A Kinetic Mechanism of the Allosteric Control of Enzyme–Coenzyme Binding: Glutamate Dehydrogenase–NADPH–Phosphate–Acetate–Hydrogen Ion Interactions†

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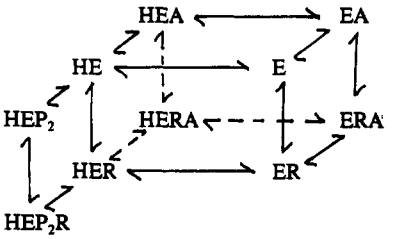
ABSTRACT: We have previously characterized the thermodynamic relationships which govern the dissociation of NADPH from bovine liver glutamate dehydrogenase and the allosteric control of that mechanically and physiologically important process by a variety of effectors. We have found that the cooperative occupancy of a specific anion binding, while the occupancy of a second allosteric acetate binding site disrupts that anion binding site and opposes those effects (Singh & Fisher, 1994). We report here the results of transient-state studies on the kinetics of the various processes involved in this complex equilibrium. We find that the only intrinsically slow steps are those of NADPH binding and dissociation, that the complex kinetic behavior of the overall system is due solely to very rapid equilibrium binding processes involving phosphate, acetate, and hydrogen ions, and that these ions exert their various effects on the kinetics of the binding process by altering the equilibrium concentrations of the two kinetically significant reactive species, E and E-NADPH. The slow intrinsic rates of NADPH association and dissociation are ascribed to a ligand-induced conformational change involving a major alteration in the degree of closure of the enzyme's active-site cleft.

The dissociation of the enzyme-reduced coenzyme complex is the rate-limiting step in the catalysis of the oxidative deamination of L-glutamate by bovine liver glutamate dehydrogenase. Any chemical entity or solvent condition which affects this particular equilibrium must result in a change in the steady-state rate of the enzyme-catalyzed reaction. This feature has been responsible for the profusion of literature reporting observations of the effects of a wide variety of nucleotides, drugs, hormones, functional group modifiers, amino and other carboxylic acids, and anions on this enzyme (Singh & Fisher, 1994; Dieter et al., 1981; Blauer & Sund, 1977; Pahlich et al., 1978; Godinot & Gautheron, 1972; Stone & Copeland, 1982; DiPrisco & Strecker, 1969). The literature on the effects of anions alone is extensive and quite contradictory. We have recently reported evidence for a specific anion binding site on the enzyme whose pattern of various positive and negative interactions with NADPH, H^+, and a variety of anions provides a comprehensive explanation for rationalizing, understanding, and predicting these many *ad hoc* observations (Singh & Fisher, 1994). The principal feature of this model is the effect of the degree of "loading" of a specific anion binding site on the enzyme which allosterically controls the pK of a coenzyme binding subsite. When "loaded", this site contains two phosphate ions and a hydrogen ion. The various interactions between the enzyme (E), the reduced coenzyme (R), phosphate (P), acetate (A), and hydrogen ion (H^+) are shown in Scheme 1 [adapted from Singh and Fisher (1994)].

Scheme 1



The population of each species indicated in the scheme is governed by a number of positive and negative interactions between the binding of various ligand pairs. Positive interactions occur between the binding of NADPH (R) and acetate (A) and between the binding of two molecules of phosphate (P, P) and a proton (H). Negative interactions are observed between the binding of NADPH and a proton, between the binding of acetate and phosphate, and between the binding of acetate and a proton.

A self-consistent set of equations defining this complex set of species in terms of the equilibrium constants indicated in the scheme has been shown to be quantitatively consistent with an extensive set of calorimetric, spectroscopic, and direct proton-displacement measurements (Singh & Fisher, 1994). In order to bring our understanding of this system to a more fundamental level, we have now carried out transient-state spectroscopic rate measurements to dissect the equilibrium constants defined in Scheme 1 into the individual forward and reverse rate constants which comprise these interconversions. We report the results of these studies here.

