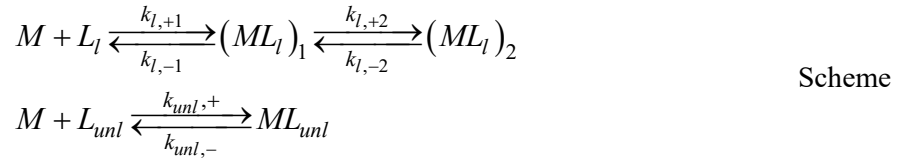**Supplemental Information**

Section S1

As an independent check of the assumptions required to fit the data in Figure 4 to a simplified two-step binding model, the mantADP binding data from Figures 3A and 4A, along with the [Gle1]-dependance of the k_{obs} for irreversible mantADP dissociation in Figure 2B, were all globally fitted using a custom MATLAB program to numerically solve for unknown parameters in Scheme 1. The relevant rate and equilibrium constants in Scheme 1 were shared across all data sets. Rate and equilibrium constants were left unconstrained and randomly initialized between 0 and 500. This fit results in the following set of parameters: $k_{45} = 1 \pm 0.4$ s^{-1}, $k_{54} = 13 \pm 6$ s^{-1}, $k_{56} = 8 \pm 7$ s^{-1}, $k_{65} = 2 \pm 1$ s^{-1}, $K_{d41} < 0.1$ µM, $K_{d52} < 0.1$ µM, $K_{d63} < 1$ µM, which are consistent with fits to the analytical solution in Figure 4B. The listed uncertainties represent the ninety-five percent confidence interval of the final converged values after many fitting iterations (n = 100).

We note that occasionally, rate constants did not converge and yielded physically unrealistic values (e.g., association rate constants far exceeding the diffusion limit). However, upon plotting it is clear that these parameters cannot match the experimental data collectively and likely represent a local, but not global minimum in the total sum of squares. Rate constants for Gle1 binding *apo*Dbp5 depend on parameter initialization, although the equilibrium constant (K_{d41}) never exceeded 0.1 µM regardless of parameter initialization (Figure S1 A, B), consistent with published data (1). The values of K_{d52} and K_{d63} never exceed ~ 1 µM, regardless of initialization (Figure S1 C-F). Together, these results validate the assumptions required for fits in Figure 4B.

Section S2

The reaction scheme for ATP and mantADP binding to Dbp5 in a kinetic competition can be described by the following general mechanism:

$$M + L_l \underset{k_{l,-1}}{\overset{k_{l,+1}}{\rightleftarrows}} (ML_l)_1 \underset{k_{l,-2}}{\overset{k_{l,+2}}{\rightleftarrows}} (ML_l)_2$$

$$M + L_{unl} \underset{k_{unl,-}}{\overset{k_{unl,+}}{\rightleftarrows}} ML_{unl}$$

Scheme

where, the unlabeled ligand L_{unl} competes to bind the macro molecule M with two-step binding of labeled ligand L_l. The time course of the reaction should follow 3 exponentials and the different equations associated with Scheme 1 are

$$\frac{d[ML_l]_1}{dt} = k_{l,+1}[M][L_l] - k_{l,-1}[ML_l]_1 - k_{l,+2}[ML_l]_1 + k_{l,-2}[ML_l]_2 \qquad (2.1)$$

$$\frac{d[ML_{unl}]}{dt} = k_{unl,+}[M][L_{unl}] - k_{unl,-}[ML_{unl}] \qquad (2.2)$$

To obtain meaningful form for unlabeled ligand concentration dependence of rate constant, suitable assumptions have to be made. Thus, we consider following 3 cases:

1. Unlabeled ligand dissociates much faster than labeled ligand binding, i.e., $k_{unl,-} \gg k_l[M][L_l]$. In this case, unlabeled ligand reaches fast equilibrium with macromolecule before labeled ligand binds.

Given rapid equilibration of L$_{unl}$ and M, $[ML_{unl}] = \dfrac{[M][L_{unl}]}{K_{unl}}$, we assume that the [L$_{unl}$] is always in excess over [M] such that:

$$[ML_{unl}] = \frac{[M][L_{unl}]_0}{K_{unl}}$$

This yields a mass balance relationship of:

$$M_{tot} = [M] + [ML_{unl}] + [ML_l]_1 + [ML_l]_2 = [M]\left(1 + \frac{[L_{unl}]_0}{K_{unl}}\right) + [ML_l]_1 + [ML_l]_2$$

$$(2.3)$$

Therefore, the two differential equations describing the reaction scheme are:

$$
\begin{pmatrix} \dfrac{d[ML_l]_1}{dt} \\[2mm] \dfrac{d[ML_l]_2}{dt} \end{pmatrix} =
$$

$$
\begin{pmatrix} -\left(k_{l,-1} + k_{l,+2} + \dfrac{k_{l,+1}[L_l]_0 K_{unl}}{[L_{unl}]_0 + K_{unl}}\right) & -\left(\dfrac{k_{l,+1}[L_l]_0 K_{unl}}{[L_{unl}]_0 + K_{unl}} - k_{l,-2}\right) \\[3mm] k_{l,+2} & -k_{l,-2} \end{pmatrix} \begin{pmatrix} [ML_l]_1 \\[2mm] [ML_l]_2 \end{pmatrix}
$$

$$
+ \begin{pmatrix} \dfrac{M_{tot} k_{l,+1} K_{unl}[L_l]_0}{[L_{unl}]_0 + K_{unl}} \\[3mm] 0 \end{pmatrix}
\qquad (2.4)
$$

The solution to Eq. 2.4 will take the following form $\overrightarrow{x(t)} = Y_c + Y_p$, where Y_c is the general solution and Y_p is the special solution. Y_c will have the following form:

$$Y_c = C_1 e^{-\lambda_1 t}\overrightarrow{\eta_1} + C_2 e^{-\lambda_2 t}\overrightarrow{\eta_2}$$

To solve for $\lambda_{1,2}$,

$$
\begin{vmatrix} -\left(k_{l,-1} + k_{l,+2} + \dfrac{k_{l,+1}[L_l]_0 K_{unl}}{[L_{unl}]_0 + K_{unl}}\right) - \lambda & -\left(\dfrac{k_{l,+1}[L_l]_0 K_{unl}}{[L_{unl}]_0 + K_{unl}} - k_{l,-2}\right) \\[3mm] k_{l,+2} & -k_{l,-2} - \lambda \end{vmatrix}
$$

$$
0 = k_{l,-1}k_{l,-2}\lambda^2 + \lambda\left(k_{l,-1} + k_{l,+2} + k_{l,-2} + \frac{k_{l,+1}[L_l]_0 K_{unl}}{[L_{unl}]_0 + K_{unl}}\right) + \frac{k_{l,+1}\left(k_{l,+2} + k_{l,-2}\right)K_{unl}[L_l]_0}{[L_{unl}]_0 + K_{unl}}
$$

Solving the roots of this equation,

$$\lambda_{1,2} = -\frac{1}{2}\left(k_{l,1} + k_{l,+2} + k_{l,-2} + \frac{k_{l,+1}[L_l]_0 K_{unl}}{[L_{unl}]_0 + K_{unl}} \pm \right.$$

$$\left. \sqrt{\left(k_{l,1} + k_{l,+2} + k_{l,-2} + \frac{k_{l,+1}[L_l]_0 K_{unl}}{[L_{unl}]_0 + K_{unl}} \right)^2 - 4\left(k_{l,-1}k_{l,-2} + \frac{k_{l,+1}k_{l,+2}K_{unl}[L_l]_0}{[L_{unl}]_0 + K_{unl}} + \frac{k_{l,+1}k_{l,-2}K_{unl}[L_l]_0}{[L_{unl}]_0 + K_{unl}} \right)} \right) \tag{2.5}$$

Now we will solve for the eigen vectors, $\overrightarrow{\eta_{1,2}}$:

$$\begin{pmatrix} -\left(k_{l,-1} + k_{l,+2} + \frac{k_{l,+1}[L_l]_0 K_{unl}}{[L_{unl}]_0 + K_{unl}} \right) - \lambda & -\left(\frac{k_{l,+1}[L_l]_0 K_{unl}}{[L_{unl}]_0 + K_{unl}} - k_{l,-2} \right) \\ k_{l,+2} & -k_{l,-2} - \lambda \end{pmatrix} \begin{pmatrix} \eta_1 \\ \eta_2 \end{pmatrix} = \begin{pmatrix} 0 \\ 0 \end{pmatrix}$$

$$\Rightarrow \eta_2 = \eta_1 \frac{k_{l,+2}}{k_{l,-2} + \lambda}$$

The eigenvector in this case is:

$$\vec{\eta}^{(1)} = \begin{pmatrix} 1 \\ \dfrac{k_{l,+2}}{k_{l,-2} + \lambda} \end{pmatrix}$$

Therefore,

$$Y_c = C_1 e^{-\lambda_1 t} \begin{pmatrix} 1 \\ \dfrac{k_{l,+2}}{k_{l,-2} + \lambda_1} \end{pmatrix} + C_2 e^{-\lambda_2 t} \begin{pmatrix} 1 \\ \dfrac{k_{l,+2}}{k_{l,-2} + \lambda_2} \end{pmatrix}$$

Now we will solve for Y_p:

$$\vec{x}' - A\vec{x} = \vec{g}, \text{ where } \vec{g} = \begin{pmatrix} \dfrac{M_{tot}k_{l,+1}K_{unl}[L_l]_0}{[L_{unl}]_0 + K_{unl}} \\ 0 \end{pmatrix}$$

