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CATION BINDING TO α_{s1}-CASEIN B. A COMPARISON OF ELECTROSTATIC MODELS

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System characteristics which determine calcium binding to and subsequent proton release from α_{s1}-casein B are reported at pH 6.6 and $[Na^+]$ equal to 0.04, 0.08 and 0.16M. Values of protein solvation, G, site bound calcium, $\bar{\nu}_{Ca,S}$, and net monomer charge, $\bar{Z}$, permitted distributed charge models to be constructed. The models examined proved inadequate in that it was impossible to keep the dielectric constant, D, within acceptable limits and/or predict measured proton release.

Three discrete charge models were constructed. At $D = 4$, all three gave good agreement between predicted and experimental data as $\bar{\nu}_{Ca,S}$ increased. The known amino acid sequence was used to make rodlet models for the whole molecule and for just the phosphate-containing acidic peptide portion. A comparison of these shows the electrostatic dominance of the acidic peptide and suggests that the electrostatic environment for the remainder of the binding sites is essentially constant as $\bar{\nu}_{Ca,S}$ increases during addition of calcium ion. The third discrete charge model bends the acidic peptide rodlet into a torus. In this case, data were matched with less assumed bond strain under conditions of high molecular charge than with the other two models. This indicates that conformation and association may be important factors to consider when constructing discrete charge models to calculate electrostatic free energy.

1. Introduction

A careful investigation of cation binding to bovine β-casein [1] and the determination of its amino acid sequence [2] permitted Slattery and Waugh [3] to construct both distributed charge and discrete charge models to account for ion binding. The calculations indicated that distributed charge models are inadequate for β-casein, probably because the net negative charge on the molecule is mainly concentrated in an acidic peptide comprising the first 25 amino acid residues at the N-terminal end [2]. However, a discrete charge model successfully accounted for cation binding and predicted certain other system characteristics such as the requirement for a low dielectric constant, D, in the region of high fixed charge.

Viscosity measurements indicate that α_s-casein [4] may be more compact than β-casein [5]. The complete primary structure for α_{s1}-casein B [6, 7] shows that most of the net negative charge is contained in an internal acidic peptide of approximately 40 residues. Although in the middle of the peptide chain, this acidic peptide participates in ion binding and is no doubt readily accessible to solvent. It would be of interest to determine whether the apparently greater compactness of the molecule and the internal position of the acidic peptide in the primary structure would now permit cation binding data to be accounted for by a distributed charge model or if a discrete charge model is again necessary as with β-casein [3]. In addition, different discrete charge models can be constructed to show some effects due to possible differences in chain conformation. Unfortunately, the calcium binding and proton release data of Waugh et al. [1] may be inapplicable since a mixture of α_s-caseins was used rather than just the α_{s1}-casein fraction. In this paper, calcium binding and proton release are reported for α_{s1}-casein B. Distributed charge and discrete charge electrostatic models are then constructed and compared.

2. Experimental

2.1. Materials and methods

2.1.1. Previously described

Bovine α_{s1}-casein B was prepared as indicated by Slattery and Evard [8]. Except for the method for determining calcium, all other procedures followed those of Waugh et al. [1]. A molecular weight of 23 615 daltons [7] for α_{s1}-casein B was used in all calculations.

2.1.2. Calcium determinations

The residual amount of calcium in the control solutions (no calcium added) was determined by means of a Perkin–Elmer 303 atomic absorption spectrophotometer. Added calcium chloride contained 3.52 μc of radioactive Ca-45 per mmole of calcium. When no precipitate was present after dialysis equilibration, one ml each of both protein solution and diffusate were added to 10 ml of liquid scintillation cocktail. The precipitates were treated as described [1] to determine the solvation and one ml of the protein solutions in 0.9 M urea were added to 10 ml of cocktail for counting. Standard solutions of Ca-45, with and without urea, were prepared for comparison. Radioactivity was measured with a Beckman LS-100-C liquid scintillation counter for 10 minutes or to a preset error of 0.5%.

