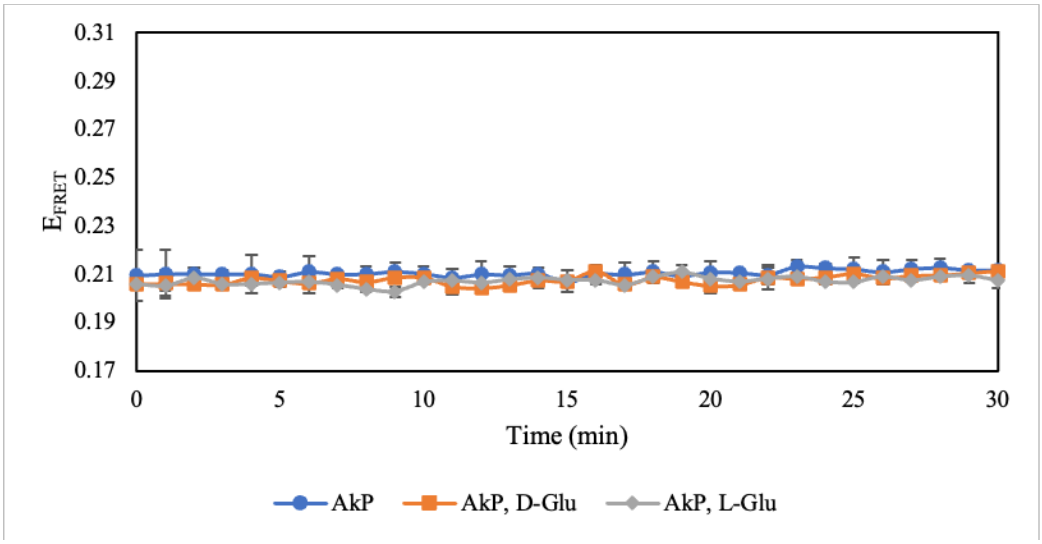

Fig. S9. FRET efficiencies of AkP with D- or L-Glu.
FRET efficiency of 0.2 µM AkP (blue circles); 0.2 µM AkP, 20 mM D-Glu (orange squares); 0.2 µM AkP, 20 mM L-Glu (gray diamonds). The buffer used to make all experimental solutions was 50 mM HEPES (pH 7). The dashed lines are to guide the eye. The error bars represent the standard deviation from two trials of the experiments.