Therefore, Y_p will be a constant vector, $\vec{B}$:

$$\vec{0} - A\vec{B} = \begin{pmatrix} \dfrac{M_{tot}k_{l,+1}K_{unl}[L_l]_0}{[L_{unl}]_0 + K_{unl}} \\ 0 \end{pmatrix} \Rightarrow \vec{B} = A^{-1} \begin{pmatrix} \dfrac{M_{tot}k_{l,+1}K_{unl}[L_l]_0}{[L_{unl}]_0 + K_{unl}} \\ 0 \end{pmatrix}$$

Therefore,

$$\vec{B} = -\frac{M_{tot}k_{l,+1}K_{unl}[L_l]_0}{k_{l,-1}k_{l,-2}\left([L_{unl}]_0 + K_{unl}\right) + k_{l,+1}[L_l]_0 K_{unl}\left(k_{l,+2} + k_{l,-2}\right)} \begin{pmatrix} k_{l,-2} \\ k_{l,+2} \end{pmatrix}$$

The general solution is:

$$\begin{pmatrix} [ML_l]_1(t) \\ [ML_l]_2(t) \end{pmatrix} = C_1 e^{-\lambda_1 t} \begin{pmatrix} 1 \\ \dfrac{k_{l,+2}}{k_{l,-2} + \lambda_1} \end{pmatrix} + C_2 e^{-\lambda_2 t} \begin{pmatrix} 1 \\ \dfrac{k_{l,+2}}{k_{l,-2} + \lambda_2} \end{pmatrix}$$

$$- \frac{M_{tot} k_{l,+1} K_{unl} [L_l]_0}{k_{l,-1} k_{l,-2} \left([L_{unl}]_0 + K_{unl} \right) + k_{l,+1} [L_l]_0 K_{unl} \left(k_{l,+2} + k_{l,-2} \right)} \begin{pmatrix} k_{l,-2} \\ k_{l,+2} \end{pmatrix}$$

(2.6)

To solve for the arbitrary constants, $C_{1,2}$, in Eq. 2.6 we use the initial conditions at $t = 0$. At equilibrium, prior to mixing (i.e., $t = 0$) $[ML_l]_1(0)$ and $[ML_l]_2(0)$ are described by the following reaction scheme:
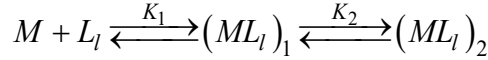
$$M + L_l \xrightleftharpoons{K_1} (ML_l)_1 \xrightleftharpoons{K_2} (ML_l)_2$$

Where $K_1 = \dfrac{k_{l,-1}}{k_{l,+1}} = \dfrac{[M][L_l]_0}{[ML_l]_1}$ and $K_2 = \dfrac{k_{l,-2}}{k_{l,+2}} = \dfrac{[ML_l]_1}{[ML_l]_2}$ if we assume $[L_l] \gg [M]_{tot}$ then, $[L_l]_{tot} \sim [L_l]_0$.

$$[ML_l]_1 + [ML_l]_2 = [ML_l]_1 + \frac{[ML_l]_1}{K_2} = \frac{1 + K_2}{K_2}[ML_l]_1$$

$$\Rightarrow [ML_l]_1 = \frac{K_2}{1 + K_2}([ML_l]_1 + [ML_l]_2)$$

(2.7)

$$\Rightarrow [ML_l]_2 = \frac{1}{1 + K_2}([ML_l]_1 + [ML_l]_2)$$

From mass balance, $[M]_{tot} = [M]+[ML_l]_1+[ML_l]_2$ and K_1, K_2, and Eq. 2.7:

$$[M]_{tot} = \frac{K_1 K_2}{1 + K_2} \frac{[ML_l]_1 + [ML_l]_2}{[L_l]_0} + [ML_l]_1 + [ML_l]_2$$

(2.8)

Solving Eq. 2.8 for $[ML_l]_1 + [ML_l]_2$ yields,

$$[ML_l]_1 + [ML_l]_2 = \frac{[M]_{tot}[L_l]_0}{[L_l]_0 + K_2 + K_1 K_2}$$

(2.9)

Substituting Eq. 2.9 into K_1 and K_2 yields,

$$[ML_l]_1(0) = \frac{[M]_{tot}[L_l]_0 K_2}{(1 + K_2)([L_l]_0 + K_2 + K_1 K_2)}$$

$$[ML_l]_2(0) = \frac{[M]_{tot}[L_l]_0}{(1 + K_2)([L_l]_0 + K_2 + K_1 K_2)}$$

(2.10)

Substituting Eq. 2.10 into Eq. 2.6 at $t = 0$ yields,

$$\begin{pmatrix} [ML_l]_1(0) \\ [ML_l]_2(0) \end{pmatrix} = \begin{pmatrix} \dfrac{[M]_{tot}[L_l]_0 K_2}{(1+K_2)([L_l]_0 + K_2 + K_1 K_2)} \\ \dfrac{[M]_{tot}[L_l]_0}{(1+K_2)([L_l]_0 + K_2 + K_1 K_2)} \end{pmatrix} = C_1 e^{-\lambda_1 t} \begin{pmatrix} 1 \\ \dfrac{k_{l,+2}}{k_{l,-2} + \lambda_1} \end{pmatrix} + C_2 e^{-\lambda_2 t} \begin{pmatrix} 1 \\ \dfrac{k_{l,+2}}{k_{l,-2} + \lambda_2} \end{pmatrix} \quad (2.11)$$

$$- \frac{M_{tot} k_{l,+1} K_{unl}[L_l]_0}{k_{l,-1} k_{l,-2}\left([L_{unl}]_0 + K_{unl}\right) + k_{l,+1}[L_l]_0 K_{unl}\left(k_{l,+2} + k_{l,-2}\right)} \begin{pmatrix} k_{l,-2} \\ k_{l,+2} \end{pmatrix}$$

Solving Eq. 2.11 for $C_{1,2}$ yields,

$$C_1 =$$
$$-\frac{M_{tot} k_{l,+1}(k_{l,-2} + \lambda_1)\lambda_2[L_l]_0\left([L_{unl}]_0 k_{l,-1} k_{l,+2} k_{l,-2} + k_{l,-1} k_{l,-2}(2k_{l,+2} + k_{l,-2})K_{unl} + k_{l,+1}(k_{l,+2} + k_{l,-2})K_{unl}(k_{l,-2} + 2k_{l,+2}[L_l]_0)\right)}{(k_{l,+2} + k_{l,-2})(\lambda_1 - \lambda_2)([L_{unl}]_0 k_{l,-1} k_{l,-2} + k_{l,-1} k_{l,-2} K_{unl} + k_{l,+1}(k_{l,+2} + k_{l,-2})K_{unl}[L_l]_0)(k_{l,-1} k_{l,-2} + k_{l,+1}(k_{l,-2} + k_{l,+2}[L_l]_0))}$$
$$C_2 =$$
$$-\frac{M_{tot} k_{l,+1}(k_{l,-2} + \lambda_2)\lambda_1[L_l]_0\left([L_{unl}]_0 k_{l,-1} k_{l,+2} k_{l,-2} + k_{l,-1} k_{l,-2}(2k_{l,+2} + k_{l,-2})K_{unl} + k_{l,+1}(k_{l,+2} + k_{l,-2})K_{unl}(k_{l,-2} + 2k_{l,+2}[L_l]_0)\right)}{(k_{l,+2} + k_{l,-2})(\lambda_1 - \lambda_2)([L_{unl}]_0 k_{l,-1} k_{l,-2} + k_{l,-1} k_{l,-2} K_{unl} + k_{l,+1}(k_{l,+2} + k_{l,-2})K_{unl}[L_l]_0)(k_{l,-1} k_{l,-2} + k_{l,+1}(k_{l,-2} + k_{l,+2}[L_l]_0))}$$

2. Unlabeled ligand binding is comparable to first binding step of labeled ligand, i.e., $k_{unl,+} \sim k_{l,+1}$, and both dissociation of unlabeled ligand and the first step of labeled ligand is much faster than labeled ligand second step binding, i.e., $k_{unl,-}, k_{l,-1} \gg [ML_l] k_{l,+2}$. In this case, the macromolecule reaches equilibrium with $[ML_l]_1$ and $[M_{unl}]$ before $[ML_l]_2$ is significantly populated. Therefore, in the time region after the first step of labeled ligand binding and unlabeled ligand binding reach equilibrium, but before labeled ligand second step binding significantly starts, according to mass conservation, the total macromolecule is approximated to

$$[M]_{tot} = [M] + [ML_{unl}] + [ML_l]_1 + [ML_l]_2 \sim [M] + [ML_{unl}] + [ML_l]_1 \quad (2.12)$$

Eq. 2.1 is reduced to

$$\frac{d[ML_l]_1}{dt} \sim k_{l,+1}[M][L_l] - \left(k_{l,-1} + k_{l,+2}\right)[ML_l]_1$$
$$= k_{l,+1}\left([M]_{tot} - [ML_l]_1 - [ML_{unl}]\right)[L_l] - \left(k_{l,-1} + k_{l,+2}\right)[ML_l]_1$$
$$= k_{l,+1}[L_l][M]_{tot} - \left(k_{l,+1}[L_l] + k_{l,-1} + k_{l,+2}\right)[ML_l]_1 - k_{l,+1}[L_l][ML_{unl}]$$

i.e.,

$$\left(\frac{d}{dt} + k_{l,+1}[L_l] + k_{l,-1} + k_{l,+2}\right)[ML_l]_1 + k_{l,+1}[L_l][ML_{unl}] = k_{l,+1}[L_l][M]_{tot} \quad (2.13)$$

