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The Role of H^+ in the K^+ Activation of
Rabbit Muscle 5'AMP Aminohydrolase

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John C.W. Campbell, Jr.

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of the requirements for

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C H Suelter

Major professor

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THE ROLE OF H^+ IN THE K^+ ACTIVATION OF RABBIT MUSCLE 5'AMP AMINOHYDROLASE

By

John C. W. Campbell, Jr.

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ABSTRACT

THE ROLE OF H^+ IN THE K^+ ACTIVATION OF RABBIT MUSCLE 5'AMP AMINOHYDROLASE

By

John C. W. Campbell, Jr.

This study is an examination of the role of H^+ in the K^+ mediated activation of 5'AMP aminohydrolase. Both kinetic and equilibrium experiments were performed in defining this role. Kinetic experiments consisted of observing changes in K_m, V_{max}, and the Hill slope as functions of pH, while equilibrium experiments observed changes in numbers of H^+ bound to enzyme upon K^+ binding. Data are presented which indicates that K^+ binding is linked to H^+ binding. Thus activation of enzyme is observed to take place when these H^+ binding sites undergo either a change in degree of ionization or protonation.

To My Parents

ACKNOWLEDGMENTS

I wish to acknowledge the assistance of Dr. Norman Good of the Botany Department. Also, the help and advice of Mark Brody, Ann Aust and Shyn-Long Yun.

In addition, I especially wish to thank Dr. Clarence Suelter for his guidance and inspiration, without which this thesis would not have been possible.

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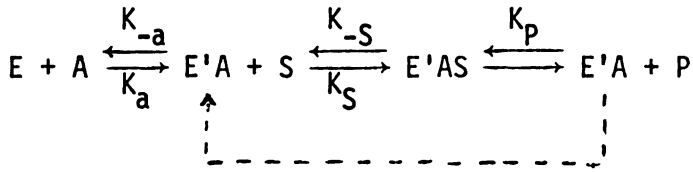
LIST OF ABBREVIATIONS

5'AMP	Adenosine 5'-Phosphate
BSA	Bovine Serum Albumin
EDTA	Ethylenediaminetetraacetic Acid
K_m	Concentration of substrate required for 50% saturation
K_A	Concentration of activator required for 50% activation
MES	2-(N-morpholino)Ethanesulfonic Acid
Tris	Tris(hydroxylmethyl)Aminomethane
V_{max}	Velocity at saturating concentrations of substrate

INTRODUCTION

The goal of this work was to examine the role of H^+ in the K^+ mediated activation of 5'AMP aminohydrolase. Previous data (77) were consistent with the following activation scheme.



where A is activator, S, substrate, P, product and E and E'A enzyme and activated enzyme, respectively. Activator A can take a variety of forms, e.g., 5'AMP, K^+, ATP, ADP, and H^+.

In studying the H^+ activation, both equilibrium and kinetic experiments will be performed. In addition, the relationship between the K^+ and H^+ activation processes will also be examined.

LITERATURE REVIEW

Occurrence of 5'AMP aminohydrolase

5'AMP aminohydrolase (E.C. 3.5.4.6) activity is found throughout the animal and plant kingdoms. Activity is present in rabbit (1-6), rat (8-12), mouse, guinea pig (8), calf (13-15), humans (20-23), cat, dog, (20), pigeon (8), chicken (31, 32, 35), duck, goose, turkey, turtle, horse, cow, goat, gerbil, hamster (37), frog, toad (33), snail (17), abalone (7), unfertilized fish eggs (24), elasmobranch fish (30), salmon, scallops, crab (34), pea seeds (29), and Aspergillus oryzae (36, 37). Tissues possessing activity include skeletal muscle (3-6, 8, 9, 11), lung (9, 13), brain (12, 13, 14), liver spleen, intestine, heart (9), erythrocyte (20-23), and human placenta (39).

Within the cell the enzyme is located both in the structural and soluble fractions of the cell. In rat brain and liver (12, 40), the enzyme is associated with the nuclear, microsomal, and mitochondrial fractions of the cell. The rabbit muscle enzyme appears to be associated with myosin (41). In heart tissue 5'AMP aminohydrolase is located in the cytoplasm (42), or with mitochondrial, microsomal, and nuclear cell fractions (43). In frog muscle the enzyme was found within the sarcolemna of the muscle fibers (19). Erythrocyles contain two forms of the enzyme, a soluble and a membrane-bound form. Approximately 15% of the total is of the latter type (32).

Purification

5'AMP aminohydrolase has been obtained in purified form from the following sources: rabbit muscle (5), pigeon muscle (8), chicken breast muscle (31), elasmobranch fish muscle (30), carp muscle (26), A. oryzae (36), and calf duodenal (15). Purifications involved combinations of salt fractionizations, ion-exchange, and gel-filtration chromatographic steps.

The procedure described by Smiley et al. (5) for rabbit muscle provides a simple one-step procedure, which has been adapted for use with other tissue sources (26, 30, 31), making it a highly useful procedure.

Structure

The purified enzyme exhibits different molecular weight and quatenary structure depending upon the source. Rabbit muscle, chicken muscle, calf brain, and A. oryzae enzymes show multiple subunit structure. The rabbit muscle enzyme and the chicken muscle enzyme have four subunits which form a dimer of 560,000 molecular weight. The enzyme from A. oryzae is a dimer of 217,000 molecular weight with two subnits of 103,000 apiece (38).

Characterization As a Metalloenzyme

5'AMP aminohydrolase from rat and rabbit muscle has been characterized as metalloenzymes having 2.0 (50) and 2.6 (51) moles of zinc, respectively, per mole of enzyme. Enzyme from other sources is inactivated by chelating agents which is consistent with a metalloenzyme structure (46, 52).

Activation and Inhibition

5'AMP aminohydrolase from many sources is activated by monovalent cations. Cations shown to be effective are K^+, Na^+, Li^+, NH_4^+, Rb^+, and Cs^+. K^+, Na^+, and Li^+ are usually the most effective as activators, but the relative order of effectiveness is dependent upon the source of enzyme. In each case, the monovalent cation decreases the K_m for 5'AMP (8, 9, 22, 26, 40, 44-48).

Metabolites such as ATP, GTP, GDP, and ADP modulate activity. These metabolites either activate or inhibit, depending upon source of enzyme, pH, and concentration of cation (8, 40, 44-48). Binding studies with ATP and GTP (6) and kinetic studies with substrate analogs (49) suggest that there are separate binding sites for 5'AMP and these metabolite activators.

In general, anions inhibit the enzyme. These include F^-, I^-, Br^-, and Cl^-, with F^- being the best inhibitor (8, 45, 47, 48).

Chemical Modifications

1. Sulfhydryl Groups. The native rat muscle enzyme has 12 sulfhydryl groups which are accessible to reaction with DTNB or N-ethylmaleimide. These react without loss of enzyme activity (62). An additional 16 to 18 sulfhydryl groups react when zinc is removed. The latter are necessary for successful reconstitution of the enzyme with zinc (62). Some sulfhydryl groups of native rabbit muscle are involved in the binding of GTP. After treatment of the enzyme with 6.5 molar equivalents of p-mercuribenzoate, GTP binding was abolished (6).

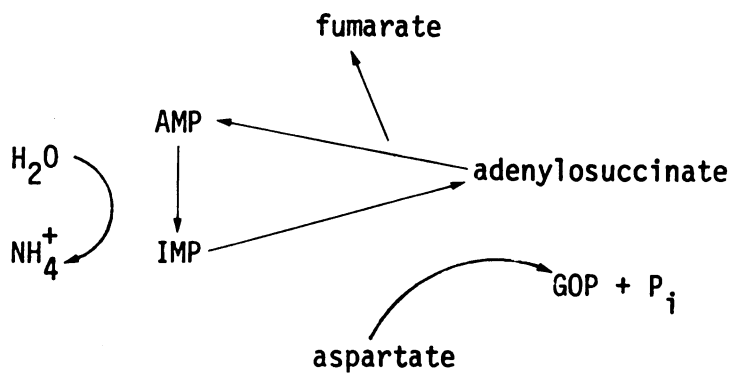
2. Tyrosine. Through the use of 1-fluoro-2,4,-dinitrobenzene, arylation of the tyrosine residues was achieved. After short exposure to this reagent, the enzyme exhibited a higher K_m for 5'AMP, with little decrease in maximum velocity. It was concluded that tyrosine residues are important in substrate binding (63).

3. Lysine. Six to seven lysine groups present in the rat muscle enzyme may be reacted with pyridoxal 5'-phosphate, and fixed by reduction with $NaBH_4$. ATP and GTP protected against this reaction. It was suggested that the lysine residues are important for GTP and ATP binding (64).

Physiological Role

The physiological role of 5'AMP aminohydrolase at this time remains obscure. Several workers have postulated possible roles. These are listed below.

1. Catalyzing a reaction in the purine nucleotide cycle.



Purine Nycleotide Cycle, after M. J. Lowenstein (1972), Physiological Review, 52, 382.

This cycle allows, at least in muscle, the regulation of fumarate.

2. Another suggested role of 5'AMP aminohydrolase is the regulation of phosphofructokinase activity, an important control in glycolysis (60). The NH_4^+ producued by the deamination reaction has been shown to activate phosphofructokinase (PFK). PFK has a K_A of 0.33 mM for NH_4^+. Another aspect of this activation is that NH_4^+ could serve to raise the pH. PFK is inhibited by ATP at pH 7.1, but not at pH 7.3. These combined effects would increase the activity of PFK (53).

