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Equilibrium Constants for Association of Guanidinium and Ammonium Ions with Oxyanions

The Effect of Changing Basicity of the Oxyanion

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Using guanidinium and *n*-butylammonium cations (C^+) as models for the positively charged side chains in arginine and lysine, we have determined the association constants with various oxyanions by potentiometric titration. For a dibasic acid, H_2A, three association complexes may exist: $K_{1M} = [CHA]/[C^+]\,[HA^-]$; $K_{1D} = [CA^-]/[C^+]\,[A^{2-}]$; $K_{2D} = [C_2A]/[C^+]\,[CA^-]$. For guanidinium ion and phosphate, $K_{1M} = 1.4$, $K_{1D} = 2.6$, and $K_{2D} = 5.1$. The data for carboxylates indicate that the basicity of the oxyanion does not affect the association constant: acetate, $pK_a = 4.8$, $K_{1M} = 0.37$; formate, $pK_a = 3.8$, $K_{1M} = 0.32$; and chloroacetate, $pK_a = 2.9$, $K_{1M} = 0.43$, all with guanidinium ion. Association constants are also reported for carbonate, dimethylphosphinate, benzylphosphonate, and adenylate anions.

INTRODUCTION

The hydrogen-bonding ability of the guanidinium group of arginine has been found to have important functions in proteins, including bonding to carboxylate and phosphate anions at the functional site of enzymes, in nucleoproteins, in hapten–antibody complexes, and in the tertiary structure of proteins. In lactate dehydrogenase, three arginine residues appear to be involved in binding the carboxylic substrate and the pyrophosphate coenzyme (*1*).

In order to gain quantitative information which will be useful in interpreting these interactions, we have determined association constants between guanidinium and *n*-butylammonium cations and anions such as carboxylates, carbonate, and phosphate. We used guanidinium and *n*-butylammonium as models for the side chains of arginine and lysine, respectively.

In a solution of a weak acid, formation of a complex with the anionic conjugate base of the acid decreases the pK_a of the acid. This observation has been the basis for determining the association constants of complexes of various phosphates with mono- and divalent cations (*2*), of guanidinium with acetate (*3*), and of guanidinium with tetraphosphate (*4*).

Theoretical basis. The relation of the apparent $pK_{a'}$ value of a weak acid to the thermodynamic pK_a is given by

$$pK_{a'} = pK_a + \log(f_A/f_{HA}),$$

where f_{HA} and f_A are the activity coefficients for HA and A, the weak acid and its conjugate base. According to the Debye–Huckel theory, $pK_{a'}$ is less than pK_a and decreases

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with an increase in ionic strength. Since tetraalkylammonium halides do not complex significantly but do contribute to ionic strength, we have used the reduction in the observed pK_a brought about by the substitution of a complexing salt for a tetraalkylammonium salt to determine the association constant independent of the effect due to ionic strength.

For association of a cation, C^+, with a monoanion, HP^-, we use

$$(10^{\Delta pK} - 1)/[C^+] = K_{1M} = [CHP]/[C^+][HP^-], \tag{1}$$

which can be derived from the acidity constants (*2*). For a dianion, the following derivation takes into account three possible complexes, K_{1M} as described, K_{1D} for one cation associating with the dianion, and K_{2D} for two cations associating with the dianion:

$$K_{a2} = \frac{[H^+][P^{2-}]}{[HP^-]} \tag{2}$$

$$K_{1D} = \frac{[CP^-]}{[C^+][P^{2-}]} \tag{3}$$

$$K_{2D} = \frac{[C_2P]}{[C^+][CP^-]} = \frac{[C_2P]}{K_{1D}[P^{2-}][C^+]^2}. \tag{4}$$

At constant ionic strength using a complexing salt, where $[C^+] \gg P^{2-}$, $K_{a2'}$ for the dianion is

$$K_{a2'} = \frac{[H^+]([P^{2-}] + [CP^-] + [C_2P])}{[HP^-] + [CHP]}. \tag{5}$$

Substituting Eqs. (1), (3), and (4) into (5) gives

$$K_{a2'} = \frac{K_{a2}(1 + K_{1D}[C^+] + K_{1D}K_{2D}[C^+]^2)}{1 + K_{1M}[C^+]}, \tag{6}$$

which can be rearranged to give

$$\frac{10^{\Delta pK} - 1}{[C^+]} + (10^{\Delta pK})K_{1M} = K_{1D} + K_{1D}K_{2D}[C^+]. \tag{7}$$