2.2. Results

2.2.1. Proton release

The measured proton release for α_{s1}-casein B differed from that reported for the α_s-caseins [1]. The results are summarized in table 1. As the protein was taken from the isoionic point to pH 6.6, $[Na^+]$ went from zero to 6×10^{-4} M and the protons lost per α_{s1}-casein B molecule, δH_I^+, equalled 19.0 ± 0.5. As NaCl was added to give $[Na^+] = 0.04$M, a further release of 1.76 protons occurred; one additional proton was released as $[Na^+]$ went from 0.04 M to 0.08 M and 0.86 proton more was released as $[Na^+]$ changed from 0.08 M to 0.16 M. When calcium chloride was added at a particular $[Na^+]$, further protons were released which had to be titrated to maintain the pH at 6.6. The third column of table 1 records ξ, the average proton release per apparent bound calcium.

Table 1
Proton release for α_{s1}-casein B

$[Na^+]$	δH_I^+	ξ
6×10^{-4} M	19.0	
0.04 M	1.76	0.41
0.08 M	1.00	0.41
0.16 M	0.86	0.37

2.2.2. Calcium binding and solvation

The extent of apparent calcium binding, $\bar{\upsilon}_{Ca,A}$ (moles Ca/mole protein), and the precipitate solvation, G (g H_2O/g protein), for α_{s1}-casein B agreed to within experimental error with those determined for the α_s-caseins [1]. The data were combined and calculations were made as described by Waugh et al. [1] to determine reasonable protein solvation in solution, the extent of site bound calcium, $\bar{\upsilon}_{Ca,S}$, and the average charge per molecule. The system characteristics are given in table 2. Column 1 of table 2 gives pCa_s, the negative logarithm of the calcium ion concentration outside the dialysis bag. The remaining columns record I_t, the total ionic strength, G, $\bar{\upsilon}_{Ca,A}$, $\bar{\upsilon}_{Ca,S}$ and $\bar{Z}$, the charge per molecule after binding.

3. Distributed charge models and binding

The distributed charge models chosen for investigation included the model of Scatchard et al. [9] and Scatchard and Yap [10] in which the protein molecule is viewed as a sphere, impenetrable to mobile ions, with $\bar{Z}$ uniformly distributed over its surface, and also the model of Tanford [11] in which only the anhydrous protein and its tightly bound solvent, U (g H_2O/g protein), are impenetrable to mobile ions. The remaining volume, $G - U$, has $\bar{Z}$ distributed uniformly throughout. Both of these models were applied to β-casein by Slattery and Waught [3] who gave the necessary equations, indicated the method of calculation of $\bar{\upsilon}_{Ca,S}$ and discussed the proper choice of intrinsic binding constants between hydrogen (H), calcium (Ca) and sodium (Na) ions and phosphate (P) and carboxylate (C) sites.

If anticipated intrinsic binding constants ($k^0_{H,P} = 3 \times 10^6$, $k^0_{Ca,P} = 120$, $k^0_{Na,P} = 1$, $k^0_{H,C} = 7 \times 10^4$

Table 2
System characteristics of α_{s1}-casein B at three [Na^+]

pCa_S	I_t	G	$\bar{\upsilon}_{Ca,A}$	$\bar{\upsilon}_{Ca,S}$	$\bar{Z}$
[Na^+] = 0.04 M					
4.627	0.0382	10.86	0.22	0.19	−20.4
4.023	0.0390	10.50	0.78	0.65	−20.0
3.857	0.0397	9.98	1.39	1.21	−19.0
3.672	0.0404	9.39	2.08	1.82	−18.0
3.598	0.0412	8.92	2.65	2.36	−17.1
3.158	0.0441	7.53	4.32	3.68	−15.1
2.734	0.0494	5.02	7.18	6.10	−11.0
2.485[a)]	0.0559	3.64	8.90	7.69	− 8.6
2.307	0.0619	2.28	9.97	8.34	− 7.8
2.180	0.0679	1.98	11.01	10.00	− 4.4
1.788	0.0980	1.64	13.41	13.65	1.8
1.442	0.1611	1.54	14.45	15.07	4.2
1.098	0.2785	1.58	15.74	17.27	8.2
[Na^+] = 0.08 M					
4.345	0.0779	7.98	0.19	0.16	−21.5
3.846	0.0786	7.73	0.76	0.68	−20.9
3.601	0.0794	7.54	1.13	0.99	−20.5
3.450	0.0801	7.17	1.76	1.58	−19.4
3.320	0.0809	7.03	2.08	1.84	−19.2
3.039	0.0838	6.16	3.62	3.22	−17.0
2.635	0.0898	5.13	5.49	4.68	−15.0
2.420[a)]	0.0958	4.15	7.24	6.22	−12.5
2.268	0.1017	3.53	8.55	7.43	−10.6
2.151	0.1077	2.45	9.56	8.21	− 9.4
1.771	0.1378	1.72	11.21	9.50	− 7.4
1.436	0.1980	1.57	13.93	14.25	1.9
1.115	0.3183	1.55	16.20	17.53	8.1
[Na^+] = 0.16 M					
4.646	0.1581	8.06	0.19	0.19	−22.6
3.738	0.1589	7.95	0.56	0.52	−22.4
3.570	0.1596	7.73	0.98	0.92	−21.8
3.375	0.1604	7.53	1.38	1.30	−21.2
3.248	0.1611	7.39	1.71	1.60	−20.9
2.909	0.1641	6.76	2.94	2.72	−19.3
2.546	0.1701	5.93	4.55	4.11	−17.3
2.341	0.1761	6.01	4.79	4.11	−17.7
2.195[a)]	0.1821	4.27	7.58	6.93	−12.5
2.060	0.1881	3.37	8.54	7.71	−11.4
1.743	0.2181	2.05	12.31	11.95	− 3.4
1.420	0.2781	1.68	13.82	13.82	0.0
1.113	0.3981	1.48	15.45	16.16	4.2