3. 5'AMP aminohydrolase may be involved in the regulation of the relative concentrations of the adenine nucleotides (54). Under conditions simulating physiological, 5'AMP aminohydrolase was shown by Chapman and Atkinson (58) to be less active at an energy charge (E.C.) 0.9, than at lower values. This value is approximately that found in the liver, and other tissues (58, 59, 65).

The depletion of ATP, during an energy-using cellular process, results in the concurrent decrease in the E.C. This activates 5'AMP aminohydrolase which converts AMP to IMP, increasing the mole fraction of ADP and ATP. The net result is the stabilization of the E.C., at lower adenine nucleotide concentrations. It was also noted (58) that the concentration of adenine nucleotides will not be lowered to a value which is not compatible with the normal functioning of the cell. This lower limit is set because ATP is an activator for most 5'AMP aminohydrolases. When ATP concentration drops, the deamination of AMP will also decrease. Thus, 5'AMP aminohydrolase would function within the cell as a protection against drastic short-term decreases in the E.C. With the additional safeguard of not

allowing complete depletion of the adenine nucleotides which would be necessary to maintain the E.C.

4. Muscular Dystrophy. The levels of 5'AMP aminohydrolase in skeletal muscle from human patients suffering from Duchenne muscular dystrophy have been shown to be lower than in normal patients. This phenomenon was also observed in mice (61). Because of the important roles which can be postulated for 5'AMP aminohydrolase, as noted above, these low levels of activity could adversely affect muscle metabolism and may, in turn, contribute to the symptoms associated with Duchenne dystrophy.

METHOD AND MATERIALS

Enzyme Purification

5'AMP aminohydrolase (E.C. 3.5.4.6) was purified from mature rabbit back muscle in the manner of Smiley et al. (5). All buffers and reagents were of the same composition as used by Smiley.

Cellulose phosphate, used in the purification procedure, was washed with 10 volumes each of 0.5 N HCl, 0.5 M NaOH, and deionized water and then soaked several days in 10 mM Tris-EDTA before washing with deionized water. It was stored until use at 4°C. Just prior to use, the cellulose phosphate was equilibrated with extraction buffer.

The purification was taken through to the cellulose phosphate step of the original procedure (5). The enzyme was then eluted with 1.0 M KCl, 1 mM 2-mercaptoethanol, pH 7.0, instead of using a 0.45 to 1.0 M KCl gradient of the original procedure. Specific activities of between 80 and 130 units per mg of protein were obtained at 50 μM 5'AMP, pH 6.3.

The enzyme was routinely stored at 4°C, in the presence of 1.0 M KCL, 1 mM 2-mercaptoethanol, pH 7.0, under a nitrogen atmosphere. There was negligible loss of activity after three weeks. In all experiments, enzyme was used within two weeks after preparation.

Enzyme Assays

5'AMP aminohydrolase activity was determined using the spectrophotometric assay of Kalckar (3). For 5'AMP concentrations above 1.0 mM the modifications by Smiley and Suelter (66) were used. The changes in optical density at 265 nm or at 285 nm were followed by the use of a Beckman DU spectrophotometer in conjunction with a Sargent-Welch recorder. Changes in optical density per minute were converted to μmoles per minute using the conversion factors given by Smiley and Suelter (66) of 8.86 μmoles ml^{-1} cm^{-1} at 265 nm and 0.30 μmoles ml^{-1} cm^{-1} at 285 nm. All assays were started by addition of enzyme.

For routine assays the following buffer was used: 50 mM Tris-MES, 150 mM KCl, 50 μM 5'AMP, pH 6.3. For K_A determinations the buffer used was 90 mM Tris-MES, 50 μM 5'AMP, 300 mM $(CH_3)_4NCl$, at the appropriate pH. One unit of enzyme activity is defined as μmoles of 5'AMP deaminated per minute at 30°C.

The Michaelis-Menten Kinetic parameters K_m, K_A, and V_{max} were calculated after weighting the data to the reciprocal of the 4th power of the initial velocities as described by Wilkinson (68). This was accomplished with a computer using the program referred to as the Wilkin program.

Removal of Activating Cations

Activating cations were removed in either of the following two ways. For determination of K_A values where protein concentrations below 0.5 mg ml^{-1} were suitable, a small volume of purified

enzyme was passed over a column of Sephadex G-25 previously equilibrated with the buffer desired. The second procedure used for removing activating cations was by dialysis. Dialysis tubing (Union Carbide Corporation) was boiled in 2 mM sodium EDTA for one hour, rinsed twice with deionized water and stored in 2 mM sodium EDTA at 4°C. Before use all tubing was washed exhaustively with deionized water. Since it was found that ionic strength was critical for enzyme stability, the following procedure was adopted. One ml of purified enzyme in 1.0 M KCl was dialyzed successively against two 500 ml volumes of 0.5 M KCl and 0.3 M KCl, each for 12 hours. Each buffer also contained 1.0 mM Tris-MES at the appropriate pH and 1.0 mM 2-mercaptoethanol. Next the enzyme was dialyzed against two 500 ml volumes of 0.5 M $(CH_3)_4NCl$, 1 mM Tris-MES, each for 12 hours. The 0.5 M $(CH_3)_4NCl$ concentration was used instead of lower concentrations because the $(CH_3)_4N^+$ ion is noted to give a lower ionic strength than normally would be expected (69).

Protein Determination

Protein concentrations were determined by the tannic acid method of Katzenellenbogen and Dobryszyck (70) or by the method of Lowry (76) or by use of an extinction coefficient of 0.920 mg cm^{-1} ml^{-1} as determined by Zielke (72). For the turbidometric method all reagents were filtered before use. BSA in 1% NaCl solution was used as a standard.

Proton Release and Uptake Experiments

Protons released or absorbed by enzyme upon addition of K^+ were detected in either of two ways. The first method involved the use of Resazurin. The second method involved the use of a hydrogen ion sensitive electrode (Sargent-Welch S-30070 combination electrode) in conjunction with a sensitive differential amplifier (Heath/Schlumberger EU-200-30) coupled to a recorder (Heath/Schlumberger EU 205-1). For either method activating cations were removed from 5'AMP aminohydrolase by extensive dialysis as described above.

K_A Determination

Purified 5'AMP aminohydrolase was freed of activating cations by passing a small volume of enzyme over a Sephadex G-25 column according to the procedure described above. Initial velocities were determined as a function of K^+ concentration using the assay procedure described previously.

An initial estimate of K_A was determined by plotting the data after the fashion of Eadie-Hofstee (73). Here the residual activity of the enzyme in the absence of cation was subtracted from the initial velocity values at each cation concentration. More precise values of K_A were then determined, by using this initial estimate of K_A to design kinetic experiments as suggested by Cleland (74). Thus, initial velocities in triplicate at 5 concentrations of cation between 20 and 80% saturation were determined.

The points were then fitted to an Eadie-Hofstee plot, using the kinetic constants obtained from the Wilkin program (see Enzyme Assays, this section, for a description of this program).

As noted by Hemphill and Suelter (75), 5'AMP aminohydrolase was inactivated at high pH, giving anomalous kinetics. This inactivation is reduced as the monovalent cation, or protein concentration is increased (75). Data when plotted as S/V versus S which showed a nonlinear response, especially at low cation concentrations, was discarded. In all cases, protein concentration in the assay was kept as high as possible.

Reagents

The Tris-Base, MES, 2-mercaptoethanol, and 5'AMP were obtained from Sigma Chemical Company (St. Louis, Missouri). The 5'AMP was either the free acid or the sodium salt.

$(CH_3)_4NCl$ and Resazurin were obtained from Eastman (Rochester, New York). The $(CH_3)_4NCl$ was recrystallized twice from isopropanol and stored at 110°C until use. Resazurin was of the certified type.

The cellulose phosphate used in the purification was obtained from Brown Corporation (Berlin, New Hampshire). Its preparation for use is described in the section entitled Enzyme Purification.

All other reagents used were of reagent grade or better.

RESULTS

Kinetic Constants for K^+ Activation of 5'AMP aminohydrolase As a Function of pH

The K^+ activation constant along with V_{max} and the Hill slope, n, were determined as a function of pH in order to establish the nature of the interaction of H^+ and K^+ with enzyme. Activating cations were removed from purified enzyme by gel-filtration, as described in Methods. Initial velocities were then measured at 30°C versus K^+ ion concentration at 50 μM 5'AMP. The Wilkin program (see Methods for a description of this program) was used to analyze data for K_A's and V_{max}. Values for n were determined from Hill plots. Values for these constants are presented in Table 1. These experiments demonstrate that the K_A for K^+ activation decreases with increasing H^+ concentration, which suggests that K^+ and H^+ are not competing for the same site. Maximum velocity and Hill slope under these conditions were independent of pH. These last observations are consistent with previous data (75).