Eq 2.2 becomes

$$\frac{d[ML_{unl}]}{dt} = k_{unl,+} \left([M]_{tot} - [ML_l]_1 - [ML_{unl}] \right)[L_{unl}] - k_{unl,-}[ML_{unl}]$$

$$= k_{unl,+}[L_{unl}][M]_{tot} - k_{unl,+}[L_{unl}][ML_l]_1 - \left(k_{unl,+}[L_{unl}] + k_{unl,-} \right)[ML_{unl}]$$

i.e.,

$$k_{unl,+}[L_{unl}][ML_l]_1 + \left(\frac{d}{dt} + k_{unl,+}[L_{unl}] + k_{unl,-} \right)[ML_{unl}] = k_{unl,+}[L_{unl}][M]_{tot} \qquad (2.14)$$

The equation to solve Eigen values of Eqs. 2.13 and 2.14 is

$$\begin{vmatrix} -\lambda + k_{l,+1}[L_l] + k_{l,-1} + k_{l,+2} & k_{l,+1}[L_l] \\ k_{unl,+}[L_{unl}] & -\lambda + k_{unl,+}[L_{unl}] + k_{unl,-} \end{vmatrix}$$

$$= \left(-\lambda + k_{l,+1}[L_l] + k_{l,-1} + k_{l,+2} \right)\left(-\lambda + k_{unl,+}[L_{unl}] + k_{unl,-} \right) - k_{l,+1}[L_l]k_{unl,+}[L_{unl}]$$

$$= \lambda^2 - \left(k_{l,+1}[L_l] + k_{l,-1} + k_{l,+2} + k_{unl,+}[L_{unl}] + k_{unl,-} \right)$$

$$+ \left(k_{l,-1} + k_{l,+2} \right)k_{unl,+}[L_{unl}] + \left(k_{l,+1}[L_l] + k_{l,-1} + k_{l,+2} \right)k_{unl,-} = 0$$

The solutions of the equation are the two observed rate constants of the period,

$$\lambda_{1,2} = \frac{1}{2}\Big(k_{l,+1}[L_l] + k_{l,-1} + k_{l,+2} + k_{unl,+}[L_{unl}] + k_{unl,-}$$

$$\pm \sqrt{\left(k_{l,+1}[L_l] + k_{l,-1} + k_{l,+2} + k_{unl,+}[L_{unl}] + k_{unl,-} \right)^2 - 4\left(k_{l,-1} + k_{l,+2} \right)k_{unl,+}[L_{unl}] - 4\left(k_{l,+1}[L_l] + k_{l,-1} + k_{l,+2} \right)k_{unl,-}} \Big)$$

$$(2.15)$$

To further characterize the features of the two observed rate constants, further approximation is needed. The faster observed rate constant of the two can be re-written as

$$\lambda_1 = \frac{1}{2}\Big(k_{l,+1}[L_l] + k_{l,-1} + k_{l,+2} + k_{unl,+}[L_{unl}] + k_{unl,-}$$

$$+ \sqrt{\left(k_{l,+1}[L_l] + k_{l,-1} + k_{l,+2} + k_{unl,+}[L_{unl}] + k_{unl,-} \right)^2 - 4\left(k_{l,-1} + k_{l,+2} \right)k_{unl,+}[L_{unl}] - 4\left(k_{l,+1}[L_l] + k_{l,-1} + k_{l,+2} \right)k_{unl,-}} \Big)$$

$$= \frac{1}{2}\Big(k_{l,+1}[L_l] + k_{l,-1} + k_{l,+2} + k_{unl,+}[L_{unl}] + k_{unl,-}$$

$$+ \sqrt{\left(k_{l,+1}[L_l] + k_{l,-1} + k_{l,+2} + k_{unl,+}[L_{unl}] - k_{unl,-} \right)^2 + 4k_{unl,+}[L_{unl}]\left(k_{unl,-} - k_{l,-1} - k_{l,+2} \right)} \Big)$$

$$(2.16)$$

or

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$$= \frac{1}{2}\Big(k_{l,+1}[L_l] + k_{l,-1} + k_{l,+2} + k_{unl,+}[L_{unl}] + k_{unl,-}$$

$$+\sqrt{\big(k_{l,+1}[L_l] - k_{l,-1} - k_{l,+2} + k_{unl,+}[L_{unl}] + k_{unl,-}\big)^2 + 4k_{l,+1}[L_l]\big(k_{l,-1} + k_{l,+2} - k_{unl,-}\big)}\Big) \qquad (2.16')$$

It means the value of λ_1 is always between $k_{l,+1}[L_l] + k_{l,-1} + k_{l,+2} + k_{unl,+}[L_{unl}]$ and $k_{l,+1}[L_l] + k_{unl,+}[L_{unl}] + k_{unl,-}$ for all titrating $[L_{unl}]$ unlabeled ligand concentration, i.e.,

$$k_{l,+1}[L_l] + k_{l,-1} + k_{l,+2} + k_{unl,+}[L_{unl}] \geq \lambda_1 > k_{l,+1}[L_l] + k_{unl,+}[L_{unl}] + k_{unl,-}, \quad \text{if } k_{l,-1} + k_{l,+2} \geq k_{unl,-}$$

$$k_{l,+1}[L_l] + k_{l,-1} + k_{l,+2} + k_{unl,+}[L_{unl}] \leq \lambda_1 < k_{l,+1}[L_l] + k_{unl,+}[L_{unl}] + k_{unl,-}, \quad \text{if } k_{l,-1} + k_{l,+2} < k_{unl,-}$$

$$(2.17)$$

At $[L_{unl}] = 0$, it should $\lambda_1 = k_{l,+1}[L_l] + k_{l,-1} + k_{l,+2}$, therefore, we choose

$$\lambda_1 \sim k_{l,+1}[L_l] + k_{l,-1} + k_{l,+2} + k_{unl,+}[L_{unl}] \qquad (2.18)$$

to be approximated faster observed rate constant for any unlabeled ligand concentration for consistency with the case when $[L_{unl}] = 0$. It is a linear function of $[L_{unl}]$ with y-incept of $k_{l,+1}[L_l] + k_{l,-1} + k_{l,+2}$ and slope of $k_{unl,+}$. With this approximation, the slower observed rate constant become

$$\lambda_2 = \frac{\lambda_1 \lambda_2}{\lambda_1} = \frac{\big(k_{l,-1} + k_{l,+2}\big)k_{unl,+}[L_{unl}] + \big(k_{l,+1}[L_l] + k_{l,-1} + k_{l,+2}\big)k_{unl,-}}{\lambda_1}$$

$$\sim \frac{\big(k_{l,-1} + k_{l,+2}\big)k_{unl,+}[L_{unl}] + \big(k_{l,+1}[L_l] + k_{l,-1} + k_{l,+2}\big)k_{unl,-}}{k_{l,+1}[L_l] + k_{l,-1} + k_{l,+2} + k_{unl,+}[L_{unl}]}$$

$$= \frac{\big(k_{l,-1} + k_{l,+2}\big)k_{unl,+}[L_{unl}] + \big(k_{l,+1}[L_l] + k_{l,-1} + k_{l,+2} + k_{unl,+}[L_{unl}] - k_{unl,+}[L_{unl}]\big)k_{unl,-}}{k_{l,+1}[L_l] + k_{l,-1} + k_{l,+2} + k_{unl,+}[L_{unl}]}$$

$$= k_{unl,-} + \frac{k_{l,-1} + k_{l,+2} - k_{unl,-}}{\dfrac{k_{l,+1}[L_l] + k_{l,-1} + k_{l,+2}}{k_{unl,+}} + [L_{unl}]}[L_{unl}] \qquad (2.19)$$

Therefore, λ_2 is approximately a hyperbola with initial and final values of $k_{unl,-}$ and $k_{l,-1} + k_{i,+2}$ and it reaches a half of maximum at $[L_{unl}] = \dfrac{k_{l,+1}[L_l] + k_{l,-1} + k_{l,+2}}{k_{unl,+}}$.