a) First precipitate.

and $k^0_{Ca,C} = 5.6$) and G values from table 2 are used while D is allowed to vary until $\bar{v}_{Ca,S}$ is calculated, both models require maximum values of D of about 130. This is far above the acceptable maximum of 75. To maintain D at 75 with these k^0 values, G must increase so as to increase spherical volume. This volume increase would need to be 3.5 times for the distributed surface charge model and 1.4 times for the distributed volume charge model. This is in a system where the protein is precipitated and would be expected to have low values for both D and G. For the data of table 3, it is assumed that the maximum D is 75 and that the G values in table 2 apply. In order to reproduce $\bar{v}_{Ca,S}$ at $[Na^+] = 0.04$ M, the low values of $k^0_{H,P} = 2.5 \times 10^5$ and $k^0_{Ca,P} = 10$ were required for the distributed surface charge model and $k^0_{H,P} = 4.5 \times 10^5$ and $k^0_{Ca,P} = 18$ for the distributed volume charge model. The series of D are non-monotonic in both cases, contrary to expectation, and $D < 1$ is the rule for the distributed volume charge model. Over the range of $\bar{v}_{Ca,S}$ considered here, the proton release calculated from $\bar{v}_H$ (bound protons) in column 2 is 1.48 protons and slightly greater than 1.28 protons from column 4. These are unacceptably low when compared with the observed proton release of 4.09 protons. From all calculations, it seems clear that ion binding to α_{s1}-casein B cannot be adequately described by these spherical electrostatic models which assume uniformly distributed charge.

Table 3
A comparison at $[Na^+] = 0.04$ of variables for reproducing calcium binding using distributed surface charge and distributed volume charge models. For surface charge $k^0_{H,P} = 2.5 \times 10^5$ and $k^0_{Ca,P} = 10$. For volume charge $k^0_{H,P} = 4.5 \times 10^5$ and $k^0_{Ca,P} = 18$

Surface		Volume	
D	$\bar{v}_{H,\,calc}$	D	$\bar{v}_{H,\,calc}$
43	2.35	<1	> 2.24
46	2.09	<1	> 2.19
38	2.42	<1	> 2.14
36	2.29	<1	> 2.09
33	2.34	<1	> 2.05
40	1.80	<1	> 1.87
41	1.39	3	1.54
39	1.19	5	1.33
75	0.87	75	0.96

4. Discrete charge models

4.1. Rodlet models

The electrostatic free energy for discrete charge models is calculated according to the theory of Harris and Rice [12, 13] and Rice and Harris [14]. It is necessary to know the exact position of each charged site and be able to calculate the distance between sites. Slattery and Waugh [3] have described the construction of a rodlet having an unstrained distance, $\Delta = 3.62$ Å between amino acid residues and an average side chain length, $\sigma = 7$ Å. The rodlet model would thus have a diameter of 14 Å and the charges would be placed on rings on the rodlet surface which are 3.62 Å apart. Two rodlet models have been used for α_{s1}-casein B. One model uses the complete primary structure (RPR model) as reported by Mercier et al. [6] and corrected by Grosclaude et al. [7]. Since there are 199 amino acid residues in the molecule, the RPR rodlet would be about 717 Å long. There are 32 carboxylates including the C-terminal, 21 amino groups including the N-terminal, 5 histidines and 8 phosphates. These are placed on their rings as indicated by the primary structure in relative positions which minimize total molecular electrostatic free energy. This RPR model should take into account all electrostatic interactions due to nearby charges along the peptide chain. Interactions due to bending or coiling of the peptide chain so that distant charges are brought together are not considered.