After determining K_{1M} using Eq. (1), K_{1D} and K_{2D} can be determined from a plot of $(10^{\Delta pK} - 1)/[C^+] + 10^{\Delta pK}K_{1M}$ vs $[C^+]$ (Eq. (7)). If there is no association of a second cation to the dianion, $K_{2D} = 0$, then Eq. (7) reduces to

$$\frac{10^{\Delta pK} - 1}{[C^+]} = K_{1D} - 10^{\Delta pK}K_{1M}. \tag{8}$$

In this case, a plot of $(10^{\Delta pK} - 1)/[C^+]$ vs $10^{\Delta pK}$ would give a plot of negative slope $= -K_{1M}$ and intercept $= K_{1D}$.

EXPERIMENTAL

Materials. Tetramethylammonium chloride (Eastman) was dried for 24 hr on an Abderhalden apparatus and stored in a desiccator. Guanidinium chloride (Sigma) was

dried *in vacuo* prior to use. *n*-Butylamine (Aldrich) was acidified with concentrated HCl and the salt was recrystallized from chloroform and benzene. Dimethylphosphinic acid was prepared from thiophosphoryl chloride (*5*) and was dried before use.

Carbonate-free sodium hydroxide solutions were made from a 50% sodium hydroxide solution and standardized with Harleco 0.1000 *N* HCl using phenolphthalein. Hydrochloric acid solutions were standardized with Harleco 0.2000 *N* NaOH.

Titration method. All titrations were performed with a Radiometer 26 pH meter using glass and saturated calomel electrodes. Solutions of 25 ml were titrated at a constant ionic strength of 1.02 *M* in a water-jacketed beaker at 30°C. Solutions above pH 6 were titrated under N_2.

Phosphate dianion, carbonate, bicarbonate, acetate, and formate solutions of 0.02 molarity were titrated with 10-μl aliquots of 1.0305 *M* HCl. Phosphate monoanion was studied at 0.12 *M* in order to maximize the accuracy because of problems associated with the low pK_a (*6*); it was titrated with 10-μl aliquots of 6.12 *M* HCl. Chloroacetic acid and dimethylphosphinic acid were titrated at 0.12 *M* with 20-μl aliquots of 3.629 *M* NaOH. AMP^- and benzylphosphonate, 0.02 *M*, were titrated with 10-μl aliquots of 0.992 *M* NaOH.

The pH meter was standardized with Harleco buffers of pH 4.01 and 7.00. Initially, calculations of pK_a were made according to the methods of Albert and Serjeant, inincluding the appropriate corrections for pH readings below 4 and above 9 (*1a*, *5*). However, further data analysis led us to find an improved method (*7*) based on nonlinear estimation using Taylor's expansion to find corrections which were added iteratively to the two unknowns, K_a and V_{ep} (the volume of titrant required to reach the equivalence point). Using this method, standard deviations decreased to an insignificant value. We noted significant errors in the expected value of V_{ep}; this problem may have originated with samples with unknown water content which caused inaccuracy in V_{ep}, a key input parameter in the Albert and Serjeant method (*6*). The calculation based on

TABLE 1

ASSOCIATION CONSTANTS FOR MONOANIONS[a]

Anion	Cation	K_a(±SD)[b]	pK	ΔpK	K_{1M}
CH_3COO^-	1 *M* TMA^+	$2.58 \pm 0.05 \times 10^{-5}$[c]	4.588		
(pK_a 4.76)	1 *M* GH^+	$3.53 \pm 0.03 \times 10^{-5}$	4.452	0.136	0.37
	1 *M* *n*-BuA^+	$3.39 \pm 0.06 \times 10^{-5}$	4.470	0.118	0.31
$HCOO^-$	1 *M* TMA^+	$2.61 \pm 0.12 \times 10^{-4}$	3.584		
(pK_a 3.75)	1 *M* GH^+	$3.45 \pm 0.14 \times 10^{-4}$	3.462	0.122	0.32
$ClCH_2COOH$	0.9 *M* TMA^+	$1.88 \pm 0.07 \times 10^{-3}$	2.727		
(pK_a 2.87)	0.9 *M* GH^+	$2.61 \pm 0.03 \times 10^{-3}$	2.584	0.143	0.43
$(CH_3)_2PO_2H$	0.9 *M* TMA^+	$8.04 \pm 0.26 \times 10^{-4}$	3.095		
(pK_a 3.08)	0.9 *M* GH^+	$1.30 \pm 0.06 \times 10^{-3}$	2.886	0.209	0.67

[a] Abbreviations used: TMA^+, tetramethylammonium chloride; GH^+, guanidinium chloride; *n*-BuA^+, *n*-butylammonium chloride.