Release or Uptake of H^+ Upon K^+ Binding to 5'AMP aminohydrolase

Resazurin, a proton sensitive dye, was used to examine uptake or release of H^+ by enzyme upon addition of K^+ to a solution of enzyme. Resazurin was first characterized as to its visible absorption spectrum and pK. Figure 1 presents the visible absorption spectrum of Resazurin at a concentration of 50 μM in 45 mM Tris-MES

TABLE 1.--Kinetic Constants for K^+ Activation of 5'AMP aminohydrolase at pHs 6.2, 6.5 and 6.8.

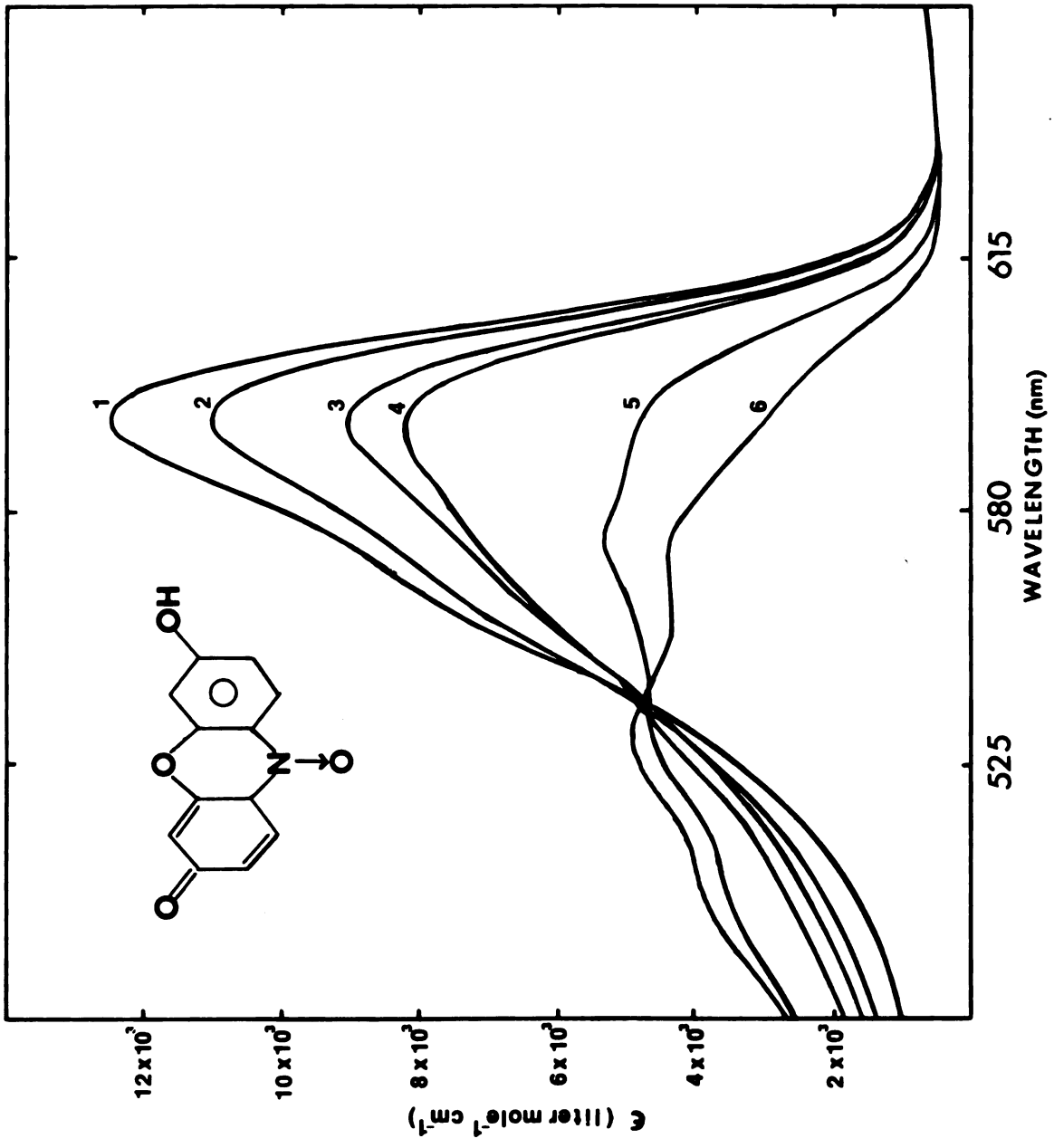
pH	K_A (mM)	V[1]	n[2]
6.2	1.96 ± 0.16	96.8 ± 13.9	0.98 ± 0.03
6.5	3.46 ± 0.25	109.0 ± 20.0	1.01 ± 0.08
6.8	6.10 ± 0.26	113.5 ± 12.0	1.02 ± 0.01

Reaction mixtures contained 90 mM Tris-MES, 300 mM $(CH_3)_4NCl$, and 50 µM 5'AMP.

[1]Velocity (µmoles min^{-1} mg^{-1}) at saturating concentrations of KCl.

[2]Hill slope as determined from plots of log (v/V-v) versus log (S).

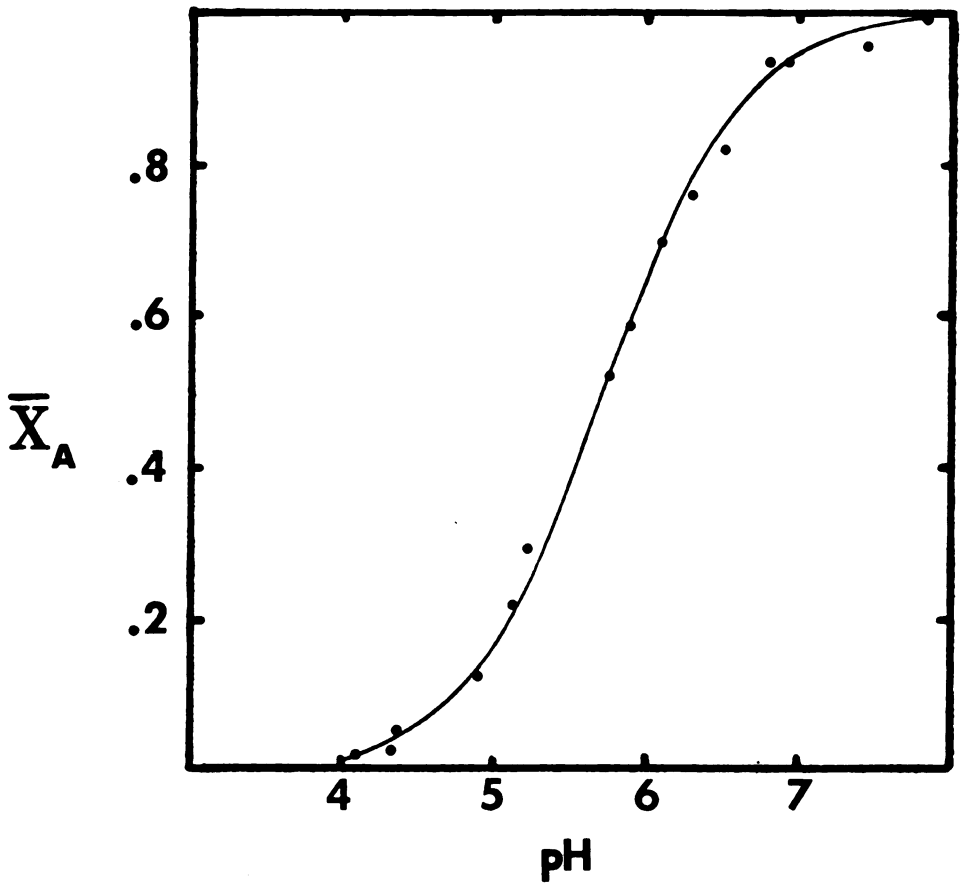
Figure 1.--Visible Absorption Spectrum of Resazurin. A Cary Model 21 Spectrophotometer with quartz cuvettes was used. Spectra of Resazurin (50 μM) dissolved in a buffer containing 45 mM Tris-MES were determined at the following pHs 6.50 (1), 6.10 (2), 5.80 (3), 5.70 (4), 5.30 (5), and 4.80 (6).


12x10³
10x10³
8x10³
6x10³
4x10³
2x10³
ε (liter mole⁻¹ cm⁻¹)
525
580
615
WAVELENGTH (nm)
1
2
3
4
5
6
OH
O
N→O
O

at various pHs. A maximum absorption at 600 nm was observed for the ionized form and 530 nm for the protonated form of dye. Titration of Resazurin with H^+ produced data which fitted a theoretical curve, calculated from the Henderson-Hasselbalch equation, using a pK of 5.7 (Figure 2).

Uptake or release of H^+ upon K^+ binding to enzyme, in the presence of Resazurin, was then examined by monitoring absorbance changes at 600 nm. Potassium was added to solutions containing 1.5 mg enzyme dissolved in one ml of buffer composed of 1.0 mM Tris-MES, 300 mM $(CH_3)_4NCl$ and between 5.0 to 15 μg of Resazurin. Amount of dye added was dependent on pH of experiment because sensitivity of dye to changes in H^+ ion concentrations is pH dependent. Experiments performed below approximately pH 6.15 shows an increase in absorbance indicating an uptake of H^+ by enzyme. At higher pHs a decrease in absorbance was observed indicating a release of H^+ by enzyme. Additional experiments at pHs below pH 6.0 were attempted but due to the precipitation of enzyme accurate absorbance readings were not possible. Experiments above pH 7.0 were not performed because earlier work demonstrated that enzyme at these pHs showed anomalous kinetics (75). As a control, K^+ was added to buffer minus enzyme resulting in a decrease in absorbance at high cation concentrations, this being due to the effect of dilution. In experiments with enzyme present, sufficiently concentrated solutions of cations were added so as to eliminate this dilution effect. In addition, levels of enzymatic activity were measured before and after each experiment and were found to change less than 5%.