The acidic peptide of α_{s1}-casein B probably dominates its electrostatic interactions with regard to ion binding. A test of that dominance can be made by comparing results from the RPR model with one in which only the acidic peptide forms a rodlet (RPE model while assuming that the groups on the remainder of the molecule (the body) bind at their k^0 values. The acidic peptide contains 40 residues numbered from 41 through 80 from the N-terminal residue. This peptide has seven phosphates in positions 6, 8, 24, 26, 27, 28 and 35, eleven carboxylates in positions 3, 7, 10, 11, 15, 16, 21, 23, 29, 30 and 37 and only four positive charges in positions 2,

18, 39 and 40, somewhat isolated from the high negative charge density. The body of the molecule contains one additional phosphate, twenty-one carboxylates and twenty-two groups capable of carrying a positive charge. When unstrained, the rodlet portion of the RPE model is about 145 Å long and 14 Å in diameter. Relative positions of the charges on their rings are again assigned so as to minimize electrostatic free energy.

4.2. Torus model

A third model changes the conformation of the acidic peptide portion by bending the rodlet into a torus (TPE model). Fig. 1 shows a cross-section of the torus on a plane passing through the origin. In spherical coordinates, ρ_x is the radius of the torus axis and σ the radius of the ring. The normal axial length is 145 Å, making $\rho_x = 23$ Å. The angle ϕ_t and distance ρ_t, characteristics of the tangent from the origin to the ring, are fixed by ρ_x and σ. Evidently

$$\rho_t = \rho_x \sin \phi_t = (\rho_x^2 - \sigma^2)^{1/2}. \quad (1)$$

The distance from the origin to a charge at j, ρ_j, is

$$\rho_j = \rho_x \left[\sin \phi_j \pm (\sin^2 \phi_j - \sin^2 \phi_t)^{1/2}\right], \quad (2)$$

where ϕ_j is the angle between the vertical axis and a line joining the origin and the charge at j. The sign in eq. (2) is positive if j is external to the tangent point. If ϕ_t is kept constant, expansion or contraction of the torus can be accomplished by alteration of the single parameter, ρ_x. Then σ will decrease proportionally as ρ_x decreases.

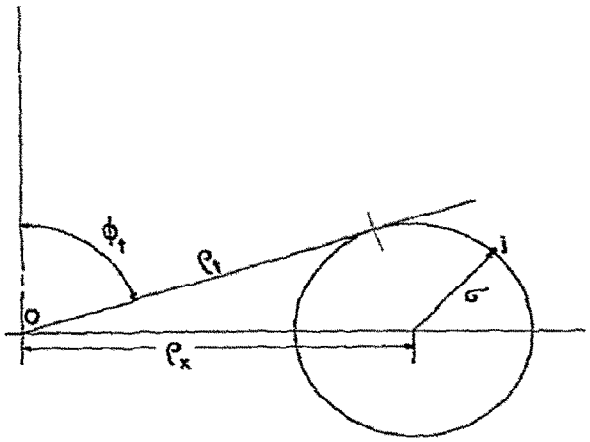

Fig. 1. Cross section through the acidic peptide torus for the TPE model.

There are 40 rings on the torus, each pair making an angle of 9° on the torus axis. One ring is placed at $\theta = 0°$, and the others at $\theta = 9°$, 18°, etc. The distance between any two charges at j and k is then

$$r_{jk} = \{\rho_j^2 - 2\rho_j\rho_k [\sin \phi_j \sin \phi_k \cos (\theta_j - \theta_k) + \cos \phi_j \cos \phi_k] + \rho_k^2\}^{1/2}. \quad (3)$$

Evidently, r_{jk} is also directly proportional to ρ_x if j and k positions remain at ϕ_j and ϕ_k, respectively, as ρ_x is altered. The charged groups are placed on their corresponding rings in succession with torus electrostatic free energy minimized at $\rho_x = 23$ Å and $[Na^+] = 0.04$ M. The first charge (lys) is placed at $\theta_1 = 0°$, $\phi_1 = 90°$, and $\rho_1 = \rho_x + \sigma = 30$ Å. The second (asp) is placed at $\theta_2 = 9°$ and trial values of ϕ_2 and ρ_2 are calculated at intervals of $\Delta\phi = 1°$. Those parameters giving a minimum electrostatic free energy are selected. Successive charges are introduced in sequence ($\theta_3 = 36°$, etc.) with minimization carried out using all preceding charges until a complete torus structure is obtained.