[b] Ionic strength maintained at 1.02 *M*.

[c] The ± values are standard deviations from the final least-squares analysis (see Experimental).

nonlinear estimation and using a digital computer program (PKNLE) provides the best fit of K_a and V_{ep} to approximately 40–50 observations. We believe that this method is a considerable improvement over previous methods (*6*); an indication of its success is given by the small standard deviations in Table 1.

RESULTS

The titration results for the oxymonoanions acetate, formate, chloroacetate, and dimethylphosphinate are shown in Table 1. The results for guanidinium and bases which can form mono- and dianions are in Table 2. The titration curves for phosphate dianion

TABLE 2

ASSOCIATION CONSTANTS OF GUANIDINIUM WITH CARBONATE, PHOSPHATE, AND PHOSPHONATE IONS

Titration[a]	Cation[b]	pK	ΔpK	Association constants
HCO_3^-/H_2CO_3	1.0 *M* TMA^+	6.296		
	1.0 *M* GH^+	6.133	0.163	$K_{1M} = 0.46$
CO_3^{2-}/HCO_3^-	1.0 *M* TMA^+	10.107		
	1.0 *M* GH^+	9.236	0.871	$K_{1D} = 4.1$
	0.75 *M* GH^+	9.379	0.728	$K_{2D} = 1.4$
	0.50 *M* GH^+	9.570	0.537	
	0.25 *M* GH^+	9.765	0.342	
$H_2PO_4^-/H_3PO_4$	0.9 *M* TMA^+	2.166		
	0.9 *M* GH^+	1.775	0.391	$K_{1M} = 1.35$
	0.7 *M* GH^+	1.873	0.293	$K_{1M} = 1.38$
				Average 1.37
$HPO_4^{-2}/H_2PO_4^-$	1.0 *M* TMA^+	6.982		
	1.0 *M* GH^+	6.122	0.860	$K_{1D} = 2.6$[c]
	1.0 *M* GH^+	6.133	0.849	$K_{2D} = 5.1$[c]
	0.8 *M* GH^+	6.239	0.743	
	0.75 *M* GH^+	6.281	0.701	
	0.6 *M* GH^+	6.388	0.594	
	0.5 *M* GH^+	6.478	0.504	
	0.25 *M* GH^+	6.700	0.282	
AMP^-/AMP^{2-}[d]	1.0 *M* TMA^+	6.549		
	1.0 *M* GH^+	5.742	0.807	$K_{1D} = 3.3$
	0.75 *M* GH^+	5.885	0.664	$K_{2D} = 3.1$
	0.5 *M* GH^+	6.055	0.494	
	0.25 *M* GH^+	6.273	0.276	
$C_6H_5CH_2PO_3H/$ $C_6H_5CH_2PO_3H_2$	1.0 *M* TMA	2.315		
	1.0 *M* GH^+	1.941	0.374	$K_{1M} = 1.4$
	0.8 *M* GH^+	1.979	0.336	1.5
	0.4 *M* GH^+	2.143	0.172	1.2
				Average 1.4

[a] For example, HCO_3^-/H_2CO_3 refers to titration of HCO_3^- to H_2CO_3.

[b] Ionic strength maintained at 1.02 *M* with $(CH_3)_4N^+Cl^-$.

[c] Determined using a value of $K_{1M} = 1.37$, the average value for phosphate monoanion.

[d] AMP = adenosine monophosphate = adenylic acid.

with 1 *M* tetramethylammonium chloride (TMA^+) and 1 *M* guanidinium chloride (GH^+) are shown in Fig. 1 as an example. The results for *n*-butylammonium are compared to guanidinium ion for association with phosphate mono- and dianions in Table 3.