In time region after unlabeled ligand binding and the fist step of the ligand binding reach equilibrium, Scheme 2 has only one differential equation left

$$\frac{d[ML_l]_2}{dt} = k_{l,+2}[ML_l]_1 - k_{l,-2}[ML_l]_2 \qquad (2.20)$$

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According to equilibrium among ligand unbound macromolecule, those with unlabeled ligand bound and the first species of labeled ligand bound, we have

$$[ML_l]_1 = \frac{[M][L_l]}{K_{l,1}}$$

$$[ML_{unl}] = \frac{[M][L_{unl}]}{K_{unl}}$$

(2.21)

and

$$[M]_{tot} = [M] + [ML_l]_1 + [ML_l]_2 + [ML_{unl}]$$

$$= \frac{K_{l,1}[ML_l]_1}{[L_l]}\left(1 + \frac{[L_{unl}]}{K_{unl}}\right) + [ML_l]_1 + [ML_l]_2$$

$$= \left(\frac{K_{l,1}}{[L_l]}\left(1 + \frac{[L_{unl}]}{K_{unl}}\right) + 1\right)[ML_l]_1 + [ML_l]_2$$

(2.22)

Substitute Eq. 2.22 into Eq. 2.20

$$\frac{d[ML_l]_2}{dt} = k_{l,+2}\frac{[M]_{tot} - [ML_l]_2}{\frac{K_{l,1}}{[L_l]}\left(1 + \frac{[L_{unl}]}{K_{unl}}\right) + 1} - k_{l,-2}[ML_l]_2$$

$$= \frac{k_{l,+2}[M]_{tot}}{\frac{K_{l,1}}{[L_l]}\left(1 + \frac{[L_{unl}]}{K_{unl}}\right) + 1} - \left(\frac{k_{l,+2}}{\frac{K_{l,1}}{[L_l]}\left(1 + \frac{[L_{unl}]}{K_{unl}}\right) + 1} + k_{l,-2}\right)[ML_l]_2$$

(2.23)

The above first order linear equation has following solution

$$[ML_l]_2 = A_3 e^{-\lambda_3 t} + \frac{\dfrac{k_{l,+2}[M]_{tot}}{\dfrac{K_{l,1}}{[L_l]}\left(1 + \dfrac{[L_{unl}]}{K_{unl}}\right) + 1}}{\dfrac{k_{l,+2}}{\dfrac{K_{l,1}}{[L_l]}\left(1 + \dfrac{[L_{unl}]}{K_{unl}}\right) + 1} + k_{l,-2}}$$

$$= A_3 e^{-\lambda_3 t} + \frac{k_{l,+2}[M]_{tot}}{k_{l,+2} + k_{l,-2}\left(\dfrac{K_{l,1}}{[L_l]}\left(1 + \dfrac{[L_{unl}]}{K_{unl}}\right) + 1\right)}$$

with rate constant

$$\lambda_3 = \frac{k_{l,+2}}{\dfrac{K_{l,1}}{[L_l]}\left(1+\dfrac{[L_{unl}]}{K_{unl}}\right)+1} + k_{l,-2}$$

$$= \frac{\dfrac{K_{unl}[L_l]}{K_{l,1}}k_{l,+2}}{K_{unl}\left(1+\dfrac{[L_l]}{K_{l,1}}\right)+[L_{unl}]} + k_{l,-2}$$

$$= \frac{\dfrac{k_{l,+2}[L_l]}{\left(K_{l,1}+[L_l]\right)}\left(K_{unl}\left(1+\dfrac{[L_l]}{K_{l,1}}\right)+[L_{unl}]-[L_{unl}]\right)}{K_{unl}\left(1+\dfrac{[L_l]}{K_{l,1}}\right)+[L_{unl}]} + k_{l,-2}$$

$$= \frac{k_{l,+2}[L_l]}{\left(K_{l,1}+[L_l]\right)} + k_{l,-2} - \frac{\dfrac{k_{l,+2}[L_l]}{K_{l,1}+[L_l]}[L_{unl}]}{K_{unl}\left(1+\dfrac{[L_l]}{K_{l,1}}\right)+[L_{unl}]}$$

(2.24)

The third observed rate constant is a downward hyperbola of $[L_{unl}]$ with initial and final values of $\dfrac{k_{l,+2}[L_l]}{\left(K_{l,1}+[L_l]\right)}+k_{l,-2}$ and $k_{l,-2}$, and it reaches a half of maximum when gaa $[L_{unl}]=K_{unl}\left(1+\dfrac{[L_l]}{K_{l,1}}\right)$.

In short, in this case that unlabeled ligand binding is comparable to the first step of labeled ligand the binding, the time course follows 3 exponentials and the fastest observed rate constant scales with unlabeled ligand concentration, while the two slower observed phases are hyperbolic functions.

Time courses in Figure 5A were fit to a sum of two or three exponential functions (continuous lines) and the observed rate constants were globally fit (Figure 5B, solid lines) to Eq (A) below (from Eqs. S2.18, S2.19 and S2.24):

$$\lambda_1 \sim k_{l,+1}[L_l] + k_{l,-1} + k_{l,+2} + k_{unl,+}[L_{unl}]$$

$$\lambda_2 = k_{unl,-} + \frac{k_{l,-1}+k_{l,+2}-k_{unl,-}}{\dfrac{k_{l,+1}[L_l]+k_{l,-1}+k_{l,+2}}{k_{unl,+}}+[L_{unl}]}[L_{unl}] \qquad (A)$$

$$\lambda_3 = \frac{k_{l,+2}}{\dfrac{K_{l,1}}{[L_l]}\left(1+\dfrac{[L_{unl}]}{K_{unl}}\right)+1} + k_{l,-2}$$

Where L_l is mantADP, L_{unl} is ATP, $K_{l,1}$ is the equilibrium constant for the first mantADP binding step, K_{unl} is the equilibrium constant for ATP binding, and $k_{unl,-}$, $k_{unl,+}$, $k_{l,+1}$, $k_{l,-1}$, $k_{l,+2}$, $k_{l,-2}$ correspond to k_{-ATP}, k_{+ATP}, k_{45}, k_{54}, k_{56}, and k_{65} in Scheme 2. The best fit estimates for k_{+ATP} and k_{-ATP} from Eq.(A) are 0.6 ± 0.1 μM^{-1} s^{-1} and 3.3 ± 1.1 s^{-1} (Table 1), respectively, consistent with MATLAB fittings. We note that

modeling the ATP kinetic competition data with Scheme 2 is valid as little hydrolysis occurs within the time scales of the mantADP binding time courses (see **Results**).

3. Unlabeled ligand binding is comparable to the slow step of labeled ligand binding and both are much slower than the first step binding of labeled ligand, In this case, $k_{unl,+}[L_{unl}] \sim k_{l,+2} << k_{l,-1}$, the labeled ligand first binding step reaches equilibrium before labeled ligand second step binding and unlabeled ligand binding taking place. The equations for this case are

$$\frac{d[ML_l]_2}{dt} = k_{l,+2}[ML_l]_1 - k_{l,-2}[ML_l]_2 \tag{2.25}$$

$$\frac{d[ML_{unl}]}{dt} = k_{unl,+}[M][L_{unl}] - k_{unl,-}[ML_{unl}] \tag{2.26}$$

$$[ML_l]_1 = \frac{[M][L_l]}{K_{l,1}} \tag{2.27}$$

$$\begin{aligned}
[M]_{tot} &= [M] + [ML_l]_1 + [ML_l]_2 + [ML_{unl}] \\
&= \left(1 + \frac{[L_l]}{K_{l,1}}\right)[M] + [ML_l]_2 + [ML_{unl}] \\
&= \left(\frac{K_{l,1}}{[L_l]} + 1\right)[ML_l]_1 + [ML_l]_2 + [ML_{unl}]
\end{aligned} \tag{2.28}$$

Equation 2.25 and 2.26 can be re-written as

$$\begin{aligned}
\frac{d[ML_l]_2}{dt} &= k_{l,+2}\frac{[M]_{tot} - [ML_l]_2 - [ML_{unl}]}{1 + \dfrac{K_{l,1}}{[L_l]}} - k_{l,-2}[ML_l]_2 \\
&= \frac{k_{l,+2}[M]_{tot}}{1 + \dfrac{K_{l,1}}{[L_l]}} - \left(\frac{k_{l,+2}}{1 + \dfrac{K_{l,1}}{[L_l]}} + k_{l,-2}\right)[ML_l]_2 - k_{l,+2}\frac{[ML_{unl}]}{1 + \dfrac{K_{l,1}}{[L_l]}}
\end{aligned} \tag{2.29}$$

$$\begin{aligned}
\frac{d[ML_{unl}]}{dt} &= k_{unl,+}[L_{unl}]\frac{[M]_{tot} - [ML_l]_2 - [ML_{unl}]}{1 + \dfrac{[L_l]}{K_{l,1}}} - k_{unl,-}[ML_{unl}] \\
&= \frac{k_{unl,+}[L_{unl}][M]_{tot}}{1 + \dfrac{[L_l]}{K_{l,1}}} - \frac{k_{unl,+}[L_{unl}][ML_l]_2}{1 + \dfrac{[L_l]}{K_{l,1}}} - \left(\frac{k_{unl,+}[L_{unl}]}{1 + \dfrac{[L_l]}{K_{l,1}}} + k_{unl,-}\right)[ML_{unl}]
\end{aligned} \tag{2.30}$$

In this case, the time course follows 2 exponentials and according to Eqs. 2.29 and 2.30, the equation for the Eigen values of differential equations Eqs. 2.29 and 2.30 is

$$\begin{vmatrix} -\lambda + \dfrac{k_{l,+2}}{1+\dfrac{K_{l,1}}{[L_l]}} + k_{l,-2} & \dfrac{k_{l,+2}}{1+\dfrac{K_{l,1}}{[L_l]}} \\[4ex] \dfrac{k_{unl,+}[L_{unl}]}{1+\dfrac{[L_l]}{K_{l,1}}} & -\lambda + \dfrac{k_{unl,+}[L_{unl}]}{1+\dfrac{[L_l]}{K_{l,1}}} + k_{unl,-} \end{vmatrix}$$

$$= \left(-\lambda + \dfrac{k_{l,+2}}{1+\dfrac{K_{l,1}}{[L_l]}} + k_{l,-2} \right)\left(-\lambda + \dfrac{k_{unl,+}[L_{unl}]}{1+\dfrac{[L_l]}{K_{l,1}}} + k_{unl,-} \right) - \dfrac{k_{l,+2}}{1+\dfrac{K_{l,1}}{[L_l]}} \dfrac{k_{unl,+}[L_{unl}]}{1+\dfrac{[L_l]}{K_{l,1}}}$$

$$= \lambda^2 - \left(\dfrac{k_{l,+2}}{1+\dfrac{K_{l,1}}{[L_l]}} + k_{l,-2} + \dfrac{k_{unl,+}[L_{unl}]}{1+\dfrac{[L_l]}{K_{l,1}}} + k_{unl,-} \right)\lambda + k_{l,-2}\dfrac{k_{unl,+}[L_{unl}]}{1+\dfrac{[L_l]}{K_{l,1}}} + \left(\dfrac{k_{l,+2}}{1+\dfrac{K_{l,1}}{[L_l]}} + k_{l,-2} \right)k_{unl,-} = 0$$