Figure 2.--Titration Curve of Resazurin. The fraction of change in absorbance, X_A at 600 nm was determined for a solution of 2.5 μg ml^{-1}. Resazurin dissolved in 100 mM Tris-MES as a function of pH. Solid line is a theoretical curve calculated using the Henderson-Hasselbalch equation with a pK of 5.7.


$\overline{X}_A$
.8
.6
.4
.2
4
5
6
7
pH

Data from these titration experiments were analyzed as follows. At zero cation concentrations, the absorbance at 600 nm was noted and was then subtracted from absorbance at 600 nm obtained after each addition of cation to give a net change in absorbance. Net changes in absorbances showed hyperbolic saturation with increasing K^+ concentration and were found to fit equation 1

$$\Delta A = \frac{\Delta A_{max} [K^+]}{K_A + [K^+]} \qquad (1)$$

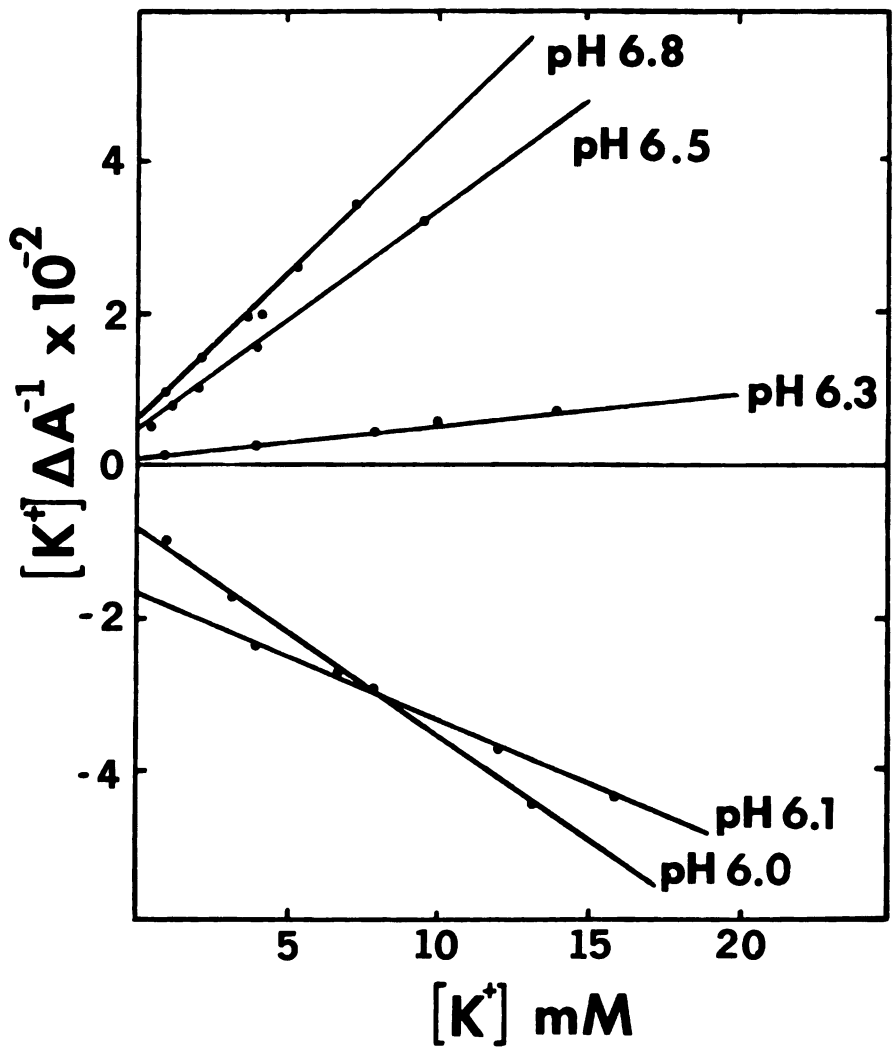
where ΔA is change in absorbance, and ΔA_{max} change in absorbance at saturating concentrations of K^+. Equation 1 may be transformed into equation 2 giving a linear relationship between ΔA and K^+ concentration.

$$\frac{[K^+]}{\Delta A} = \frac{[K^+]}{\Delta A_{max}} + \frac{K_A}{\Delta A_{max}} \qquad (2)$$

Likewise, equation 2 may also be transformed into an equation having the same functional form as the Lineweaver-Burk equation. Thus, the Wilkin program may be used to analyze data for K_A's and ΔA_{max}. These constants were then used to plot data after equation 2 (Figure 3). Titration experiments were performed and analyzed in this manner over a pH range of 6.0 to 7.0. (The K_A's are summarized in Figure 5, page 34).

The slopes of plots shown in Figure 3 would normally be used to obtain information concerning the nature of interactions between two molecules for the same or different binding sites,

Figure 3.--Presentation of Change in Absorbance of Resazurin, Upon K^+ Binding to 5'AMP aminohydrolase (1 mg ml^{-1}) as per Equation 2. Buffer included 1.0 mM Tris-MES, and 300 mM $(CH_3)_4NCl$. See text for explanation of data treatment.


pH 6.8
pH 6.5
pH 6.3
pH 6.1
pH 6.0
4
2
0
-2
-4
5
10
15
20
[K+] ΔA-1 x 10-2
[K+] mM

however, due to the varying sensitivity of Resazurin with pH, and absorption of H^+ by byffer and enzyme, slopes at each pH cannot be compared. In order to overcome this, attempts were made to back-titrate the total change in absorbance so that the exact nature of these variables could be determined and the data corrected. Unfortunately, all attempts to back-titrate ended in precipitation of enzyme. This was probable due to locally high concentrations of base, in the cuvette, following addition of stock base solutions, prior to mixing. Lowering stock concentration of base to prevent this necessitated the addition of larger volumes of base which, in this case, was technically not possible due to volume limitations of cuvettes being used.

It is important to note that these experiments were done in absence of substrate. Thus, K^+ binds to free form of enzyme suggesting that the K^+ mediated activation does not involve K^+ binding to substrate or to the enzyme-substrate complex. This distinction becomes important when considering an activation scheme.

Finally, binding of K^+ to enzyme causes a release or an absorption of H^+s depending on the pH of the solution. This would indicate that there are several types of functional groups present on the enzyme surface which are affected by K^+ binding.

Stoichiometry of H^+ Released or Absorbed Upon K^+ Binding to 5'AMP aminohydrolase As a Function of pH

A H^+ sensitive glass electrode coupled to a differential amplifier (see Methods for a description of equipment used) was used

to determine the pH change following addition of K^+ to a solution of enzyme. Here, back-titration could be performed without any of the problems associated with the Resazurin experiments. In these experiments the enzyme was freed of activating cations by dialysis, according to the procedure outlined in Methods. The pHs of solutions containing 1.5 mg/ml of enzyme in a buffer composed of 1.0 mM Tris-MES, and 300 mM $(CH_3)_4NCl$ at the appropriate pH, were then monitored upon addition of K^+. A control experiment was performed where pH of the above buffer solution, minus enzyme, was monitored upon addition of K^+, resulting in no detectable change in pH. All experiments were performed under a nitrogen atmosphere to prevent absorption of CO_2 by buffer. Data generated in this manner showed hyperbolic saturation with increasing K^+ concentration and were found to fit equation 3

$$\Delta pH = \frac{(\Delta pH)_{max}\,[K^+]}{K_A + [K^+]}$$

where $(\Delta pH)_{max}$ is the change in pH at saturating concentrations of K^+. Equation 3 may be transformed into an equation having the same functional form as that of the Lineweaver-Burke equation. Thus, the Wilkin program may be used to analyze data for K_A's and $(\Delta pH)_{max}$ as an amount of OH^- or H^+. This was done by back-titrating pH changes. After saturating levels of K^+ had been reached (100 x K_A) several aliquots of $(CH_3)_4NOH$ or HCl were added with pH change being noted after each addition. From such data standard curves of ΔpH versus OH^- or H^+ concentrations were constructed. The slopes,

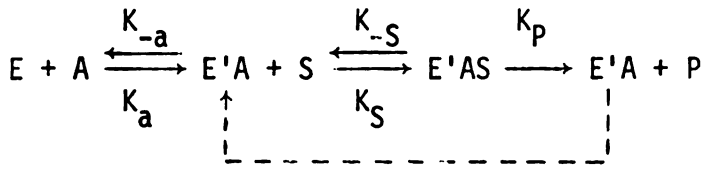
$\Delta pH/[OH^-]$ or $\Delta pH/[H^+]$ calculated from a least squares fit of data to a straight line, were then used, knowing volume of enzyme solution, to express $(\Delta pH)_{max}$ as an amount of OH^- or H^+. The following formula was then employed to calculate numbers of H^+ released or absorbed per molecule of enzyme:

$$\Delta \bar{V}^n_{H^+} = \frac{(B)(270,000)}{1.5 \times 10^{-3}} \qquad (4)$$

where B represents amount of base or acid added to neutralize $(\Delta pH)_{max}$, 270,000 the molecular weight of enzyme (6) and the factor of 1.5×10^{-3} grams represents amount of enzyme used in each experiment. These experiments were repeated over the pH range of 6.0 to 7.0 and Figure 4 (page 28) summarizes the data.