MATERIALS AND METHODS

Materials. Beef liver L-glutamate dehydrogenase (L-glutamate:NAD(P)$^+$ oxidoreductase (deaminating), EC 1.4.1.3) was purchased from Boehringer Mannheim as an ammonium

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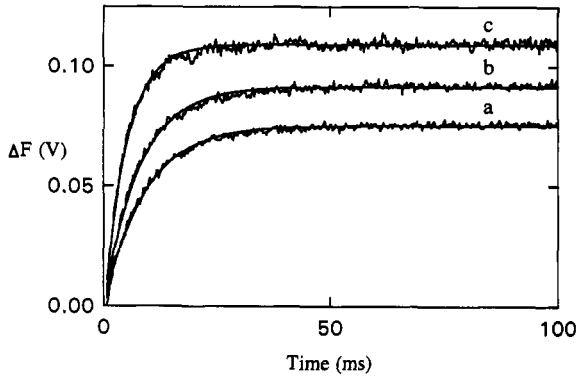


FIGURE 1: Typical fluorescence time courses of the binding of NADPH to enzyme. Solid curves are fits of eq 1 to the data. The reactions were run under condition U (as defined in the text) at pH 6.8, 20 °C. The excitation wavelength was 320 nm. The enzyme concentration in each case was 5 μM. NADPH concentrations were curve a, 5 μM; curve b, 7.5 μM; curve c, 20 μM. The k_{obs} values for curves a–c were calculated to be 112, 130, and 200 s^{-1}, respectively.

sulfate suspension and prepared as described previously (Fisher, 1985). NADPH was purchased from Sigma. Concentrations were determined on a HP8450A spectrophotometer using ϵ_{340} = 6.22 cm^{-1} mM^{-1} for NADPH and ϵ_{280} = 54.417 cm^{-1} mM^{-1} for enzyme.

Kinetic Measurements. Most of the stopped flow kinetic measurements were performed in a Durrum-Gibson instrument arranged for fluorescence with the photomultiplier positioned over the long side of a 1.71 × 0.5 × 0.5 cm^3 cell. The cell was irradiated by a 320 nm arc lamp beam passed through a 2 mm slit. A Corning C.S. 0–52 filter, with a transmittance <5% at 340 nm, was used betwen the photomultiplier and the cell. The instrument had a dead time of 5 ms and was interfaced to a Digital DECLAB 11/MNC computer. Some experiments were done in a HiTech SF-51 instrument having a dead time of 2 ms. The results from the two instruments showed no significant difference.

All experiments were carried out with a solution of enzyme in one mixing syringe and a solution of NADPH in the other, and enzyme and NADPH kinetic base lines were subtracted. Order-of-addition experiments were done in both the fluorescence and absorbance modes.

The sets of buffer conditions are symbolized as follows: L (loaded) = 0.1 M potassium phosphate; U (unloaded) = 0.01 M potassium phosphate; LA (loaded, acetate) = 0.1 M potassium phosphate, 0.1 M potassium acetate; UA (unloaded, acetate) = 0.01 M potassium phosphate, 0.1 M potassium acetate. The final concentration of enzyme was 5 ± 0.01 μM unless otherwise stated. The solution temperature was maintained at 20 ± 0.1 °C.

Determination of Kinetic Constants. The kinetic scans are fitted to a single exponential equation weighted more heavily toward the final base line. k'_{on} and k'_{off} are obtained from a linear dependence based on $k_{obs} = k'_{off} + k'_{on}([E]_{eq} + [R]_{eq})$ without assuming any value of K_d. The dynamic equilibrium concentrations of E and R are obtained by an iterative procedure. In this procedure, a crude estimate of k'_{off} and k'_{on} from a linear fit of k_{obs} vs $([E]_t + [R]_t)$ results in a more refined K_d whose use provides a more accurate value of $[E]_{eq}$ + $[R]_{eq}$. The process is repeated until invariant k'_{on} and k'_{off} values are obtained.[1]

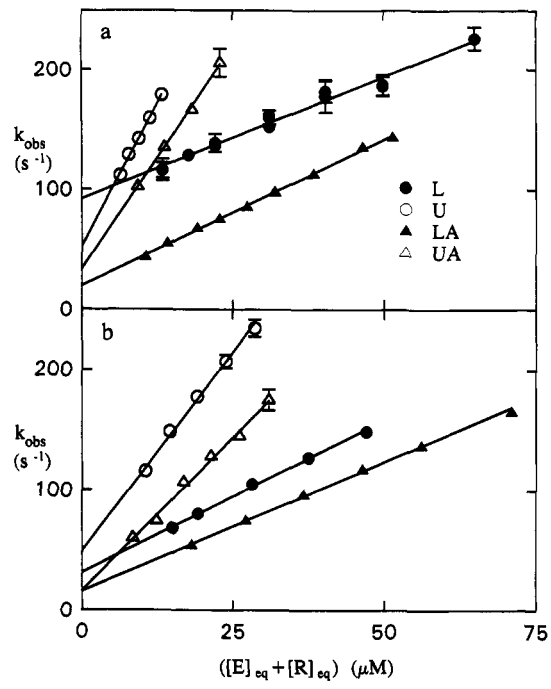


FIGURE 2: Dependence of k_{obs} on the sum of the equilibrium reactant concentrations (calculated as described under Materials and Methods). The four cases noted refer to the state of "loading" of the anion binding: L for loaded, U for unloaded, and A indicating the simultaneous presence of acetate. Specific conditions are described in the text. Panel a corresponds to pH = 6.8 and panel b to pH = 7.8.