TABLE 3

COMPARISON OF ASSOCIATION CONSTANTS OF BUTYLAMMONIUM AND GUANIDINIUM IONS WITH PHOSPHATE IONS

Phosphate	Cation[a]	p*K*	*Δ*p*K*	Association constants
$H_2PO_4^-/H_3PO_4$	0.9 *M* TMA^+	2.166		
	0.5 *M* *n*-BuA^+	2.016	0.150	$K_{1M} = 0.83$
	0.2 *M* *n*-BuA^+	2.085	0.081	1.03
	GH^+	Table 2		1.37
$HPO_4^{2-}/H_2PO_4^-$	1.0 *M* TMA^+	6.982		
	1.0 *M* *n*-BuA^+	6.372	0.610	$K_{1D} = 2.0$
	0.75 *M* *n*-BuA^+	6.482	0.500	$K_{2D} = 2.2$
	0.5 *M* *n*-BuA^+	6.645	0.337	
	0.25 *M* *n*-BuA^+	6.815	0.167	
	GH^+	Table 2		$K_{1D} = 2.6$
				$K_{2D} = 5.1$

[a] Ionic strength maintained at 1.02 *M* with $(CH_3)_4N^+Cl^-$.

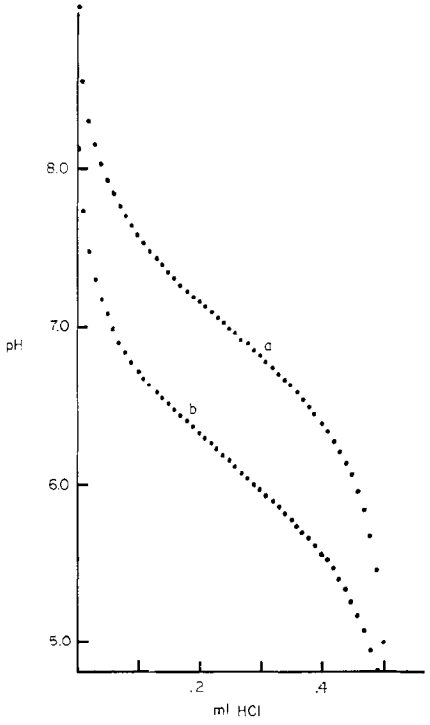


FIG. 1. Titration curve of HPO_4^{2-} with (a) 1 *M* TMA^+ and (b) 1 *M* GH^+.

The experimental data for $HPO_4^{2-}/H_2PO_4^-$ were inconsistent with Eq. (8), which assumes $K_{2D} = 0$, because we found a positive slope; therefore, we included K_{2D} (Eq. (7)) and found that the data fit well (Fig. 2). The slopes and intercepts were obtained by the method of least squares.

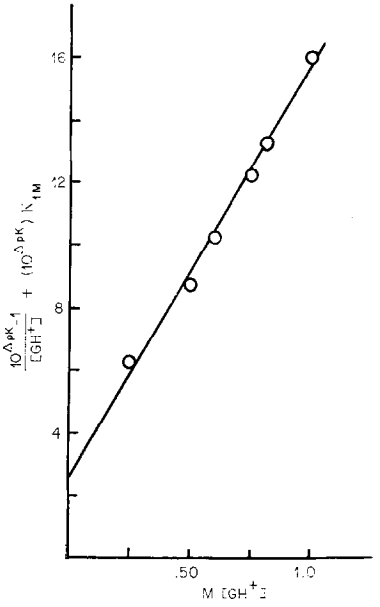


FIG. 2. Plot of data using Eq. (7) for the titration of HPO_4^{2-} with GH^+.

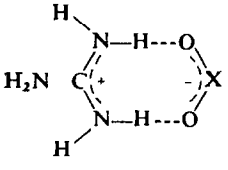
The pK's were determined with appropriate corrections (*6*) at low pH and with correction for reaction with the complexing agent (*7*):

$$CH^+ + A^- \rightleftharpoons HA + C. \tag{9}$$

The pH values in titration curves were reproducible within ±0.01 pH unit. The typical standard deviation for the pK_a's determined from the titrations of acetate anion, formate anion, bicarbonate anion, carbonate dianion, phosphate dianion, and AMP dianion are within ±0.01. However, the values of K_{1D} and K_{2D} have considerably greater error because they are determined from Eq. (7) and graphs such as Fig. 2.