The solutions are

$$\lambda_{\pm} = \dfrac{1}{2}\left(\dfrac{k_{l,+2}}{1+\dfrac{K_{l,1}}{[L_l]}} + k_{l,-2} + \dfrac{k_{unl,+}[L_{unl}]}{1+\dfrac{[L_l]}{K_{l,1}}} + k_{unl,-} \right.$$

$$\left. \pm \sqrt{\left(\dfrac{k_{l,+2}}{1+\dfrac{K_{l,1}}{[L_l]}} + k_{l,-2} + \dfrac{k_{unl,+}[L_{unl}]}{1+\dfrac{[L_l]}{K_{l,1}}} + k_{unl,-} \right)^2 - 4k_{l,-2}\dfrac{k_{unl,+}[L_{unl}]}{1+\dfrac{[L_l]}{K_{l,1}}} - 4\left(\dfrac{k_{l,+2}}{1+\dfrac{K_{l,1}}{[L_l]}} + k_{l,-2} \right)k_{unl,-}} \right)$$

$$(2.31)$$

For further approximation, the fast rate constant in Eq. 2.31 can be re-written as

$$\lambda_+ = \frac{1}{2}\left(\frac{k_{l,+2}}{1+\dfrac{K_{l,1}}{[L_l]}} + k_{l,-2} + \frac{k_{unl,+}[L_{unl}]}{1+\dfrac{[L_l]}{K_{l,1}}} + k_{unl,-} \right.$$

$$\left. + \sqrt{\left(\frac{k_{l,+2}}{1+\dfrac{K_{l,1}}{[L_l]}} + k_{l,-2} + \frac{k_{unl,+}[L_{unl}]}{1+\dfrac{[L_l]}{K_{l,1}}} - k_{unl,-} \right)^2 + 4\left(k_{unl,-} - 4k_{l,-2}\right)\frac{k_{unl,+}[L_{unl}]}{1+\dfrac{[L_l]}{K_{l,1}}}} \right) \qquad (2.32)$$

or

$$= \frac{1}{2}\left(\frac{k_{l,+2}}{1+\dfrac{K_{l,1}}{[L_l]}} + k_{l,-2} + \frac{k_{unl,+}[L_{unl}]}{1+\dfrac{[L_l]}{K_{l,1}}} + k_{unl,-} \right.$$

$$\left. + \sqrt{\left(\frac{k_{l,+2}}{1+\dfrac{K_{l,1}}{[L_l]}} - k_{l,-2} + \frac{k_{unl,+}[L_{unl}]}{1+\dfrac{[L_l]}{K_{l,1}}} + k_{unl,-} \right)^2 + 4\frac{k_{l,+2}}{1+\dfrac{K_{l,1}}{[L_l]}}\left(k_{l,-2} - k_{unl,-}\right)} \right) \qquad (2.32')$$

Apparently, the fast observed rate constant in this case is between $\dfrac{k_{l,+2}}{1+\dfrac{K_{l,1}}{[L_l]}} + k_{l,-2} + \dfrac{k_{unl,+}[L_{unl}]}{1+\dfrac{[L_l]}{K_{l,1}}}$

and $\dfrac{k_{l,+2}}{1+\dfrac{K_{l,1}}{[L_l]}} + \dfrac{k_{unl,+}[L_{unl}]}{1+\dfrac{[L_l]}{K_{l,1}}} + k_{unl,-}$, i.e.,

$$\frac{k_{l,+2}}{1+\dfrac{K_{l,1}}{[L_l]}} + k_{l,-2} + \frac{k_{unl,+}[L_{unl}]}{1+\dfrac{[L_l]}{K_{l,1}}} \geq \lambda_+ > \frac{k_{l,+2}}{1+\dfrac{K_{l,1}}{[L_l]}} + \frac{k_{unl,+}[L_{unl}]}{1+\dfrac{[L_l]}{K_{l,1}}} + k_{unl,-}, \quad \text{if } k_{l,-2} \geq k_{unl,-}$$

$$\frac{k_{l,+2}}{1+\dfrac{K_{l,1}}{[L_l]}} + k_{l,-2} + \frac{k_{unl,+}[L_{unl}]}{1+\dfrac{[L_l]}{K_{l,1}}} \leq \lambda_+ < \frac{k_{l,+2}}{1+\dfrac{K_{l,1}}{[L_l]}} + \frac{k_{unl,+}[L_{unl}]}{1+\dfrac{[L_l]}{K_{l,1}}} + k_{unl,-}, \quad \text{if } k_{l,-2} < k_{unl,-}$$

$$(2.33)$$

To be consistent with $\lambda_+ = \dfrac{k_{l,+2}}{1+\dfrac{K_{l,1}}{[L_l]}} + k_{l,-2}$, when $[L_{unl}] = 0$, we choose the approximated form of

the fast rate constant to be

$$\lambda_+ \sim \frac{k_{l,+2}}{1+\dfrac{K_{l,1}}{[L_l]}} + k_{l,-2} + \frac{k_{unl,+}[L_{unl}]}{1+\dfrac{[L_l]}{K_{l,1}}} \tag{2.34}$$

at any unlabeled ligand concentration. It is a linear function of unlabeled ligand concentration with y-intercept of $\dfrac{k_{l,+2}}{1+\dfrac{K_{l,1}}{[L_l]}} + k_{l,-2}$ and slope of $\dfrac{k_{unl,+}}{1+\dfrac{[L_l]}{K_{l,1}}}$. Under this fast phase approximation, the slow phase is approximated to

$$\lambda_- = \frac{\lambda_-\lambda_+}{\lambda_+} \sim \frac{k_{l,-2}\dfrac{k_{unl,+}[L_{unl}]}{1+\dfrac{[L_l]}{K_{l,1}}} + \left(\dfrac{k_{l,+2}}{1+\dfrac{K_{l,1}}{[L_l]}}+k_{l,-2}\right)k_{unl,-}}{\dfrac{k_{l,+2}}{1+\dfrac{K_{l,1}}{[L_l]}} + k_{l,-2} + \dfrac{k_{unl,+}[L_{unl}]}{1+\dfrac{[L_l]}{K_{l,1}}}}$$

$$= \frac{k_{l,-2}\dfrac{k_{unl,+}[L_{unl}]}{1+\dfrac{[L_l]}{K_{l,1}}} + \left(\dfrac{k_{l,+2}}{1+\dfrac{K_{l,1}}{[L_l]}}+k_{l,-2}+\dfrac{k_{unl,+}[L_{unl}]}{1+\dfrac{[L_l]}{K_{l,1}}}-\dfrac{k_{unl,+}[L_{unl}]}{1+\dfrac{[L_l]}{K_{l,1}}}\right)k_{unl,-}}{\dfrac{k_{l,+2}}{1+\dfrac{K_{l,1}}{[L_l]}} + k_{l,-2} + \dfrac{k_{unl,+}[L_{unl}]}{1+\dfrac{[L_l]}{K_{l,1}}}}$$

$$= k_{unl,-} + \frac{k_{l,-2}\dfrac{k_{unl,+}[L_{unl}]}{1+\dfrac{[L_l]}{K_{l,1}}} - \dfrac{k_{unl,+}[L_{unl}]}{1+\dfrac{[L_l]}{K_{l,1}}}k_{unl,-}}{\dfrac{k_{l,+2}}{1+\dfrac{K_{l,1}}{[L_l]}} + k_{l,-2} + \dfrac{k_{unl,+}[L_{unl}]}{1+\dfrac{[L_l]}{K_{l,1}}}}$$

$$= k_{unl,-} + \frac{\left(k_{l,-2}-k_{unl,-}\right)[L_{unl}]}{\dfrac{K_{l,1}+[L_l]}{K_{l,1}k_{unl,+}}\left(\dfrac{k_{l,+2}[L_l]}{[L_l]+K_{l,1}}+k_{l,-2}\right)+[L_{unl}]} \tag{2.35}$$

The approximated slow phase is a hyperbola with initial and final values of $k_{unl,-}$ and $k_{l,-2}$, and it reaches a half of maximum at $[L_{unl}] = \dfrac{K_{l,1}+[L_l]}{K_{l,1}k_{unl,+}}\left(\dfrac{k_{l,+2}[L_l]}{[L_l]+K_{l,1}}+k_{l,-2}\right)$. In summary, in the case both unlabeled

ligand binding and the second step of labeled ligand binding is much slower than the equilibrium of the labeled ligand first step binding, the time course follows 2 exponentials. The fast phase rate constant scales linearly with unlabeled ligand concentration, whereas the slow phase concentration is a hyperbola of unlabeled ligand concentration.