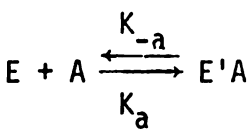
DISCUSSION

The goal of this work was to delineate the importance of H^+ in the K^+ mediated activation of 5'AMP aminohydrolase. A mechanism for K^+ activation represented by the following scheme has been proposed by Suelter et al. (77):



Scheme 1

where A is activator, S, substrate, P, product and E and E'A enzyme and activated enzyme, respectively. Activator, A, can take a variety of forms, e.g., 5'AMP, K^+, ATP, ADP, and H^+. Hydrogen ion is included because previous data (77) were consistent with such an activation. As results show, K_A's for K^+ activation generated by measuring H^+ uptake or release using Resazurin and a Hydrogen ion electrode in the absence of substrate are simular to those obtained kinetically in presence of substrate (Figure 5). Thus, K^+ activation of the enzyme can be portrayed by the first step in the above scheme.



(1)

Changes in the equilibrium of equation 1 observed as a decrease in K_A with decreasing pH implies a cooperative interaction between H^+ and K^+ binding sites.

The stoichiometry of H^+ binding can be estimated by fitting difference titration data to equation 2 (see Appendix A for the derivation of this equation)

$$\Delta\bar{\nu}^{n}_{H^+} = \sum_{i=1}^{\ell} \left(\frac{n_i a_H}{a_H + K_i} - \frac{n_i a_H}{a_H + K_i^{(A)}} \right) \qquad (2)$$

where $\Delta\bar{\nu}^{n}_{H^+}$ is the net difference in numbers of H^+ released or absorbed by enzyme upon addition of saturating levels of K^+ and K_i and $K_i^{(A)}$ are H^+ ion dissociation constants in presence and absence of K^+, respectively. Figure 4 shows difference titration data along with theoretical curves calculated from equation 2. It became apparent in early attempts to fit data that there are large numbers of groups undergoing pK changes. With so many variables (n_1, n_2, . . . , n_ℓ; k_1, k_2, . . . , k_ℓ; $k_1^{(A)}$, $k_2^{(A)}$, . . . , $k_\ell^{(A)}$) it is impossible to obtain a unique fit to data and only the cases using the least numbers of variable constants are presented. This leads to a postulation of at least 18 H^+ binding sites per subunit which are affected by K^+ binding (Figure 4). A further conclusion which can be reached from these experiments is that the enzyme undergoes a conformational change upon K^+ binding. Such a conclusion can be reached for the following reason. An anomalous pK for a group can result from hydrogen bonding (82), or from strong near-neighbor

Figure 4.--Difference Titration Data and Theoretical Curves as Calculated from Equation 4. Changes in pH, using equipment as described in Methods, of solutions containing 1.5 mg ml^{-1} 5'AMP aminohydrolase dissolved in 1.0 mM Tris-MES, and 300 mM $(CH_3)_4NCl$ upon addition of KCl. All experiments were performed under an atmosphere of N_2. Theoretical curves were calculated from equation 4 of Discussion for cases 1 (——), 2 (-·-), 3 (---), and 4 (···) using constants given in Table 2.

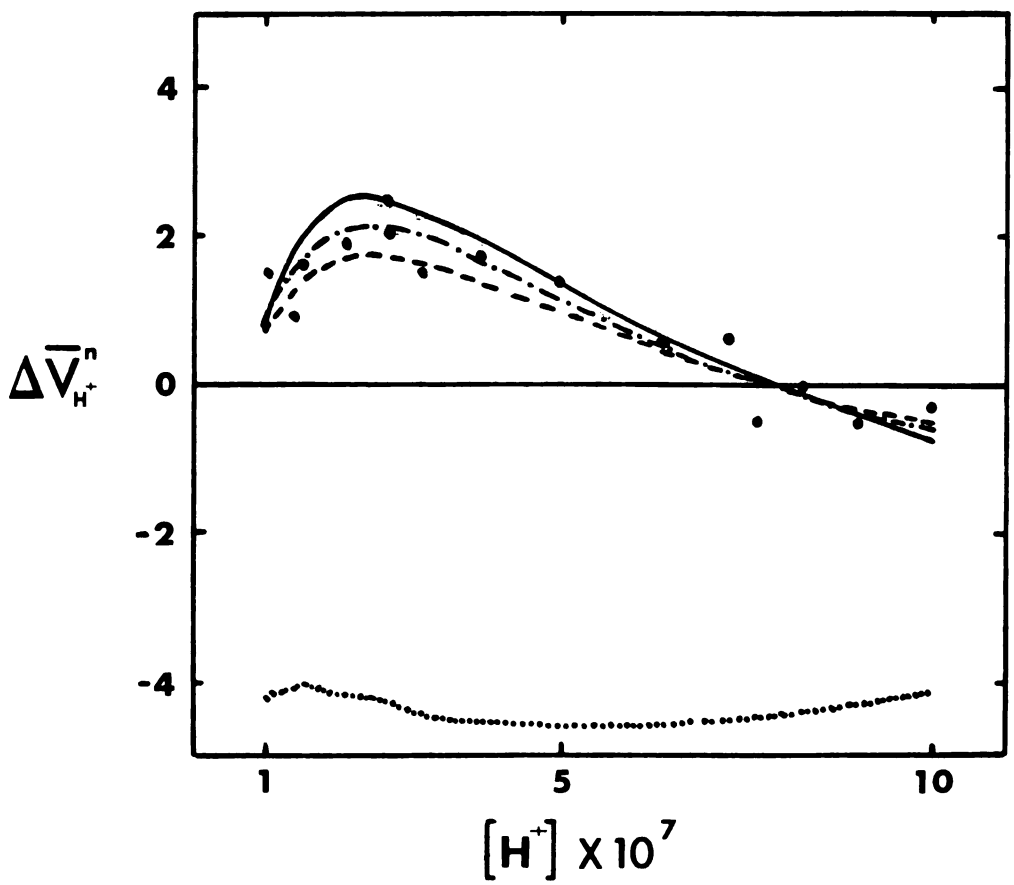

4
2
0
-2
-4
$\Delta\bar{V}^n_{H^+}$
1
5
10
$[H^+] \times 10^7$

TABLE 2.--Constants* Used in Equation 4 of Disucssion for Calculation of Theoretical Curves as Depicted in Figure 4.

Case	pK	$pK^{(A)}$	n
	5.78	6.22	7
1	6.75	6.20	7
	7.70	8.20	7
	5.78	6.22	6
2	6.75	6.20	6
	7.70	8.20	6
	5.78	6.22	5
3	6.75	6.20	5
	7.70	8.20	5
	6.10	6.80	6
4	6.90	6.45	6
	7.65	8.20	6

*See Discussion for explanation of constants.

electrostatic interactions (83) between this group and other nearby groups, or by the group itself being buried in a hydrophobic region (84). Thus, any conformational change will modify these types of effects producing a pK change.

Now, by considering Wyman's theory of linked functions, K_A versus pH data may be shown to satisfy a linkage equation (80), thus implying cooperative interactions between H^+ and K^+ binding sites. Wyman shows, from mass action considerations, that the basic linkage equation for binding of two ligands A and B is given by

$$\left(\frac{\partial \bar{V}_A}{\partial \ln a_B}\right)_{a_A} = \left(\frac{\partial \bar{V}_B}{\partial \ln a_A}\right)_{a_B} \tag{3}$$

where $\bar{V}_A$ and $\bar{V}_B$ are numbers of A and B ligands bound to enzyme, respectively, and a_A and a_B are activities of A and B ions, respectively. A further linkage equation (which is suitable for integration) may be derived from equation 3 and in our case for H^+ and K^+ binding is given by

$$\left(\frac{\partial \bar{V}_{K^+}}{\partial \bar{V}_B}\right)_{a_B} = -\left(\frac{\partial \ln a_B}{\partial \ln a_{K^+}}\right)_{\bar{V}_{K^+}} \tag{4}$$

(see Appendix B for derivation of equation 4), where $\bar{V}_{K^+}$ and $\bar{V}_B$ are numbers of K^+ are the sum of all other ions bound to enzyme, respectively, and a_{K^+} is activity of K^+ ion and a_B is a composite activity of all other ions in equilibrium with enzyme. This equation must be

written in this form because there is no reason to suspect that K^+ does not affect all types of ion equilibria. Given this, one can introduce the relationship

$$a_B = a_{H_+^+}\, a_{H_-^+}\, a_{A_1^-}\, a_{A_2^-} \tag{5}$$

where $a_{H_+^+}$ and $a_{H_-^-}$ are activities of H^+s absorbed and released by enzyme, rexpectively, and $a_{A_1^-}$ and $a_{A_2^-}$ are activities of buffer anions which are in equilibrium with enzyme. Introduction equation 4 into equation 5 results in equation 6.