DISCUSSION

We have evaluated association constants for the interactions depicted in **1** and **2**. Since these association constants were measured in aqueous solution, solvation of the



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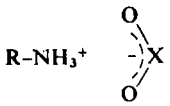

2

complex and the separated ions will play an important role. Since

$$\Delta G(\text{electrostatic}) = Q_1 Q_2 e^2/Dr \tag{10}$$

by classical electrostatic theory, we can expect that the effective charges (Q_1 and Q_2), the effective dielectric constant (D) separating the charges, and the distance (r) between charges will all influence the energy of interaction. In addition, nonclassical, quantum mechanical effects are possible, notably the contribution from a double minimum potential well. Some conclusions are possible from the data and they are discussed below.

Effect of basicity of the oxyanion. The data in Table 1 demonstrate that the basicity of the oxyanion has no large effect on the strength of coordination to guanidinium ion. We explain this as follows: The basicity of guanidine (*8*) is so much greater than any of the oxyanions that there will be little contribution to the strength of hydrogen bonding from simultaneous bonding of the protons to both heteroatoms which can occur through a double minimum potential well. This effect appears partly responsible for the strength of hydrogen bonding in dimers of carboxylic acids. However, in the RCO_2^- $[H_2NC(NH_2)_2]^+$ interaction, guanidine is approximately 10^9 more basic than carboxylates, so we may expect that the major force of interaction will be classical electrostatic (Eq. (10)) and will therefore be similar if the charge densities on the oxygen atoms of various RCO_2^- are similar. That is, the hydrogen-bonding protons will be bonded to nitrogen and will not tunnel to within bonding distance of the carboxylate oxygens. The three K_{1M} values for carboxylates in Table 1 are probably within experimental error of being equal. Since the three carboxylate anions differ considerably in basicity, it appears that basicity is a minor factor in these association constants.

Association constants for oxydianions. For the dibasic acids it is observed, as expected from electrostatic forces, that the association of a monocation is greater to a dianion than a monoanion, $K_{2D} > K_{1M}$. It might also be expected from electrostatic forces that $K_{2D} < K_{1D}$; in Table 2, $K_{2D} < K_{1D}$ for CO_3^{2-} and AMP^{2-}. However, the association of phosphate with guanidinium (Table 2) or *n*-butylammonium (Table 3) cations shows $K_{2D} > K_{1D}$. For the other dianions, K_{1D}, K_{1M}, and K_{2D} are similar in magnitude. We suggest four possible reasons for this surprising comparison and for $K_{2D} > K_{1D}$ for phosphate dianion in which the experimental error appears small enough so that this comparison is reliable.

1. Electrostatics. As discussed above, it seems likely that the primary force of interaction is electrostatic (*9*). Since electrostatic forces diminish with distance, the primary force of interaction should be with the two oxygens at the edge of the oxyanion directed toward the cation (**1**). The electronic structure in the rest of the oxyanion may be insignificant compared to this primary interaction, which may not change much for K_{1M}, K_{1D}, and K_{2D}.

2. Statistical effects. Assuming tetrahedral symmetry of the phosphate ions, there are six equivalent edges to which a guanidinium ion may bind but there is only one site for binding to acetate ion and the other ions in Table 1. In RPO_3^{2-} and RPO_3H^-, and CO_3^{2-} there will be three equivalent edges for complexation to guanidinium ion. If RNH_3^+ interacts with only one oxygen atom, the statistical factor will change to 4 for phosphate ions. In understanding the fundamental chemistry which underlies the relative values of association constants, statistical effects will have to be considered.

3. Sites of binding of two guanidinium ions to dianions. Due to proton exchange, the phosphate dianion may be considered to have tetrahedral symmetry; it will, therefore, be possible for two guanidinium ions to bind to opposite edges of the tetrahedron so that the presence of the first guanidinium ion will not inhibit the binding of the second. On the other hand, in carbonate and AMP dianions, binding of the two guanidinium ions must occur at adjacent edges and share one of the three oxygen atoms.

Although a recently published crystal structure of bis(methylguanidinium) monohydrogen phosphate shows four hydrogen bonds to three oxygens (*10*), rapid proton exchange in solution may render the results of crystal studies inapplicable to solution studies.

4. Solvation effects. Solvation effects can be controlling when there are such small differences in equilibrium constants. This may be particularly important in comparing K_{2D} to K_{1D} and K_{1M}; the binding of one guanidinium ion to an oxydianion may disrupt the solvation envelope so that there is less solvation barrier to binding the second guanidinium ion.