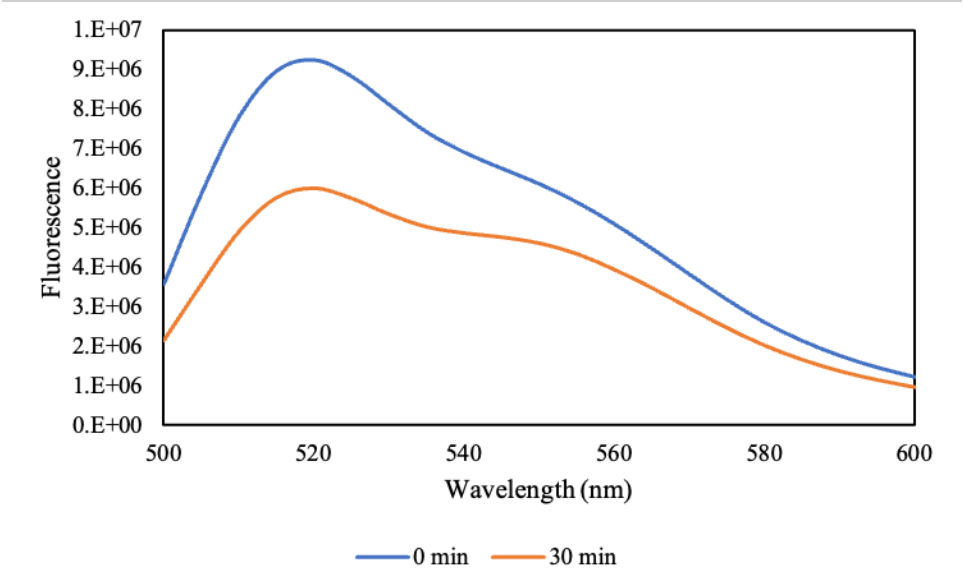

Fig. S10. Emission spectra with and without FRET.
The fluorescence spectra for an experiment that shows aggregation (0.2 μM AkP, 0.5 mM $Zn(NO_3)_2$, 0.5 mM $Mg(NO_3)_2$ and Na_2HPO_4) at 0 minutes and 30 minutes. When the dye-tagged enzymes are far apart, there is very little emission at 555 nm. When the dye-tagged enzymes come closer and begin to aggregate, the emission at 555 nm becomes more apparent.

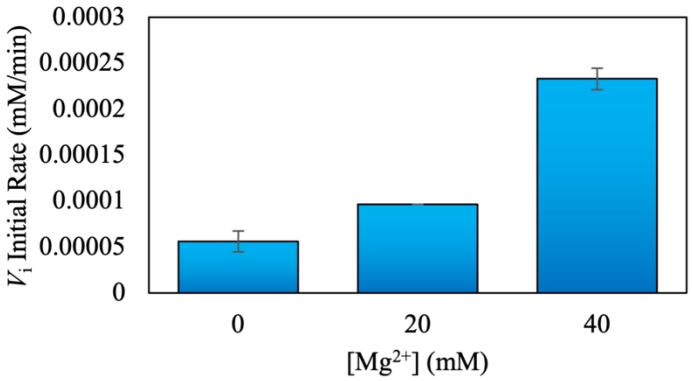

Fig. S11. Activity of HK over 60 min time period upon catalysis.
Activity of 0.2 μM tagged hexokinase from 0-60 min with 20 mM Glu, 20 mM ATP, 2.5 mM $NADP^+$ and 10 units of G6PDH while increasing the concentration of $MgCl_2$ from 0 to 40 mM. The buffer used to make all experimental solutions was 50 mM HEPES (pH 7). The error bars represent the standard deviation from two trials. The activity at 40 mM Mg^{2+} is statistically significant compared to the activity at 0 mM Mg^{2+} ($P < 0.05$; see Materials and Methods).

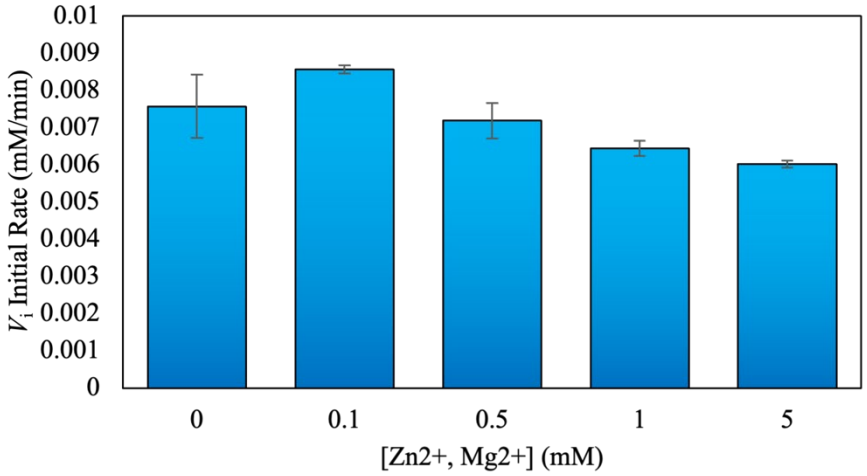

Fig. S12. Activity of AkP over 30 min time period with increasing concentrations of Zn^{2+} and Mg^{2+}.