$$-\left[\frac{\partial \bar{V}_{K^+}}{\partial \bar{V}_B}\right]_{a_{B^+}} = \left[\frac{\partial \ln a_{H_+^+}}{\partial \ln a_{K^+}}\right]_{\bar{V}_{K^+}} + \left[\frac{\partial \ln a_{H_-^+}}{\partial \ln a_{K^+}}\right]_{\bar{V}_{K^+}} + \left[\frac{\partial \ln a_{A_1^-}}{\partial \ln a_{K^+}}\right]_{\bar{V}_{K^+}} + \left[\frac{\partial \ln a_{A_2^-}}{\partial \ln a_{K^+}}\right]_{\bar{V}_{K^+}} \tag{6}$$

Experimentally, all H^+s and all buffer ions in equilibrium with enzyme are indistinguishable, thus, equation 6 is rewritten as

$$\left[\frac{\partial \bar{V}_{K^+}}{\partial \bar{V}_B}\right]_{a_{B^+}} = \left[\frac{\partial \ln a_{H^+}}{\partial \ln a_{K^+}}\right]_{\bar{V}_{K^+}} + \left[\frac{\partial \ln a_{A^-}}{\partial \ln a_{K^+}}\right]_{\bar{V}_{K^+}} \tag{7}$$

An equation similar to equation 7 has been derived by Wyman describing the Bohr effect of Hemoglobin (80). In that case it was shown that the effect of anions on O_2 binding was, within experimental

error, to be zero. If this approximation is used in our case, equation 7 becomes

$$\left(\frac{\partial \bar{V}_{K^+}}{\partial \bar{V}_{H^+}}\right)_{a_{H^+}} = -\left(\frac{\partial \ln a_{H^+}}{\partial \ln a_{K^+}}\right)_{\bar{V}_{K^+}} \tag{8}$$

which says that K^+ binding is now solely dependent upon numbers of H^+ bound to enzyme. Wyman (80) describes a procedure by which equation 8 can be integrated (integration is detailed in Appendix C) to give an equation showing K_A as a function of a_{H^+}. This is given as

$$K_A = K_A''\left[\left(\frac{a_{H^+} K_1}{a_{H^+} K_1^{(A)}}\right)^{n_1/q}\left(\frac{a_{H^+} K_2}{a_{H^+} K_2^{(A)}}\right)^{n_2/q} \cdots \left(\frac{a_{H^+} K_\ell}{a_{H^+} K_\ell^{(A)}}\right)^{n_\ell/q}\right] \tag{9}$$

where K_A'' is K_A at infinite a_{H^+}, and K_1 and $K_1^{(A)}$ are dissociation constants for dissociation of H^+ from group ℓ in presence and absence of saturating concentrations of K^+, respectively, and q and n being numbers of K^+ and H^+ binding sites, respectively. It should be noted here that equation 9 is written in a form which cannot be used to predict whether those H^+ binding sites which release H^+s cause a decrease in K_A with decreasing pH or whether those H^+ binding sites which absorb H^+s cause such decrease (see Appendix C for elaboration of this point). This question will be answered later by using Weber's principle of conservation of free-energy.

Figure 5 shows K_A's generated from equilibrium and kinetic experiments versus H^+ concentration along with theoretical curves

Figure 5.--The pH Dependence of K_A for K^+ Activation of 5'AMP aminohydrolase and Theoretical Curves as Calculated from Equation 10. Activation constants, K_As were determined from Equilibrium (▲) or Kinetic (●) experiments (experimental conditions and data treatment are given in Results). Theoretical curves were calculated from equation 10 of Discussion for cases 1 (——), 2 (···), 3 (-··-), and 4 (---) using constants given in Table 3.

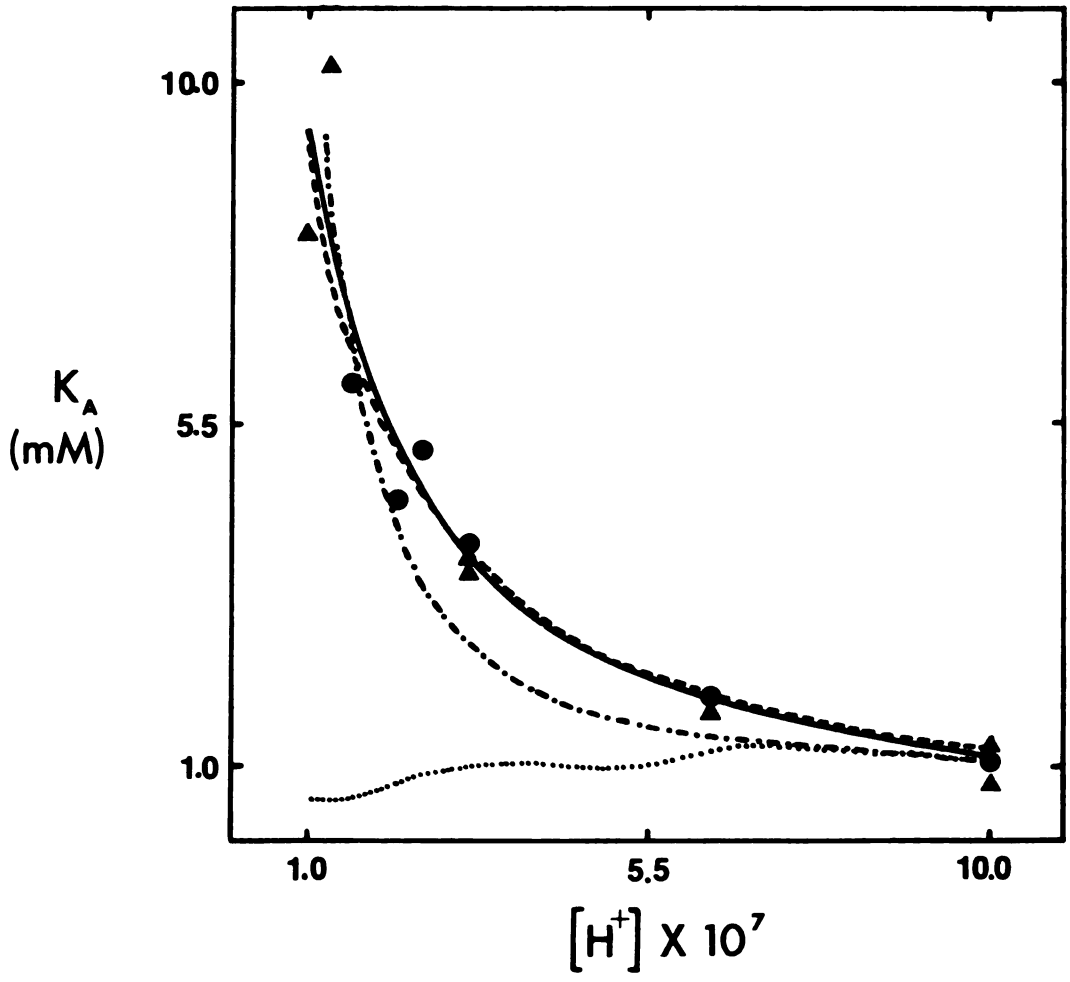

10.0
5.5
1.0
K_A
(mM)
1.0
5.5
10.0
$[H^+] \times 10^7$

TABLE 3.--Constants* Used in Equation 10 of Discussion for Calculation of Theoretical Curves as Depicted in Figure 5.

Case	pK	$pK^{(A)}$	n/q
	6.10	6.80	6
1	6.90	6.45	6
	7.65	8.20	6
	5.78	6.22	6
2	6.75	6.20	6
	7.70	8.20	6
	6.10	6.75	12
3	6.65	6.10	12
	7.65	8.05	12
	6.10	6.80	4
4	6.90	6.50	4
	7.65	8.20	4

*See Discussion for explanation of constants.

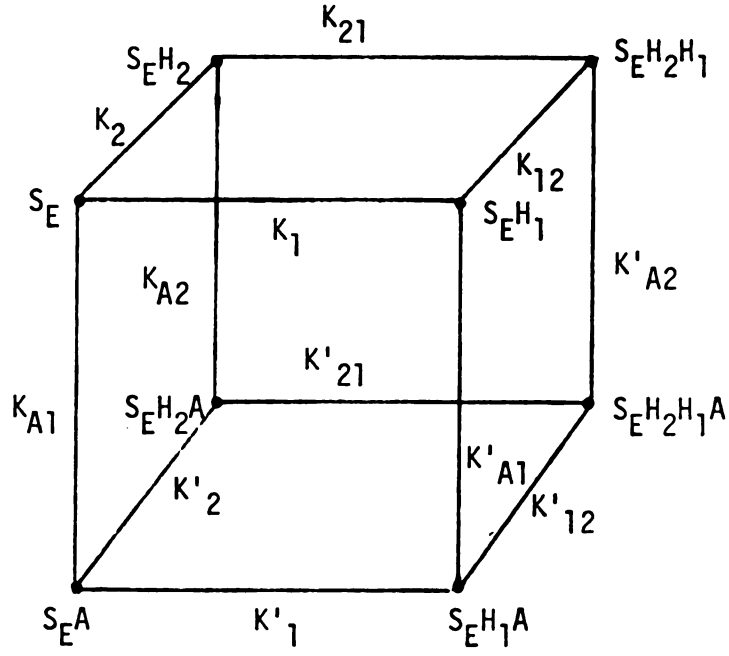
calculated using equation 9. Again, data suggests that there are large numbers of groups undergoing pK changes. With so many variable constants (K''_A; n_1, n_2, . . . , n_ℓ; k_1, k_2, . . . , k_ℓ; $k_1^{(A)}$, $k_2^{(A)}$, . . . , $k_\ell^{(A)}$), it is impossible to determine unique values for these constants. As a result, the data may be fit in either of the following two ways: either by allowing a small number of pKs to vary with a large number of sites having the same pK or, alternatively, a large number of pKs varying with perhaps only 2 or 3 sites having identical pKs. This second case is the more probable but embodies a larger number of variable constants which makes fitting of data impossible. In either case, theoretical lines were calculated using 18 H^+ binding sites per subunit because this was the minimum number of sites shown to fit difference titration data (Figure 4). Although the stoichiometry is complex, the data do fit a linkage equation, thus implying cooperative interactions between H^+ and K^+ binding sites.