Applications to biological chemistry. These results give some insight into binding of biologically important phosphates and carboxylates to the active sites of enzymes and receptors.

The large ΔpK values indicate that ionizable substrate and coenzyme groups will have their pK's lowered significantly when bound by Arg or Lys side chains at active sites; for example, a phosphate monoester will be bound as a dianion due to the lowering of the pK for $ROPO_3H^-$ (Table 2). At an active site, the extent of lowering of pK_a will be very large because of total complexing.

In contrast to these ammonium cations, the alkali metal ions do not form complexes with monoanions such as acetate (*3*) or phosphate (*2*). With phosphate dianion, K^+, Na^+, and Li^+ have K_{1D}'s of 3.1, 4.0, and 5.2, respectively, and no K_{2D}. Similarly, their K_{1D}'s with AMP are 1.6, 2.9, and 4.1. For these cations, decreasing the radius increases complex formation, as expected for electrostatic interactions.

Our evidence for the formation of a C_2P complex, two cations associated with a dianion, is important with regard to the active site of the enzyme staphyloccocal nuclease (*1c*, *11*). X-ray crystallographic studies indicate that the 5′-phosphate of the inhibitor deoxythymidine-3′,5′-diphosphate is rigidly held in position by two sets of parallel hydrogen bonds from Arg-35 and -87. The four hydrogen bonds are to three oxygens. Thus, one oxygen has two hydrogen bonds. A model for this interaction, crystalline bis(methylguanidinium) monohydrogen phosphate, shows a similar arrangement of four hydrogen bonds to three oxygens (*10*).

In applying these results to biochemical systems, the effective medium of biological binding sites must be kept in mind. Since the important interactions are electrostatic, a decrease in the effective dielectric constant will result in stronger binding (Eq. (10)).

It is significant that there are not great differences in the association constants for complexing phosphate mono- and dianions to guanidinium and *n*-butyl ammonium cations (Table 3). We suggest that this is due to two important concepts: (1) Electrostatic effects are predominant in these hydrogen bonded complexes, as discussed above. (2) Formation of two hydrogen bonds to a guanidinium ion (as in **1**) will cause considerably more loss of entropy than in formation of the *n*-butyl ammonium complex (**2**). Although it is not clear how strong the barriers will be to movement of a guanidi-

nium group within an ion pair of a guanidinium cation and an oxyanion, it seems likely that the barriers are large enough that there will be greater loss of entropy of rotation in the guanidinium complexes than in the butylammonium ones. The single hydrogen bond in the butyl–ammonium complex leaves the cation and anion free to rotate around the ^{+}N–C and ^{-}O–P or ^{-}O–C bonds, respectively. This rotation is not possible, however, with guanidinium complexes where there are two parallel hydrogen bonds. Therefore, we expect that the force of interaction ($-\Delta H$) with guanidinium ions is greater than with $C_4H_9NH_3^+$ because of the formation of two hydrogen bonds, but the entropy effect compensates, yielding similar association constants.

These concepts have important consequences for enzymes. In binding a XO_2^- group at an active site, a guanidinium ion will have the orienting effect which can be expected to be an important part of the biological role of the guanidinium group in arginine because of the formation of two hydrogen bonds (**1**). Such orientation of substrate now appears to be one of the most important parts of enzymic catalysis (*12*). Due to the presence of arginine at an active site, an XO_2^- group could be specifically oriented. In addition, if the arginine side chain were restricted before binding by its nonbonded interactions to the rest of the protein, one would expect no loss of rotational entropy upon formation of a hydrogen-bonded complex to a guanidinium ion at the active site of an enzyme. Therefore, in contrast to aqueous solution, at an active site binding of XO_2^- to arginine could be considerably stronger than binding to lysine.

Kinetic determination of association constants. In other papers (*13*), we will describe the kinetic consequences of complexation of both a carboxylate and a phosphate to guanidinium ion. Each study also reveals an association constant:

$$K_{1M} = 0.55\ M^{-1} \text{ for } {}^{-}O_2CCH_2CH_2CO_2C_6H_5,$$

$$K_{1M} = 0.27\ M^{-1} \text{ for } (O_2N\text{—}C_6H_4\text{—}O)_2PO_2^-.$$

These appear to be in reasonable agreement with the potentiometric values reported in this paper and give us confidence in both methods.

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