RESULTS

As a basis for our approach, we define four specific initial conditions of the reaction mixture. Case U: Here, the anion binding site of the enzyme initially may be considered to be "unloaded" for all practical purposes because of the low phosphate concentration employed. Case L: Here, the anion binding site of the enzyme initially may be considered as essentially "loaded" (containing two phosphate ions) due to the high concentration of phosphate employed. Cases UA and LA represent conditions in which acetate has been added to enzyme solutions which were initially in the U and L states, respectively. The time dependence of the binding of NADPH (R) to enzyme was measured at various R concentrations over the available pH range, in each of the four defined cases. Examples of the results of several such experiments are shown in Figure 1. We have found that throughout the entire range of phosphate and NADPH concentrations, and at all pH's studied, the time course of the binding step can be fitted with a single exponential:

$$\Delta F(t) = F_0 e^{-k_{obs}} + \text{base} \qquad (1)$$

where F_0 and ΔF are the initial value and the specific value of the fluorescence signal at any time t, respectively.

The experimentally obtained relaxation constants (k_{obs}) have been plotted vs the sum of $[E]_{eq}$ and $[R]_{eq}$ (calculated as described under Materials and Methods) for cases U, L, UA, and LA and are shown in Figure 2. A linear dependence on total reactant concentration is observed in each case. On this basis, we write the reaction as:

[1] The forward and reverse rate constants can be reliably obtained by the relaxation kinetic method (even when pseudo-first-order conditions are not met) provided the equilibrium concentrations of the reactants are precisely calculated by an iterative procedure (Bernasconi, 1976). We have further validated our approach by fitting our data to a formal bimolecular binding equation (Appelman et al., 1988). However, this method results in larger errors in the calculated rate constants.

$$R + E \underset{k'_{off}}{\overset{k'_{on}}{\rightleftharpoons}} ER$$

express the concentration dependence of k_{obs} as

$$k_{obs} = k'_{off} + k'_{on}([E]_{eq} + [R]_{eq}) \quad (2)$$

and evaluate k'_{on} from the slope of the plot k_{obs} vs ($[E]_{eq}$ + $[R]_{eq}$) and k'_{off} from the intercept at a zero reactant concentration.

Comparing the results for the four cases, U, L, UA, and LA, the following conclusions can be reached by inspection of Figure 2: (1) an increase in phosphate concentration lowers k'_{on} in either the presence or absence of phosphate and (2) an increase in acetate concentration lowers k'_{off} in either the presence or absence of phosphate. Thus phosphate affects k'_{on} most strongly, while acetate, on the other hand, exerts its effect principally on k'_{off}.

Examination of the pH dependence of the resolved k'_{on} and k'_{off} values indicates the following: The k'_{on} values, in all four cases, show no significant pH dependence. However, the reverse rate constant, k'_{off}, is pH-dependent in the L and U cases, where it decreases in value on increasing pH. A pK value of 6.8 ± 0.4 is indicated in both cases. There is no pH dependence of k'_{off} in the LA and UA cases, implying that the presence of acetate ion removes the pH dependence of k'_{off}, irrespective of whether the phosphate ion is present or not.

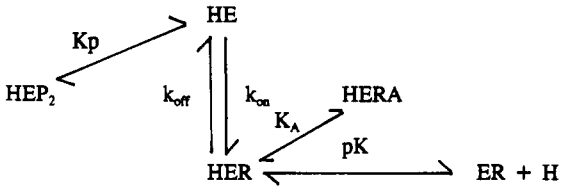
DISCUSSION

Kinetic Model. Since phosphate affects only k'_{on}, and acetate and pH affect only k'_{off}, we can account for all of the phenomena reported here between pH 6.8 and 8.5 using Scheme 2, where k_{on} and k_{off} are intrinsic forward and reverse rate constants, respectively, and K_p, K_A, and pK are the assigned equilibrium constants.