Section S3

The effects of Gle1 on ADP binding were measured through kinetic competition by incubating 1 µM Dbp5 with 10 µM Gle1 for at least 2 hours at room temperature and subsequently mixing with 20 µM mantADP with various concentrations of ADP. Time courses of mantADP binding in the presence of ADP are best fit by a sum of two exponential functions (Figure S3A), indicating that one of the three binding steps equilibrates much faster than the other two (Scheme 2). Given that the rate constants for mantADP binding are independently known, Gle1-Dbp5 binding ADP is likely a rapid equilibration. Under these conditions, the fast and slow phase observed rate constants should display an [ADP]-dependent decrease (**Supplemental Information**, section **S2-1**). However, the noise associated with fitting the fast phase is significant. Therefore, we utilize only the [ADP]-dependance of the slow phase to estimate the ADP affinity (Figure S3B) with Eq. 2.5 above (see **Supplemental Information** section **S2-1**), yielding an affinity (K_{d94}) of 240 ± 15 µM for Gle1-Dbp5 binding ADP.

Section S4

Inclusion of up to 10 mM free phosphate does not significantly alter steady-state ATP hydrolysis by Dbp5 under saturating ATP and Gle1 conditions. This indicates that the apparent binding affinity of free phosphate for Gle1-Dbp5 complex during steady state ATP hydrolysis $K_{Pi, SS} \geq 10$ mM, because the equilibrium binding affinity between Gle1-Dbp5 and free phosphate ($K_{Pi,eq}$) is always greater than $K_{Pi, SS}$ (2), $K_{Pi,eq} > K_{Pi, SS} \geq 10$ mM. Therefore, phosphate rebinding does not need to be considered as no more than 80 µM ($\ll$ 10 mM) free phosphate is present in solution during the time scale of the quench-flow experiment.

Section S5

The maximum solution ionic strength (I, calculated from molality, so is unitless) change in our study < 2-fold over the [ATP] range examined, ranging from $I = 0.14$ (in assay buffer without other components added) to $I = 0.21$ with 15 mM Mg-ATP (50% change; Fig. 1) or $I = 0.23$ with 15 mM Mg-ATP and 10 mM phosphate in the buffer solution (64.3% change; see below). At the high ATP used (~15 mM), this change has notable effect on the activity coefficient of ATP and reduces the effect ATP concentration < 23%. For example, the effective concentration of 15 mM ATP in the assay buffer with 10 mM phosphate is 11.6 mM. Consequently, there is no effect on the observed steady-state ATPase behaviors, which is the only experiment such high [ATP] is used. This is supported by the fact that the ATPase activity in the presence of saturating [Gle1] is comparable in 0.1 mM Mg-ATP (~ $k_{cat} = 0.15$, saturated; Fig. 7; $I \sim 0.14$ assay buffer ionic strength; see below) and 15 mM Mg-ATP (~ $k_{cat} = 0.16$; Fig. 1; $I = 0.21$; see below). In addition, the Dbp5 ATPase, both in the presence (this work) and absence (previous work from our group (2)) of Gle1 were not affected by inclusion of 10 mM phosphate. Nevertheless, we provide calculations of our solution ionic strength here to inform readers.

    1. Ionic strength of assay buffer: The ionic strength of the assay buffer is mainly from 1 of each K^+ and Cl^+ in KCl (100 mM), 1 Mg^{2+} and 2 Cl^+ in MgCl₂ (2 mM), and 1.5 charge from HEPES (30 mM). The ionic strength is $I \sim 0.5([KCl] \times (1^2+1^2) + [MgCl_2] \times (2^2+2 \times 1) + [HEPES] \times 1^2 = 0.14$

    2. Concentration of ions in our 100 mM ATP stock solution: To make ATP stock solution at pH 7.0, we dissolve disodium ATP powder (Sigma-Aldrich, cat# A7699) in ddH₂O and immediately bring the pH up to 7.0 with KOH. In neutral pH solution, a ATP loses 3 protons and the affinity of the 4th proton is $\log(K) = 6.51$ (3). Therefore, in pH 7.0, the ratio of ATP with the 4th proton off and on is

$$\log\left(\frac{\left[ATP^{4-}\right]}{\left[HATP^{3-}\right]}\right) = pH - pKa = 7 - 6.51 = 0.49$$

i.e.,

$$\frac{\left[ATP^{4-}\right]}{\left[HATP^{3-}\right]} = 10^{0.49} = 3.09.$$

For 100 mM ATP stock solution in pH 7.0, 100/4.09 ~ 24.4 mM of it is HATP^{3-} and 75.6 mM of it is ATP^{4-}. Consequently, 3[HATP^{3-}]+4[ATP^{4-}] = 3×24.4+4×75.6 = 375.6 mM proton H$^+$ is dissociated from disodium ATP into solution in pH 7.0 and it needs the same molar equivalent KOH to neutralize the released H$^+$. In conclusion, in 100 mM ATP stock solution, there is 375.6 mM of K$^+$ ion in the solution from KOH added apart from 2×100 = 200 mM Na^{2+} from disodium ATP complex.

3. *Ionic strength of 15 mM MgATP in the assay buffer*: 15 mM MgATP solution was made by adding 15 mM ATP from 100 mM K$^+$ATP pH 7 stock and 15 mM MgCl₂. When adding 15 mM ATP from stock solution, 375.6×15/100 ~ 56 mM K$^+$ and 200×15/100 = 30 mM Na$^+$ are also carried into the solution from K$^+$ATP stock. Then the ion concentration in 15 mM ATP buffer solution includes:

K$^+$: 100 + 56 =156 mM

Na$^+$: 30 mM

Monovalent metal ion: 156+30 = 186 mM, adjusted to 176.4 mM free (see below)

Cl$^-$: 100 + 2×2 + 2×15 = 134 mM

Mg^{2+}: 2+15 = 17 mM, adjusted to 2 mM free (see below)

HEPES$^{1.5-}$: 30 mM

MgATP^{2-}: 15-9.6 = 5.4 mM (see below)

KMgATP$^-$: 9.6 mM (see below).

However, not all the ions above are free. K$^+$ and Na$^+$ have the same charge and their affinities for ATP are similar ~ 100 mM (3). Therefore, we treat K$^+$ and Na$^+$ the same in their conjugating to ATP and in calculation of ionic strength, i.e., the total monovalent metal concentration is 156 of K$^+$ + 30 of Na$^+$ = 186 mM in which

$$\left[K^+ ATP\right] = \frac{1}{2}\left(\left[ATP\right]_{tot} + \left[K^+\right]_{tot} + K_{K^+} - \sqrt{\left(\left[ATP\right]_{tot} + \left[K^+\right]_{tot} + K_{K^+}\right)^2 - 4\left[ATP\right]_{tot}\left[K\right]_{tot}}\right)$$

9.6 mM is conjugated with ATP and 176.4 mM is free ([K$^+$]$_{tot}$ is treated as total of both K$^+$ and Na$^+$).

In the presence of ~100 mM KCl or NaCl, the affinity of Mg^{2+} for ATP is reduced to ~0.21 mM (3), but is still tight. Thus, in the presence of 17 mM Mg^{2+} and 15 mM ATP, almost all the ATP has a Mg^{2+} complexed.

In summary, in our experimental condition and buffer, 2 of 4 negative charges in ATP are neutralized by Mg^{2+} conjugation to become $MgATP^{2-}$. 1 of 2 negative charges in the majority of $MgATP^{2-}$ (9.6 mM) is further neutralized by a K^+ or Na^+ binding, becoming $KMgATP^-$ and the rest of $MgATP^{2-}$ is most possibly staying as $MgATP^{2-}$ since pK_a of $MgATP^{2-}$ is 4.55 (3). Finally, in all consideration,

the ionic strength in 15 mM MgATP buffer is:

$$I = \frac{1}{2}\left(0.1764 \times 1^2 + 0.134 \times 1^2 + 0.002 \times 2^2 + 0.03 \times 1.5^2 + 0.0096 \times 1^2 + 0.0054 \times 2^2\right) = 0.21.$$

15 mM MgATP adds additional 0.21-0.14 = 0.07 to the assay buffer strength and it is ~ 0.07/0.14=50% change in ionic strength from assay buffer alone. According to Debye-Hückel equation (4), the population weighted average activity coefficient for MgATP is:

$$\log \gamma_{MgATP} = -0.509\sqrt{I}\,\overline{z_{MgATP}^2}$$
$$= -0.509 \times \sqrt{0.21} \times 2.08 = -0.48$$

where the population averaged charge square is:

$$\overline{z_{MgATP}^2} = \frac{[KMgATP^-] \times (-1)^2 + [MgATP^{2-}] \times (-2)^2}{[KMgATP^-] + [MgATP^{2-}]}$$
$$= \frac{9.6 \times (-1)^2 + 5.4 \times (-2)^2}{15} = 2.08$$

Therefore, the MgATP activity coefficient in 15 mM MgATP buffer is $\gamma_{MgATP} = 0.33$ and it is slight change compared to $\gamma_{MgATP} = 0.40$ if the MgATP is in buffer without ATP ($I = 0.14$; $\overline{z_{MgATP}^2} = 2.08$).