5. Discrete charge binding

For the discrete charge models given here, $\bar{v}_{Ca,S}$ can be calculated by the iterative process described by Slattery and Waugh [3]. As binding increases to reduce the net charge on the molecule, electrostatic repulsion will decrease and conformational changes will occur which will bring the charges closer together. For the distributed charge models, these changes are represented by changes in G (or in spherical volume). Solvation does not explicitly enter into the calculations for discrete charge models. All that is necessary is that the molecular dimensions be accommodated within the physical limitations of the solution volume. All changes in site density, including conformational changes and the effects of molecular association, are accounted for by changes in Δ (or ρ_x) with a proportionate change in σ. Thus the decrease in Δ can be larger than one would expect from a molecular model. However, the maximum value for Δ can reasonably be assumed from molecular dimensions. Accepting $\Delta = 3.85$ Å as a reasonable maximum value [3] and using

Table 4
Calcium binding, proton binding and proton release for α_{s1}-casein B

RPR				RPE				TPE				δH^+ obs.
$\bar{\upsilon}_{Ca,S}$	Δ	$\bar{\upsilon}_H$	δH^+	$\bar{\upsilon}_{Ca,S}$	Δ	$\bar{\upsilon}_H$	δH^+	$\bar{\upsilon}_{Ca,S}$	Δ	$\bar{\upsilon}_H$	δH^+	
$[Na^+]$ = 0.04 M												
0.22	3.85	5.38	–	0.22	3.85	5.41	–	0.21	3.67	5.39	–	–
0.75	3.85	4.97	0.41	0.75	3.85	5.01	0.40	0.73	3.67	5.05	0.34	0.32
1.19	3.69	4.86	0.52	1.18	3.69	4.89	0.52	1.26	3.44	5.04	0.35	0.57
1.86	3.53	4.60	0.78	1.80	3.53	4.63	0.78	1.83	3.37	4.75	0.64	0.85
2.37	3.38	4.44	0.94	2.33	3.38	4.46	0.95	2.35	3.22	4.61	0.78	1.09
3.69	3.38	3.56	1.42	3.65	3.38	3.55	1.86	3.74	3.22	3.66	1.73	1.77
6.14	3.06	2.44	2.94	6.06	3.06	2.53	2.88	6.11	2.99	2.62	2.77	2.94
7.78	2.75	1.92	3.46	7.69	2.75	2.03	3.38	7.62	2.77	2.14	3.25	3.65
8.28	3.30	1.60	3.78	8.34	3.61	1.78	3.63	8.38	3.44	1.78	3.61	4.09
$[Na^+]$ = 0.08 M												
0.22	2.91	5.30	–	0.22	2.91	5.34	–	0.22	2.77	5.31	–	–
0.65	2.91	4.90	0.40	0.65	2.91	4.94	0.40	0.70	2.69	5.10	0.21	0.31
1.02	2.91	4.64	0.66	1.04	2.91	4.68	0.66	0.99	2.77	4.74	0.57	0.46
1.65	2.75	4.50	0.80	1.64	2.75	4.54	0.80	1.62	2.62	4.65	0.66	0.72
1.99	2.75	4.27	1.03	1.97	2.75	4.31	1.03	1.97	2.62	4.42	0.89	0.85
3.34	2.59	3.65	1.65	3.29	2.59	3.69	1.65	3.36	2.47	3.82	1.49	1.48
4.57	2.67	2.85	2.45	4.58	2.67	2.94	2.40	4.70	2.54	2.99	2.32	2.25
6.25	2.43	2.29	3.01	6.23	2.43	2.39	2.95	6.20	2.39	2.48	2.83	2.97
7.37	2.28	1.96	3.34	7.33	2.28	2.07	3.27	7.38	2.24	2.15	3.16	3.51
$[Na^+]$ = 0.16 M												
0.07	2.12	5.21	–	0.07	2.12	5.25	–	0.06	2.02	5.24	–	–
0.58	2.04	5.09	0.12	0.58	2.04	5.10	0.15	0.55	1.94	5.20	0.04	0.21
1.01	1.96	5.00	0.21	1.04	1.96	5.00	0.25	1.00	1.87	5.17	0.07	0.36
1.43	1.96	4.72	0.49	1.41	1.96	4.72	0.53	1.38	1.87	4.91	0.33	0.51
1.60	1.96	4.52	0.69	1.70	1.96	4.52	0.73	1.67	1.87	4.70	0.54	0.63
2.65	1.96	3.90	1.31	2.65	1.96	3.92	1.33	2.64	1.87	4.08	1.16	1.09
4.30	1.88	3.09	2.12	4.20	1.88	3.14	2.11	3.99	1.87	3.33	1.91	1.68
7.09	1.65	2.17	3.04	6.80	1.65	2.29	2.96	6.78	1.65	2.42	2.82	2.80
7.74	1.65	1.86	3.35	7.67	1.65	1.99	3.26	7.69	1.65	2.09	3.15	3.16