In all four cases, the linear dependence of k'_{on} on [R] implies that the time dependence of each of the binding reactions is completely dominated by a single kinetic step, that this step must involve the bimolecular binding of R to some species of the free enzyme, and that all other steps must equilibrate rapidly. The absence of any observed order-of-addition effects under all four experimental conditions supports these conclusions. The effects of acetate, phosphate, and pH on the observed kinetics of the coenzyme-binding reaction (cases L, LA, and UA) are now easily explained. Since there is only a single forward rate-limiting step and a single reverse rate-limiting step, any observed effect of acetate, phosphate, or pH on either HE $\overset{k_{on}}{\rightharpoonup}$ or HER $\overset{k_{off}}{\rightharpoonup}$ must be exerted by altering the concentrations of HE or HER, respectively. As shown in the Appendix, the agreement of the observed behavior of k'_{on}, k'_{off}, and K'_D with eqs 3, 4, and 7 establishes the validity of Scheme 2 as at least a proper quantitative explanation of the principal phenomena observed over the experimental range of the work reported here.

In addition to these qualitative kinetic conclusions, the comparison of the measured dynamic equilibrium constants (k'_{off}/k'_{on}) in each of the U, L, UA, and LA cases permits the quantitative evaluation of certain intrinsic equilibrium constants in eqs 5 and 6 (see Appendix). The binding constant, K_p, is estimated to be 2.8×10^3 M^{-2} from eq 5, using data at pH = 6.8. This K_p estimated from the kinetic data agrees within error limits with the value of 5×10^3 M^{-2} obtained from equilibrium measurements (Singh & Fisher, 1994). This is a validation of the one-step kinetic model. In a similar way, we calculate K_A in case L at pH = 6.8 to be K_A = 17 ± 5 mM

Scheme 2



from eq 6, which is comparable with the value of 32 ± 5 mM reported by Srinivasan (1991).

While the findings described here are very limited in scope, they do provide a much needed linkage between several well-known but, thus far, isolated phenomena involving the kinetic, energetic, and structural aspects of this enzyme.

Linkage of NADPH-Binding Kinetics to Thermodynamic Properties and pH Behavior of the Binary Complex. Some 20 years ago, we showed that the binding of NADPH to bovine liver glutamate dehydrogenase is a two-step process, in which the reduced nicotinamide ring binds first and is followed by the binding of the ribose–phosphate–phosphate–ribose moiety (Colen et al., 1974). Frieden's studies of the effects of various nucleotides on this same process at that time also implied a two-step process involving a conformational change as one of the steps (Huang & Frieden, 1972). More recently we showed that one of the steps in this same binding process involves the deprotonization of a single specific enzyme functional group having an initial pK of 8.5, a $\Delta H°$ = 19 kcal mol^{-1}, and a $\Delta S°$ = 23 eu. The final pK of this group in the enzyme–coenzyme complex is 7.0 ± 0.3 (Fisher et al., 1986). The results described here now make it clear that the initial step (the binding of the reduced nicotinamide ring) is rate-limiting and responsible for the unusual thermodynamic effects, while the subsequent isomerization step is responsible for the specific proton ionization.

Baker et al. (1992) have reported a crystal structure of the closely homologous *Clostridium symbosium* glutamate dehydrogenase–NAD complex which shows that each subunit consists of two domains separated by a deep cleft. The NAD is bound in the open form with the nicotinamide ring buried deep in the (presumably) narrower interior end of the cleft, while the adenylate moiety is strung out in the direction of the subunit surface. Simple consideration of this gross geometry makes it obvious that the only physically feasible way in which such a process could occur requires that the nicotinamide ring must bind first to the narrow end of the cleft and that the remainder of the coenzyme, which occupies the less constrained outer portion of the cleft, must bind in a subsequent step. Such a picture is, of course, in detailed accord with our kinetic results.