4. *Ionic strength of 10 mM phosphate (PO₄) in assay buffer*: The highest phosphate (PO_4) concentration used in this study is 10 mM, which was made by the assay buffer pH 7.5 supplemented with 10 mM phosphate taken from 1 M phosphate stock solution with H_2O pH 7.5. The phosphate stock solution was made by dissolving different amounts of monobasic (KH_2PO_4, acid) and dibasic (K_2HPO_4, base) potassium phosphate powder, respectively, into H_2O to final 1 M phosphate and pH 7.5. There are 3 dissociable protons in a phosphate and their pK_a values are 2.15, 6.82, and 12.38 (5). Therefore, in the final pH 7.5, the 1st proton should be all dissociated, the 2nd one partially dissociated and the 3rd one should stay bound with phosphate. In the 1 M phosphate stock solution pH 7.5, using the Henderson-Hasselbalch (HH) equation:

$$pH = pK_a + \log([Base]/[Acid])$$

with $pK_a = 6.82$, the ratio of base to acid is:

$$[Base]/[Acid] = 10^{7.5-6.82} = 4.79.$$

Since

$$[Base] + [Acid] = 1\ M$$

$$[Acid] = 1/(1+4.79) = 0.17 \text{ M and } [Base] = 1- 0.17 = 0.83 \text{ M}$$

are the final base and acid concentration in the phosphate stock solution. Since the proton contribution from solution pH change (10^{-7} to $10^{-7.5}$) is negligible, the calculated final base and acid concentration is the concentration of dibasic and monobasic potassium phosphate dissolved (5). Consequently, K^+ concentration in the phosphate stock from both mono- and dibasic potassium phosphate is:

$$[K^+] = 0.83 \times 2 + 0.17 \times 1 = 1.83 \text{ M}$$

and $1830 \times 0.01 = 18.3$ mM was carried over with 10 mM phosphate when using phosphate stock to make buffer. The ions in 10 mM phosphate assay buffer solution are:

K^+: 100 + 18.3=118.3 mM, adjusted to 118.3-2.8 = 115.5 mM (see below)

Cl^-: 100 + 4 = 104 mM

Mg^{2+}: 2 mM

$HEPES^{1.5-}$: 30 mM.

HPO_4^-: 1.7 mM

PO_4^{2-}: 8.3 mM, adjusted to 8.3–2.8 = 5.5 mM by K^+ binding (see below)

KPO_4^-: 2.8 mM

The affinity of K^+ for PO_4^{2-} is weak, $\log K = 0.64$ or $K_d \sim 230$ mM (3). The concentration of K^+ bound at the 2nd phosphate proton position of PO_4^{2-} (8.3 mM) is:

$$\left[KPO_4^-\right] = \frac{1}{2}\left(\left[PO_4^{2-}\right]_{tot} + \left[K^+\right]_{tot} + K_{K^+} - \sqrt{\left(\left[PO_4^{2-}\right]_{tot} + \left[K^+\right]_{tot} + K_{K^+}\right)^2 - 4\left[PO_4^{2-}\right]_{tot}\left[K^+\right]_{tot}}\right)$$

$$= \frac{1}{2}\left(8.3 + 118.3 + 230 - \sqrt{(8.3 + 118.3 + 230)^2 - 4 \times 8.3 \times 118.3}\right) \sim 2.8 \text{ mM}$$

Finally, the ionic strength is:

$$I = 0.5 \times (0.1155 + 0.102 + 0.002 \times 2^2 + 0.03 \times 1.5^2 + 0.0045 + 0.0055 \times 2^2) \sim \text{-}0.16$$

It is 0.02/0.14=4.3% change from assay buffer ionic strength.

5. *Ionic strength of 15 mM MgATP with 10 mM phosphate (PO₄) in assay buffer*: similar to the case above, 15 mM MgATP in buffer adds ~0.07 to the assay buffer ionic strength, 15 mM MgATP would add ~0.07 to 10 mM phosphate buffer, such that the final ionic strength is $I \sim 0.16 + 0.07 = 0.23$ and it is 64.3% change compared to the ionic strength of buffer alone. The MgATP activity coefficient in 15 mM MgATP with 10 mM phosphate is $\gamma_{MgATP} = 0.31$ and reduced 0.09 (22.5%) from activity coefficient in assay buffer (0.40). At this activity coefficient vs. that in the assay buffer alone, the effect concentration of 15 mM MgATP reduce to:

$$[MgATP]_{eff} = 0.31 \times 15/0.4 = 11.6 \text{ mM}.$$

Section S6

Below are the formulas used to calculate unmeasured values in Scheme 3 using detailed balance (Table 1). Uncertainties were calculated with conventional error propagation methods (6).

$$K_{d107} = \frac{K_{d74}K_{d41}}{K_{d101}} = \frac{5.5 \times 0.8}{3000} = 1.5 \pm 0.8 \; nM$$

$$K_{d118} = \frac{K_{d87}K_{d107}}{K_{d1110}} = \frac{1.7 \times 0.0015}{0.004} = 0.6 \pm 0.2 \; \mu M$$

$$K_{d129} = \frac{K_{d94}K_{d41}}{K_{d121}} = \frac{240 \times 0.8}{360} = 0.5 \pm 0.2 \; \mu M$$