anticipated k^0, a value of $D = 4$ for the RPR and RPE models is required. Higher values of D would require lower k^0 values or an unacceptable increase in Δ. A lower limit of $D = 2$ would correspond to charges interacting through organic matter. This D is therefore within the expected limits [12] and is slightly lower than the value of 5 used for β-casein [3]. This is in accord with the higher charge density for α_{s1}-casein B and the fact that the acidic peptide is internal.

Using anticipated k^0 values and $D = 4$, $\bar{\upsilon}_{Ca,S}$ were matched for nine systems at each $[Na^+]$. These include systems where precipitate was present but not where there was apparent charge reversal. For each of the three models, table 4 gives calculated values of $\bar{\upsilon}_{Ca,S}$, Δ and $\bar{\upsilon}_H$. Proton release, δH^+, was

calculated from sequential values of $\bar{\upsilon}_H$ in each case and these can be compared with the observed δH^+ in the last column. The between-model agreement of δH^+ values is good as is the agreement between any single model and observed δH^+. For the 27 systems listed, the overall average difference between calculated and observed δH^+ for the three models is equal to or less than 0.16 proton while the standard deviation of the single difference is equal to or less than 0.13 proton. Any of these discrete charge models would appear to be a reasonable approximation.

A comparison between the two rodlet models reveals them to be nearly identical in terms of the Δ necessary to reproduce $\bar{\upsilon}_{Ca,S}$. The values for $\bar{\upsilon}_H$ are also nearly identical but begin to show some divergence in systems where precipitate is present. Low values for Δ and high site density are characteristic of such systems. The near identity of the RPR and RPE models demonstrates the electrostatic dominance of the acidic peptide in the α_{s1}-casein B molecule and shows that any electrostatic influence acting at the body binding sites must remain essentially constant during calcium binding.

Comparing the RPE and TPE models suggests that conformational changes can be important. The most obvious difference is that the torus conformation allows $\bar{\upsilon}_{Ca,S}$ to be matched at the minimum in I_t and pCa_s without stretching the distance between residues much beyond the normal $\Delta = 3.62$ Å while the minimum Δ is decreased only slightly. The TPE model exhibits slightly poorer matching between calculated and observed δH^+ at $[Na^+] = 0.04$ M than do the other models but slightly better matching on the average at $[Na^+] = 0.08$ M and 0.16 M. It thus appears to be at least as good as the rodlet models with the added advantage of requiring a less strained configuration at the initial binding values.

As in the case of β-casein [3], none of the models examined predicts the δH_I^+ given in table I for changes in ionic strength between $[Na^+] = 0.04$ M and 0.08 M or 0.08 M and 0.16 M. The calculated releases are less than 0.10 proton in each case or about one-tenth of the observed. This suggests that there may be groups on the body of the molecule which are, because of molecular conformation or association, in electrostatic environments sensitive to ionic strength changes but relatively constant over the I_t changes associated with the $\bar{\upsilon}_{Ca,S}$ range examined in these models. In fact, the largest percentage change in I_t occurs at $[Na^+] = 0.04$ M and the largest discrepancy between calculated and observed δH^+ is seen at high $\bar{\upsilon}_{Ca,S}$ where the contribution to I_t by $CaCl_2$ is roughly 60% of that by NaCl. It may be that some of the histidine residues are involved since they bind protons and have pk^0 near the ambient pH of 6.6. An average release of about 0.35 proton per histidine is required.

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