Linkage between the NADPH-Binding Kinetics and the Location of the Specific Acetate Binding Site. Srinivasan (1991) demonstrated that acetate is a competitive inhibitor vs α-ketoglutarate in the glutamate dehydrogenase-catalyzed reductive amination reaction. He observed that a group with a pK of 8 must be protonated for acetate to bind to the ER complex, that the K_d for this binding at pH 6.8, 25 °C, is 32 ± 5 mM, and that the binding step involved a substantial $\Delta H°$. Our work at 20 °C suggests the existence of such a group having a K_d = 17 ± 5 mM, a pK of 6.8, and a $\Delta H°$ = 17 kcal mol^{-1}. The minor discrepancy may be due to the difference in experimental temperature. On this basis, we suggest that the protonated HER complex indicated in Scheme 2 may in fact be the same species to which the competitive inhibitory form of acetate binds to form the HERA complex indicated in Scheme 1. If this assignment is correct, it

establishes the location of the acetate binding site in the active-site cleft of the enzyme. The identity of the acetate binding site with that of the α-ketoglutarate γ-carboxylate binding site would provide a clear explanation of the mechanism of the allosteric interaction between acetate and NADPH at the active site. The location of the anion binding site, however, remains undetermined.

Linkage of the Induction of Protein Two-State Transitions to the Kinetics of the Reverse Reaction. In the course of pursuing a quite different line of investigation, we discovered the existence of a high-enthalpy, proton-linked two-state transition in mammalian glutamate dehydrogenase and showed that specific ligands shifted the temperature at which this transition occurs. NADPH binding lowers this transition temperature from 43 to about −20 °C (Fisher et al., 1981). We later showed that both the ΔC_p and the pK of the proton ionization which accompanies NADPH binding are identical to those which characterize the two-state transition of the protein (Fisher et al., 1986). In an earlier paper (Singh & Fisher, 1994), we showed that the effects of occupancy of the anion binding site are the same for both phenomena. We also note that the rate constant for NADPH binding to this enzyme (and for most pyridine nucleotide dehydrogenases) is at least 3 orders of magnitude slower than that predicted for a diffusion-controlled protein–ligand-binding process. (Fersht, 1985). Transient-state studies of other enzymes have shown that this apparently low binding velocity is in fact due to a rate-limiting isomerization process following a relatively fast encounter step, as evidenced by a leveling off of k_{obs} at high ligand concentrations. The behavior observed here, however, is quite different; k_{obs} does not level off within our measurement range, and the binding of protons and phosphate and acetate ions is immeasurably fast. Those findings strongly suggest that the time-dependent process we observe in NADPH binding is in fact the conformational change induced by the formation of the NADPH–enzyme encounter complex and that this step corresponds to the cleft closing described above.

Linkage between the Kinetics of the Formation of the Enzyme–NADPH Complex and the Transduction of Ligand-Binding Energy into a Catalytic Driving Force. Our studies of the thermodynamic interaction parameters of the various stable complexes which occur along the catalytic reaction coordinate have led us to propose a scheme in which the energizing of the protein by NADPH binding, the storage of that energy through several intermediate steps, and the release of that energy during the hydride transfer step accomplish the requirements set fourth by Lumry (1986) for such a process. The NADPH-binding-induced two-state transition is the central feature of such a scheme. The observations described here which show that a high-enthalpy, proton ionization-linked, enzyme–reactant-binding step appears to be kinetically competent to be a participant in the catalytic reaction provide the first direct evidence linking energetics with the chemistry of the reaction. The further exploration of such linkages will be our next goal.

APPENDIX

Phospate, Acetate, and pH Dependence of the Binding Kinetics. In all the cases studied here, the linear dependence of k'_{on} on [R] suggests that the binding of free enzyme to NADPH involves a single kinetic step and that any other step must equilibrate rapidly. We can identify this kinetic step most simply by considering case U (where neither acetate nor phosphate loading is present), which, therefore, involves only the three species in the scheme: HE, HER, and ER. On this

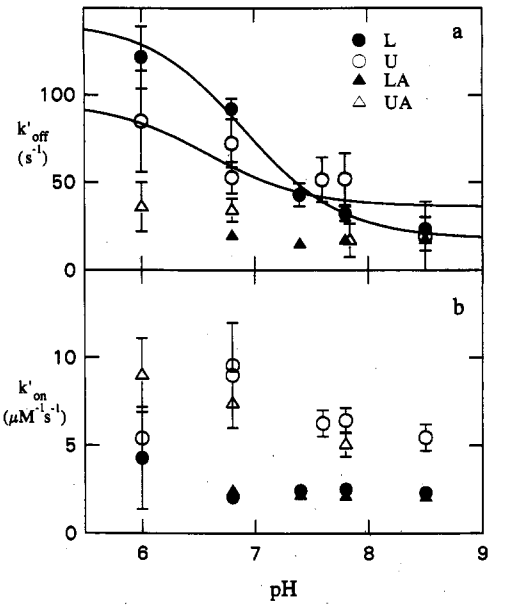


FIGURE 3: pH dependence of k'_{on} and k'_{off} for each of the four cases. k'_{on} and k'_{off} were in each case determined from the slopes and intercepts, respectively, of plots similar to those shown in Figure 2, based on eq 2. In panel a, the pH-dependent k'_{off} data in the L and U cases were fitted to the equation: $k'_{off} = C_1(1 + K/[H]) + C_2$ where C_1 and C_2 are constants and K is the ionization constant.