Supplementary Figures
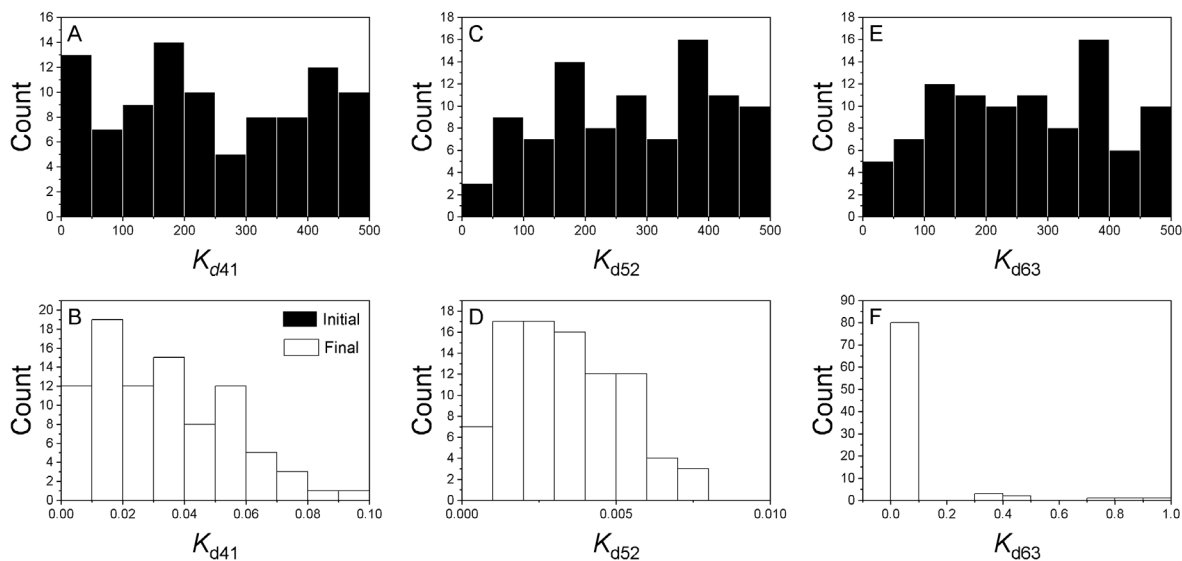
Figure S1. Distribution of initial (A, C, E) and final (B, D, F) K_{d41}, K_{d52}, and K_{d63} (Scheme 1) from global fits in Figure 2B, 3A, and 4A (dashed lines) using numerical integration techniques in a custom MATLAB program. $InsP_6$ is included in all experiments at an equimolar concentration with Gle1.
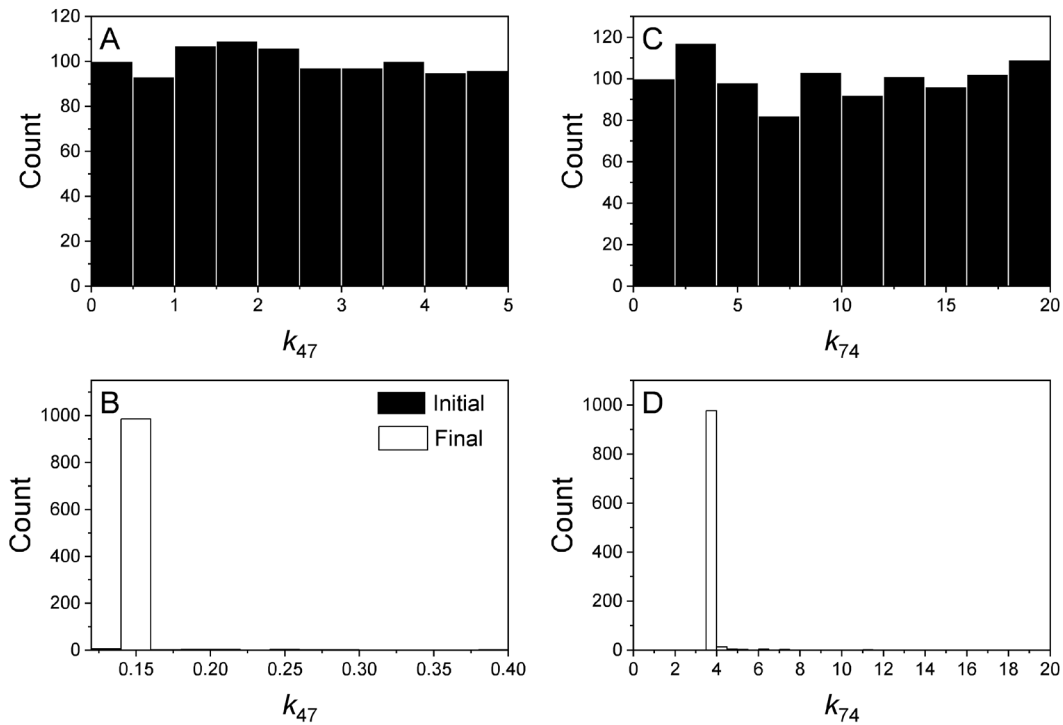
Figure S2. Distribution of final (B, D) and initial (A, C) ATP association (k_{47}) and dissociation (k_{74}) rate constants (Scheme 2) from fits to a kinetic simulation of Scheme 2 in Figure 5 using numerical integration techniques in a custom MATLAB program. Rate constants for Gle1-Dbp5 binding mantADP were fixed to values determined from fits in Figure 4. InsP$_6$ is included in all experiments at an equimolar concentration with Gle1.
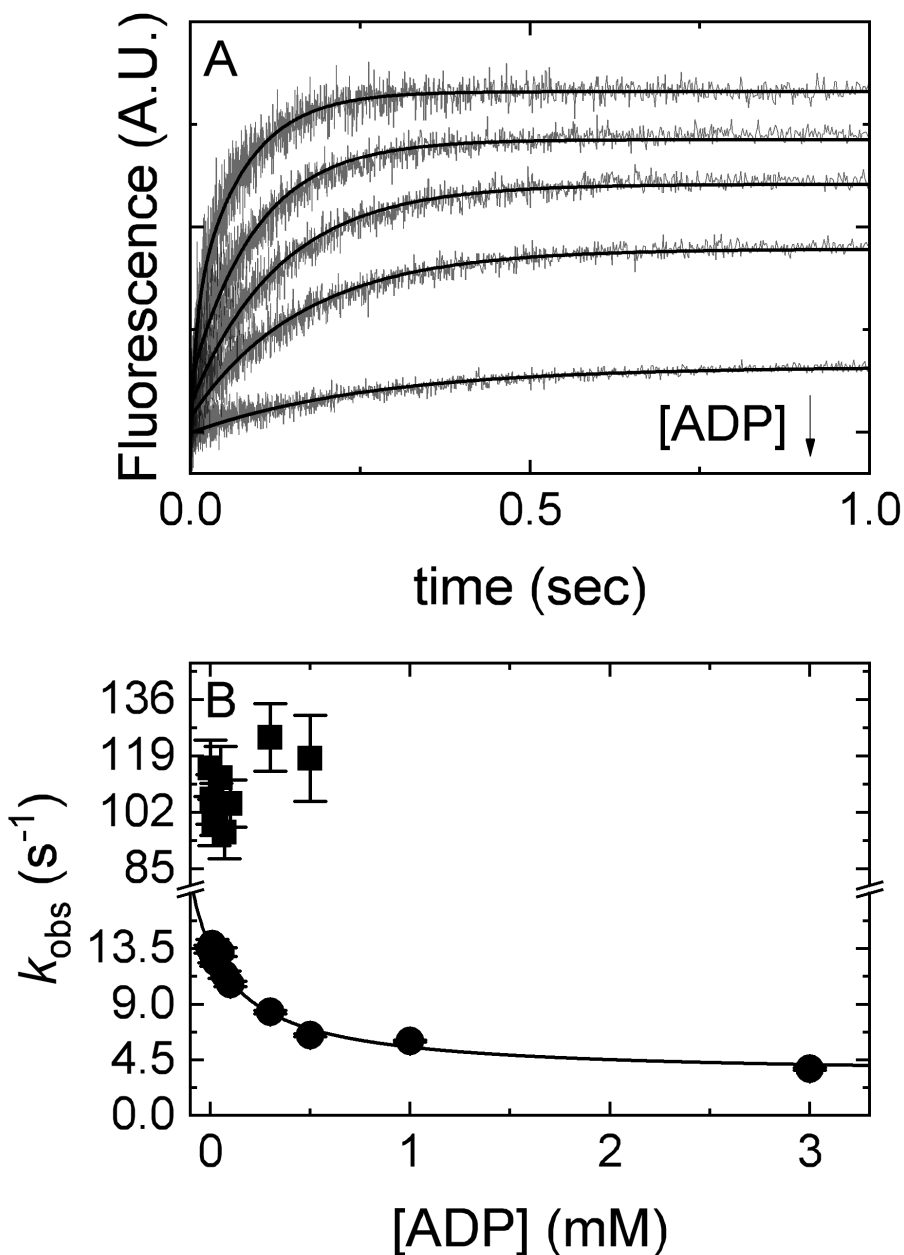
Figure S3. ADP rapidly equilibrates with Gle1-Dbp5 complex. (A). Time courses of FRET signal changes in pre-equilibrated solution of 2 µM Dbp5 (1 µM after mixing) with 20 µM Gle1 (10 µM after mixing) upon rapid mixing with an equal volume of 40 µM mantADP (20 µM after mixing) with various concentrations of ADP (from 0 to 3 mM after mixing). Continuous lines through the data are the best fits to double or single exponential functions (solid lines). (B) [ADP]-dependence of the observed rate constants from exponential fits in A (solid lines) for mantADP binding pre-formed Gle1-Dbp5 complex. The continuous line through the data is the best fit to Eq. S2.5 (Supplemental Information, section 2-1). Uncertainty bars represent standard error in the fits and are contained within the data points. The best fit yields an affinity for Gle1-Dbp5 binding ADP (K_{d94}) of 240 ± 15 µM. $InsP_6$ is included in all experiments at an equimolar concentration with Gle1.
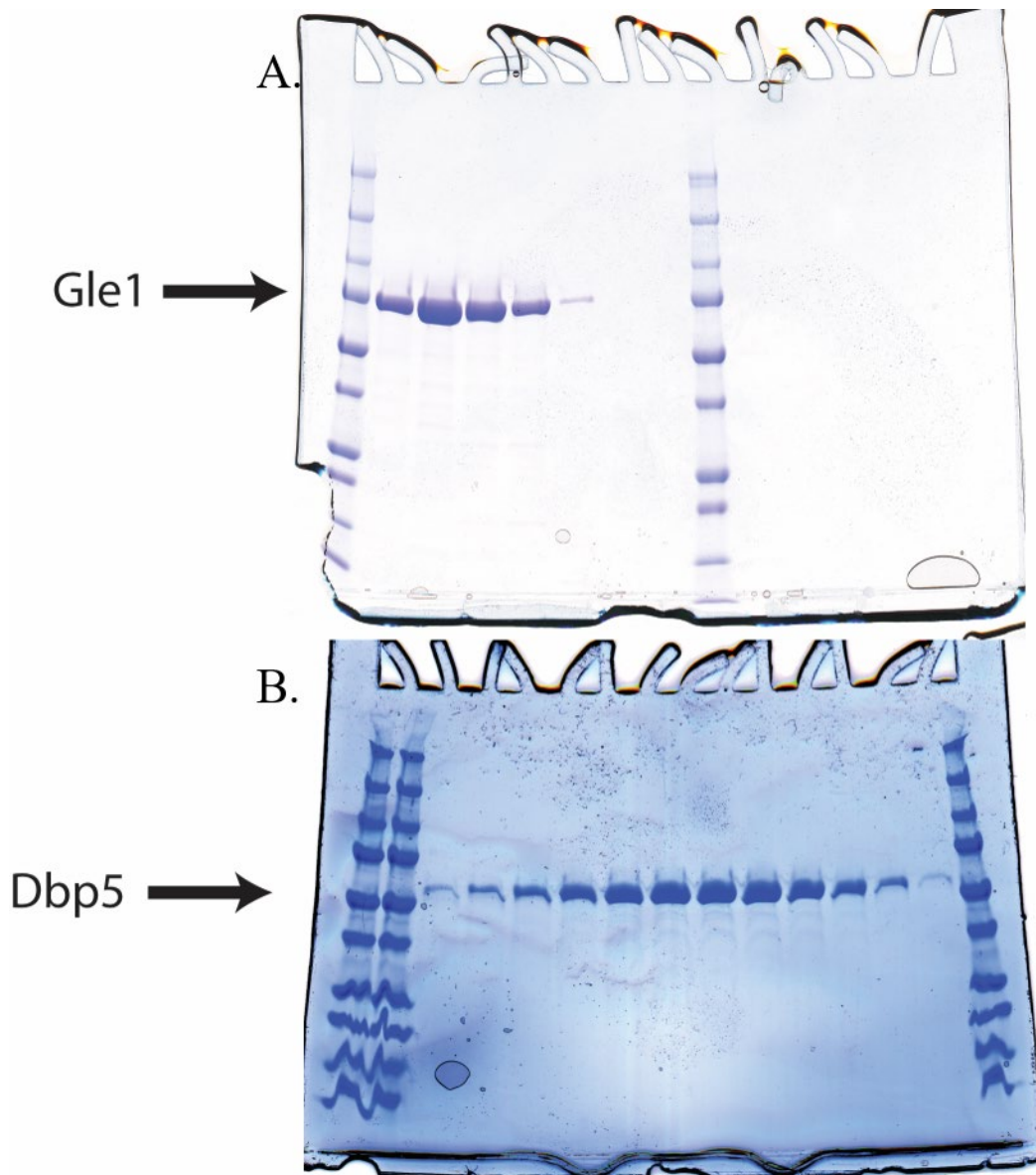
Figure S4. Representative SDS-PAGE gel of purified Dbp5 and Gle1. (A). SDS-PAGE gel of purified Gle1-MBP fractions following final FPLC separation. (B). SDS-PAGE gel of purified Dbp5 fractions following final FPLC separation. $InsP_6$ is included in all experiments at an equimolar concentration with Gle1.

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