Activity of 0.2 μM tagged alkaline phosphatase from 0-30 min with 0.5 mM p-nitrophenyl phosphate (pNPP) while increasing the concentration of zinc nitrate and magnesium nitrate from 0.1 mM to 5 mM; $[Zn^{2+}] = [Mg^{2+}]$. The buffer used to make all experimental solutions was 50 mM HEPES (pH 7). The error bars represent the standard deviation from two trials. None of the activities are statistically different from 0 mM Zn^{2+}, Mg^{2+} ($P > 0.05$; see Materials and Methods).

Table S1. Tagging parameters for the enzymes studied.
The tagging parameters for alkaline phosphatase, hexokinase, and glucose oxidase that were used for the FRET experiments. Starting concentration is the concentration of enzymes that were mixed with the dyes. The starting ratios specify the starting ratio of enzyme to dye that was used in the tagging. Additional chemicals specifies any other chemicals that were added to help the tagging, as well as the concentration used of those chemicals. The final mol dye: mol protein ratio specifies the final number of dye molecules to the number of enzyme molecules, calculated using UV-Vis spectroscopy.
* Mannose (Sigma-Aldrich) was used to prevent the tagging of the active site of HK and was added to the experimental solution at a final concentration of 10 mM.

Enzyme	Starting Conc.	Starting Enzyme:488 Dye Ratio	Starting Enzyme: 532 Dye Ratio	Additional Chemicals?	Final Mol Dye: Mol Protein
Alkaline Phosphatase	12.5 μM	1:6	1:2	No	~1.1 (488) ~1.3 (532)
Hexokinase	45.5 μM	1:2	2:1	Yes, mannose*	~2.3 (488) ~2.0 (532)
Glucose oxidase	12.5 μM	1:10	1:5	No	~ 1.1 (488) ~1.1 (532)

Table S2. pH Values of the ATP or ADP stock solutions.

A summary of the resulting pH values after adjusting the pH of the ATP or ADP solutions. pH ATP or pH ADP indicates that the pH was adjusted to be close to 7. The buffer used was 50 mM HEPES (pH 7).

Solution	Initial pH	Final pH	Volume of added 3M NaOH or Buffer
500 mM ATP	3.18	3.33	135 µL buffer
500 mM pH ATP	3.21	7.05	135 µL NaOH
500 mM ADP	3.96	4.00	64 µL buffer
500 mM pH ADP	3.94	7.06	64 µL NaOH

Table S3. pH values for the experiments outlined in Figure 7.

A summary of the pH values for **Figure 7** of the manuscript. Initial pH indicates pH at the beginning of the experiment (minute 0) and final pH indicates pH at the end of the experiment (minute 60). The colors of the table correspond to the colors used for the different FRET efficiencies in **Figure 7**.

* Indicates that hydrochloric acid (1 M; EMD) was added to lower the pH to 4.10 and 4.25 respectively, a pH similar to the experiments containing both ATP and Mg^{2+}.

Experimental Solution	Initial pH	Final pH
0.2 µM HK	7.04	7.05
0.2 µM HK*	7.00 + HCl (25 µL) 4.10	4.22
0.2 µM HK 40 mM $MgCl_2$ 20 mM pH ATP	6.91	6.93
0.2 µM HK 40 mM $MgCl_2$ 20 mM pH ATP	4.14	4.11
0.2 µM HK 40 mM $MgCl_2$ 20 mM pH ADP	6.89	6.91
0.2 µM HK 40 mM $MgCl_2$ 20 mM ADP	4.55	4.66
0.2 µM HK* 40 mM $MgCl_2$	6.93 + HCl (21 µL) 4.25	4.84

References:

(1) P.G. Wu, L. Brand. *Anal. Biochem*. 1994, **218**, 1.
(2) H.I. Okur, J. Hladílková, K.B. Rembert, Y. Cho, J. Heyda, J. Dzubiella, P.S. Cremer, P. Jungwirth. *J. Phys. Chem. B*. 2017, **121**, 1997.