basis, at a constant pH and assuming that any proton ionization steps are very rapid, we now write

$$HE + R \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} HER$$

Thus

$$k'_{on} = k_{on}\frac{[HE]}{[E_t]} \tag{3}$$

where $[E_t]$ is the total enzyme concentration. Similarly, in Scheme 2, in the reverse direction, we write

$$k'_{off} = k_{off}\frac{[HER]}{[E_t]} \tag{4}$$

The effect of phosphate on the prekinetic free enzyme species is defined by the equilibrium constant K_p:

$$k'_{on} = k_{on}\left(\frac{1}{1 + \frac{[P]^2}{K_p}}\right) \tag{5}$$

where

$$K_p = \frac{[HE][P]^2}{[HEP_2]}$$

In a similar way, the reactive species for k_{off} is HER. The effects of acetate and pH on this postkinetic species are defined by K_A and pK, respectively:

$$k'_{off} = k_{off}\left(\frac{1}{1 + K_A[A] + \frac{K}{[H^+]}}\right) \tag{6}$$

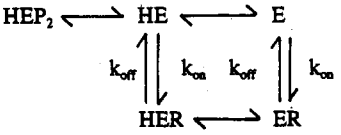
where

$$K_A = \frac{[HER][A]}{[HERA]} \quad \text{and} \quad K = \frac{[ER][H^+]}{[ERH]}$$

Finally, we define a "kinetic K'_d" as

$$K'_d = \frac{k_{off}}{K_{on}}\left(\frac{1 + \frac{[P]^2}{K_p}}{1 + K_A[A] + \frac{K}{[H^+]}}\right) \tag{7}$$

The pH behavior of the system as shown in Figure 3 indicates the occurrence of two phenomena which cannot be accounted for explicitly without some further expansion of the mechanism as depicted in Scheme 2: the finite limit reached by K_d at high pH and the pH dependence of k_{off}. The existence of a finite limit of K_d at high pH requires that the unprotonated form of E must also be capable of binding to R, leading to the following expanded scheme:



in which binding of R to either HE or E is a kinetic step contributing to the observed rate.

We have also noted that k_{on} is independent of pH, whereas k_{off} is dependent on pH. This latter pH dependence can be explained in the following way: Unprotonated ER is formed with the same kinetic constants as the protonated HER but then rapidly equilibrates with the tighter ER′ complex.[2]

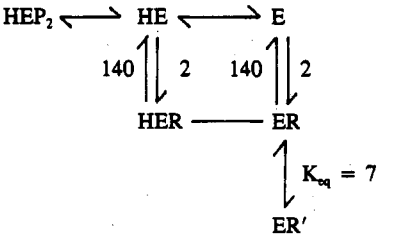
Quantitative Evaluation of Internal Equilibrium Constants in Scheme 2. The comparison of the measured dynamic equilibrium constants (k'_{off}/k'_{on}) in each of the U, L, UA, and LA cases leads to the quantitative evaluation of the intrinsic equilibrium constants, K_p and K_A.

The phosphate binding constant, K_p, for the reaction:

$$HE + 2P \overset{K_p}{\rightleftharpoons} HEP_2$$

is estimated to be 2.8×10^3 M^{-2} from eq 5, using data at pH = 6.8.

In a similar manner, we calculate K_A in case L at pH = 6.8 to be $K_A = 17 \pm 5$ mM from eq 6. Since the observed low-pH $k_{off} = 140$ and high-pH $k_{off} = 20$, we can calculate K_{eq} in Scheme 3



Scheme 3 as follows:

$$K_{eq} = \frac{k_{off}(\text{low pH})}{k_{off}(\text{high pH})} = \frac{140}{20} = 7.0 \tag{8}$$

[2] Another possible (but less likely) explanation of this feature involves a mechanism in which ER and HER are formed at the same rate as at high pH and HER rapidly equilibrates with a weaker complex, HER′.

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