Although obtaining absolute values for H^+ dissociation constants cannot be accomplished with the above data, several conclusions can be drawn in comparing Figures 4 and 5. First, the non-identity of pKs generated by the two fits shows that K^+ binding is not solely dependent upon numbers of H^+s bound. This conclusion comes from considering that difference titration experiments measure only net changes in H^+ ion equilibria upon K^+ binding, while K_A determinations are dependent upon both numbers of counterions and H^+s bound. For our case, then, in contrast to the Bohr effect for

Hemoglobin, the approximation of setting $((\partial \ln a_{A^-})/(\partial \ln a_{K^+}))_{\bar{V}_{A^-}}$ equal to zero is a poor one. This could be a reflection of the 9-fold difference in numbers of changes in H^+ equilibrium seen here as compared to those seen for O_2 binding to hemoglobin. In general it was observed that fits to equation 9 gave pKs which were of larger values than those obtained from fits to equation 2. In addition, when pKs obtained from best fits from each of these equations were interchanged and used to calculate theoretical lines, very poor fits were obtained (this is demonstrated in case 2 of Figure 5, and case 4 of Figure 4). The high pKs used in fitting data to equation 9 says that there are fewer numbers of H^+ bound than difference titration data indicates. Thus, some of the positive effect on K^+ binding exerted by H^+ binding is not observed. This suggests that the term $((\partial \ln a_{A^-})/(\partial \ln a_{K^+}))_{\bar{V}_{A^-}}$ of equation 7 is negative. From this, one would predict anions would inhibit K^+ binding and, thus, the activation process, with the net result being apparent inhibition of activity by anions. Such a conclusion is supported by experimental evidence which shows anions to inhibit 5'AMP aminohydrolase activity (8, 47, 50, 51).

Since it is apparent that stoichiometry of of H^+ release and uptake is extremely complex, a quantitative model cannot be given. However, using Weber's analysis of cooperative binding (78), the cooperative interactions between H^+ and K^+ binding sites can be described on a thermodynamic basis. This will allow formulation of a qualitative scheme which models experimental results without

having to deal with the complexities of stoichiometry. Applying Weber's analysis to our case results in the following model:



Scheme 2

where all equilibrium constants will be defined as dissociation constants. One subunit of enzyme, S_E, is depicted because, as discussed above, subunits are independent of one another, and identical. It should be noted that true stoichiometry is not portrayed. Thus, Scheme 2 is a qualitative model meant to depict one H^+ binding site either releasing or absorbing a H^+.

Applying the principle of free-energy conservation (78) to Scheme 2 results in 6 identities, each showing either linkage relationships between K^+ and H^+ binding site or between H^+ binding sites. The linkage relationships between H^+ binding sites are as follows:

in the absence of A

$$k_2k_{21} = k_{12}k_1 \tag{10}$$

and

in the presence of A

$$k_2'k_{21}' = k_{21}'k_1' \tag{11}$$

When $A(K^+)$ binds to enzyme the descriptions of H^+ equilibria of Scheme 2 pass from equilibrium constants of equation 10 to those of equation 11. Depending upon the relative magnitudes between these old and new equilibrium constants, either a release or uptake of H^+s will be observed. Which of these alternative observations takes place may be established by considering that uptake of H^+s implies $k_1' < k_1$. This inequality in turn implies $k_{21} < k_{21}'$, $k_2' < k_2$, and $k_{12}' < k_{12}$. (It is realized that conditions set forth by $k_1' < k_1$ can be met by a combination of equalities and inequalities between these constants. The case which is considered is the most general and thus will be used.) These last three inequalities now have the following consequences, $k_2' < k_2$, and $k_{21} < k_{21}'$ uptake of H^+s and $k_{21} < k_{21}'$ release of H^+s. Such uptake and release of H^+s is observed in difference titration experiments (Figure 4).

For Scheme 2 the linkage relationships between H^+ and K^+ binding sites are given by the following identities:

$$k_{A2}k_{21}' = k_{2A}'k_{21} \tag{12}$$

$$k_{A1}k'_1 = k'_{A1}k_1 \tag{13}$$

$$k'_{A1}k'_{12} = k'_{A2}k_{12} \tag{14}$$

$$k_{A1}k'_2 = k_{A2}k_2 \tag{15}$$

The behavior of K_A with changing pH can now be shown by applying the following inequalities $k'_A < k_2$, $k'_1 < k_1$, $k_{21} < k'_{21}$, and $k'_1 < k_1$ to these equations. When the inequalities $k'_{12} < k_{12}$, $k'_2 < k_2$, and $k'_1 < k_1$ are applied to equations 14, 15, and 13, respectively, it can be seen that over certain pH ranges the protonated form of enzyme will bind K^+ better than the unprotonated form of enzyme, resulting in a decrease in K_A with increasing pH. While the inequality $k_{21} < k'_{21}$ as applied to equation 12 demonstrates that over certain pH ranges the unprotonated form of enzyme binds K^+ better than the protonated form of enzyme, resulting in an increase in K_A with decreasing pH. This raises the question, which one of these effects dominated the behavior of K_A with pH? The answer to this question lies in the theory of linked functions where it was demonstrated that for our case it is the ratio between a H^+ equilibrium constant and its shifted value that determines such behavior. Thus, from equation 9 it can be seen that a small pK and its shifted value will have a greater effect on K^+ binding than a large pK and its shifted value. It should also be noted that such an effect will increase with increased numbers of H^+ binding sites involved. For our case, then, the H^+ binding sites which absorb a H^+ upon K^+

binding will determine the behavior of K_A with pH. This implies a decrease in K_A with decreasing pH. Such decrease in K_A is observed experimentally over the entire pH range examined.

In summary, stoichiometry of H^+ binding would indicate that K^+ activation, on a microscopic scale, is extremely complex. This being the case, only a qualitative scheme for K^+ activation can be given (Scheme 2). Such a scheme has as its basis cooperative interactions between H^+ and K^+ binding sites and, as demonstrated above, models experimental results. Thus, over the pH range studied, it is consistent to state that activation of 5'AMP aminohydrolase comes about by H^+s binding to sites on enzyme to give activated form of enzyme and K^+ is observed to activate because binding of K^+ promotes binding of these H^+s.

SUMMARY

The goal of this work was to examine the role of H^+ in the K^+ mediated activation of 5'AMP aminohydrolase. Both kinetic and equilibrium experiments were performed in defining this role. Kinetic experiments demonstrated that V_{max} and the Hill slope were, over the pH range examined, independent of pH, while the K_m decreased with decreasing pH. Equilibrium experiments demonstrated that the numbers of H^+ bound to enzyme changed upon K^+ binding. Data indicate linkage exists between the H^+ and K^+ binding sites. Thus, activation of enzyme is observed to take place when the H^+ binding sites undergo either a change in degree of ionization or protonation.

APPENDICES

APPENDIX A

DERIVATION OF EQUATION 2 OF THE DISCUSSION

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DERIVATION OF EQUATION 2 OF THE DISCUSSION

The net difference in numbers of H^+ bound to enzyme in the presence and absence of saturating concentrations of K^+, $\Delta\bar{V}^n_{H^+}$, is obtained from difference titration experiments and can be written as

$$\Delta\bar{V}^n_{H^+} = \bar{V}_{H^+}^{\bar{X}_{K^+}=1} - \bar{V}_{H^+}^{\bar{X}_{K^+}=0} \qquad (A1)$$

where $\bar{V}_{H^+}^{\bar{X}_{K^+}=1}$ and $\bar{V}_{H^+}^{\bar{X}_{K^+}=0}$ are numbers of H^+ bound to enzyme at a fractional saturation in K^+ of 1 and 0, respectively. Equation A1 can be rewritten in terms of a_{H^+} and dissociation constants for each group. This gives equation A2

$$\Delta\bar{V}^n_{H^+} = \sum_{i=1}^{\ell} \left(\frac{n_i a_H}{a_{H^+} k_i} - \frac{n_i a_H}{a_{H^+} k_i^{(A)}} \right) \qquad (A2)$$

with ℓ number of groups undergoing a pK change, each having n numbers of sites.

APPENDIX B

DERIVATION OF EQUATION 4 OF THE DISCUSSION

APPENDIX B

DERIVATION OF EQUATION 4 OF THE DISCUSSION

Wyman (80) shows from mass action considerations that the basic linkage equation for the binding of two ligands (in this case, K^+ and B) to a macromolecule, at constant temperature and pressure, is given by equation B1

$$\left(\frac{\partial \bar{V}_{K^+}}{\partial \ln a_B}\right)_{a_{K^+}} = \left(\frac{\partial \bar{V}_B}{\partial \ln a_{K^+}}\right)_{a_B} \tag{B1}$$

where $\bar{V}_{K^+}$ and $\bar{V}_B$ are numbers of K^+ and B ions bound to enzyme, respectively, and a_{K^+} and a_B are activities of K^+ and of B ions, respectively.

The total differential equations, at constant temperature and pressure, showing the dependency of $\bar{V}_{K^+}$ and $\bar{V}_B$ on a_{K^+} and a_B are

$$d\bar{V}_{K^+} = \left(\frac{\partial \bar{V}_K}{\partial \ln a_{K^+}}\right)_{a_B} d \ln a_{K^+} + \left(\frac{\partial \bar{V}_{K^+}}{\partial \ln a_B}\right) d \ln a_B \tag{B2}$$

and

$$d\bar{V}_B = \left(\frac{\partial \bar{V}_B}{\partial \ln a_{K^+}}\right)_{a_B} d \ln a_{K^+} + \left(\frac{\partial \bar{V}_B}{\partial \ln a_B}\right)_{a_{K^+}} d \ln a_B \tag{B3}$$

Experimentally, one observes changes in $\bar{V}_B$ at constant $\bar{V}_{K^+}$, thus setting $d\bar{V}_{K^+} = 0$ in equation B2 gives

$$\left(\frac{\partial \bar{V}_{K^+}}{\partial \ln a_{K^+}}\right)_{a_B} d \ln a_{K^+} = - \left(\frac{\partial \bar{V}_{K^+}}{\partial \ln a_B}\right)_{a_K} d \ln a_B \qquad (B4)$$

Now, substituting equation B1 into equation B4 gives

$$\left(\frac{\partial \bar{V}_{K^+}}{\partial \ln a_K}\right)_{a_B} d \ln a_K = - \left(\frac{\partial \bar{V}}{\partial \ln a_{K^+}}\right)_{a_B} d \ln a_B \qquad (B5)$$

This equation can be rearranged (remembering $d\bar{V}_{K^+} = 0$) to give the linkage equation of interest.

$$\left(\frac{\partial \bar{V}_{K^+}}{\partial \bar{V}_B}\right)_{a_B} = - \left(\frac{\partial \ln a_B}{\partial \ln a_{K^+}}\right)_{V_{K^+}} \qquad (B6)$$

APPENDIX C

INTEGRATION OF EQUATION 9 OF THE DISCUSSION

APPENDIX C

INTEGRATION OF EQUATION 9 OF THE DISCUSSION

Integration of equation 9 can be accomplished by using a procedure outlined by Wyman (80) which is as follows. Defining the fractional saturation, $\bar{X}_{K^+}$ of enzyme K^+ as

$$\frac{\bar{V}_{K^+}}{q} = \bar{X}_{K^+} \tag{C1}$$

where $\bar{V}_{K^+}$ is number of K^+ bound to enzyme, and q the number of K^+ binding sites. Differentiating this equation gives

$$\frac{1}{q} d\bar{V}_{K^+} = d\bar{X}_{K^+} \tag{C2}$$

then multiplying equation 9 by above equation C2 results in

$$\frac{1}{q} (d\bar{V}_{H^+}) = - \left(\frac{\partial \ln a_{K^+}}{\partial \ln a_{H^+}}\right)_{\bar{V}_{K^+}} d\bar{X}_{K^+} \tag{C3}$$

Integration between the limits of $\bar{X}_{K^+} = 0$ and $\bar{X}_{K^+} = 1$ results in

$$\frac{1}{q} \int_{\bar{X}_{K^+}=0}^{\bar{X}_{K^+}=1} d\bar{V}_{H^+} = - \int_{\bar{X}_{K^+}=0}^{\bar{X}_{K^+}=1} \left(\frac{\partial \ln a_{K^+}}{\partial \ln a_{K^+}}\right)_{\bar{V}_{K^+}} d\bar{X}_{K^+} \tag{C4}$$

Integrating the left side of this equation is straightforward while the right side can be rearranged to give

$$\frac{1}{q}\left[\bar{V}_{H^+}^{\bar{X}_{K^+}=1} - \bar{V}_{H^+}^{\bar{X}_{K^+}=0}\right] = -\frac{\partial}{\partial \ln a_{H^+}} \int_{\bar{X}_{K^+}=0}^{\bar{X}_{K^+}=1} \ln a_{K^+}\, d\bar{X}_{K^+} \qquad \text{(C5)}$$

where $\bar{V}_{H^+}^{\bar{X}_{K^+}=1}$ and $\bar{V}_{H^+}^{\bar{X}_{K^+}=0}$ are numbers of K^+ bound to enzyme in the presence and absence of saturating levels of K^+, respectively. Now, considering the total free-energy change upon K^+ binding to a macromolecule to be given by

$$\Delta F_T = RT \int_{\bar{X}_{K^+}=0}^{\bar{X}_{K^+}=1} (\ln a_{K^+}) d\bar{X}_{K^+}$$

Whyman (80) shows that the left side of equation C6 is equal to RT ln $(a_{K^+})_{1/2}$ where $(a_{K^+})_{1/2}$ is the median ligand (K^+) activity. This results in

$$\int_{\bar{X}_{K^+}=0}^{\bar{X}_{K^+}=1} \ln(a_{K^+}) d\bar{X}_{K^+} = \ln(a_{K^+})_{1/2} \qquad \text{(C7)}$$

Substituting this relationship in equation C6 and identifying K_A with $(a_{K^+})_{1/2}$ gives

$$\frac{\ln K_A}{\ln a_{H^+}} = -\frac{1}{q}\left[\bar{V}_{H^+}^{\bar{X}_{K^+}=1} - \bar{V}_{H^+}^{\bar{X}_{K^+}=0}\right] \qquad \text{(C8)}$$

As shown in Appendix A, the terms $\bar{V}_{H^+}^{\bar{X}_{K^+=1}}$ and $\bar{V}_{H}^{\bar{X}_{K^+=0}}$ may be replaced by $(na_H)/(a_{H^+}K^{(A)})$ and $(na_H)/(a_{H^+}K)$, respectively. Since there is more than one group undergoing a pK change, equation C8 along with the above substitutions is now rewritten for ℓ groups undergoing a pK change as

$$\frac{\partial \ln k_A}{\partial \ln a_{H^+}} = \frac{1}{q} \left[\sum_{i=1}^{\ell} \left(\frac{n_i a_H}{a_{H^+} k_i} - \frac{n_i a_H}{a_{H^+} k_i^{(A)}} \right) \right] \tag{C9}$$

Rearranging and taking the indefinite integral by substitution (by letting $\mu^1 = a_{H^+}k_1$, $\mu^{1A} = (a_{H^+})k_1^{(A)}$, $\mu^2 = (a_{H^+})k_2$, $\mu^{2A} = (a_{H^+})k_2^{(A)}$, . . . , $\mu^{\ell} = (a_{H^+})k_{\ell}$, $\mu^{\ell A} = (a_{H^+})k_{\ell}^{(A)}$)

gives

$$\ln k_A + C = \frac{n_1}{q} \ln \frac{(a_{H^+}k_1)}{(a_{H^+}k_1^{(A)})} + \frac{n_2}{q} \ln \frac{(a_{H^+}k_2)}{(a_{H^+}k_2^{(A)})} + \cdots$$

$$+ \frac{n_{\ell}}{q} \ln \frac{(a_{H^+}k_{\ell})}{(a_{H^+}k_{\ell}^{(A)})} \tag{C10}$$

Now, letting K_A'' be K_A at infinite a_{H^+}, equation C10 becomes

$$\ln k_A = \ln k''_A + \frac{n_1}{q} \ln \frac{(a_{H^+} k_1)}{(a_{H^+} k_1^{(A)})} + \frac{n_2}{q} \ln \frac{(a_{H^+} k_2)}{(a_{H^+} k_2^{(A)})} + \cdots$$

$$+ \frac{n_\ell}{q} \ln \frac{(a_{H^+} k_\ell)}{(a_{H^+} k_\ell^{(A)})} \qquad \text{(C11)}$$

or, in nonlogarithmic terms,

$$k_A = k''_A \left[\left(\frac{a_{H^+} k_1}{a_{H^+} k_1^{(A)}} \right)^{n_1/q} \left(\frac{a_{H^+} k_2}{a_{H^+} k_2^{(A)}} \right)^{n_2/q} \cdots \left(\frac{a_{H^+} k_\ell}{a_{H^+} k_\ell^{(A)}} \right)^{n_\ell/q} \right] \qquad \text{(C12)}$$

It should be noted that in order to obtain a value of C (the constant of integration) it is necessary to arbitrarily assign uptake of H^+s as having a negative effect on K^+ binding and release of H^+s as having a positive effect on K^+ binding. Thus, the pK shifts of equation C12 cannot predict whether an uptake or release of H^+s will be observed upon K^+ binding. This will not make equation C12 any less valid for our case because, as it can be seen, it is the ratio of a pK and its shifted value which determines K^+ binding. In order to clarify this, the conservation of free-energy must be applied, as done in the Discussion.

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