CHEMICAL RELAXATION STUDIES ON BOVINE SERUM ALBUMIN

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1. Introduction

Recent studies utilizing lanthanide(III) substitution for the chemically similar Ca(II) ions have demonstrated that these ions have the same binding sites in biochemical systems [2–6]. Because the spectroscopic and magnetic properties, as well as sensitivity to X-rays, are superior for the lanthanides, this isomorphous replacement may provide the most sensitive determination of calcium binding sites. Only for bovine serum albumin was there evidence that the two metal ions bind at different sites [7, 8], but our earlier investigation [1] and the spectroscopic measurements of Birnbaum et al. [9] indicated that BSA was not an exception to the observation that when both cations bind to a biological molecule, they do so at the same sites. These measurements extend the earlier results to higher pH where the mechanism that was proposed earlier is no longer valid [1]. The chemicals, equipment and techniques are similar to those already described [1].

2. Results and discussion

The relaxation mechanism that describe the data at pH values between 6 and about 7.5 is given by reactions (1) through (4).

$$ImH^+ \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} Im + H^+ \quad (1)$$

$$ImH^+ + OH^- \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} Im + H_2O \quad (2)$$

$$HIn = H^+ + In^- \quad (3)$$

$$H_2O = H^+ + OH^- \quad (4)$$

Reactions (3) and (4) were assumed to be in rapid equilibrium with (1) and (2). * The measurements reported here at pH's up to 8.65 did not fall on the relaxation curve derived for this mechanism, requiring the presence of at least one additional step.

The existence of a conformational change around pH 7 has been reported with a corresponding change in the pK_a of the imidazole groups [10]. However, the addition of steps corresponding to a conformational change (conformation constant varied from 0.001 to 1000) was inconsistent with the relaxation data. This does not rule out this change, it merely states that the difference in pK values for the two conformers is not great enough to be observable within the experimental errors of the relaxation data. Another step which was tried and which did not agree with the data was one involving the self-association of BSA. The mechanism which was found to fit the data over the entire pH range from 5.3 to 8.7 involved the ionization of groups in BSA which have higher pK values [11], thereby introducing two new reaction steps into the mechanism. *

$$Im \underset{k_{43}}{\overset{k_{34}}{\rightleftharpoons}} Im^- + H^+ \quad (5)$$

$$Im + OH^- \underset{k_5}{\overset{k_{45}}{\rightleftharpoons}} Im^- + H_2O \quad (6)$$

* Symbols: ImH^+ represents the protonated imidazole group, Im the deprotonated group, Im^- is the ionized form of the next BSA group to dissociate, and HIn and In^- are the protonated and anionic forms of the indicator.

Table 1
Summary of relaxation data for BSA solutions at 7°C at ionic strength = 0.20 ($NaClO_4$)

10^6 BSA, M	pH	10^8 (H^+), M	10^6 Im, M	10^8 Im^-, M	10^{-6} In^-, M (Metacresol purple)	10^{-3} τ^{-1}, s^{-1}	10^{-10} $\frac{\tau^{-1}}{\theta}$, $\frac{M^{-1}}{s}$	10^{-7} ϕ, M^{-1}
5.51	7.65	2.97	4.23	2.25	1.98	22.4 ± 0.6	11.1	3.37
6.60	8.60	0.334	6.11	28.9	6.97	39.8 ± 2.3	37.8	29.9
11.0	8.26	0.791	10.0	20.0	4.85	24.5 ± 2.4	19.0	12.6
13.7	7.39	5.41	8.87	2.59	1.19	17.4 ± 1.0	3.12	1.85
13.7	7.70	2.64	10.8	6.44	2.17	26.2 ± 1.5	9.60	3.78
13.7	7.99	1.36	11.9	13.9	3.51	27.1 ± 1.8	15.9	7.36
13.7	8.15	0.947	12.3	20.6	4.39	22.4 ± 1.5	15.7	10.6
13.7	8.35	0.593	12.6	33.6	5.58	23.1 ± 2.2	19.1	16.9
16.5	7.20	8.30	9.01	1.71	0.806	19.7 ± 0.6	1.91	1.20
16.5	7.80	2.11	13.5	10.1	2.58	28.5 ± 0.8	11.6	4.74
16.5	7.83	1.96	13.7	11.1	2.72	20.0 ± 0.5	8.60	5.10
16.5	7.83	1.96	13.7	11.1	2.73	24.1 ± 0.6	10.4	5.11
16.5	8.18	0.874	14.9	27.0	4.59	26.9 ± 3.3	18.8	11.5
16.5	8.20	0.846	15.0	27.9	4.68	23.0 ± 2.3	16.3	11.8
16.5	8.25	0.742	15.1	32.1	5.01	16.3 ± 0.3	12.2	13.5
16.5	8.54	0.383	15.3	63.1	6.66	28.2 ± 2.1	26.4	26.1
17.9	8.65	0.298	16.5	87.7	7.22	33.3 ± 0.4	33.7	33.6
22.0	8.06	1.15	19.5	26.9	3.91	17.2 ± 1.2	9.68	8.71
33.0	8.35	0.595	30.4	80.7	5.58	33.4 ± 1.0	25.3	16.8
33.0	8.54	0.381	30.6	127	6.67	40.5 ± 0.4	38.2	26.2
The following data are calculated based on the assumption of no Gd(III) binding. [Gd(III)] = 8.80×10^{-4} M.								
7.16	8.53	0.392	6.64	26.7	6.57	25.2 ± 1.1	23.5	25.5
11.1	7.75	2.36	8.93	5.97	2.35	21.5	9.49	4.23
14.3	8.39	0.548	13.2	38.1	5.75	27.7 ± 1.7	23.4	15.3
17.9	8.43	0.497	16.6	52.7	5.99	23.0 ± 0.8	19.8	16.6
17.9	8.63	0.315	16.6	83.1	7.06	25.5 ± 1.2	25.4	31.8
21.5	8.24	0.759	19.6	40.8	4.92	26.3 ± 0.7	18.7	13.2
35.8	8.33	0.619	32.9	84.2	5.44	26.7 ± 1.4	19.6	16.2

The relaxation equations based upon the mechanism involving reactions (1) and (2) as the slow steps in the sequence are:

$$\tau^{-1}/\theta = k_{21} + k_{32}\,\Phi \tag{7}$$

where

$$\Phi = \{1 + K_a(1-\beta)/[H^+] + K_a(1-2\beta)\,[ImH^+]/\gamma\,[H^+]\}/\theta \tag{8}$$

$$\theta = \{[H^+] - [Im](2\beta-1)/\gamma + K_a(1-\beta)\} \tag{9}$$

$$\beta = \frac{[Im^-] - \gamma K_{Im}}{\gamma[H^+] + 2\,[Im^-]} \quad \text{and} \quad \gamma = 1 + \frac{[OH^-]}{[H^+]} + \frac{[In^-]}{K_{In} + [H^+]} \tag{10}$$

with $K_a = k_{12}/k_{21}$, $K_{Im} = k_{34}/k_{43}$ and K_{In} the indicator dissociation constant. For this mechanism to be valid, a straight line should be obtained with slope equal to k_{32} and intercept equal to k_{21} when $\tau^{-1}/$ is plotted as a function of ϕ. The results in fig. 1 show that the deviations are random and, thus, this mechanism is consistent with the data. This result occurs when Im^- is assumed to be the ϵ-amino groups, of which there are 57 with $pK_{Im} = 9.8$ [11]. If Im^- is assumed to be the single α-amino group ($pK = 7.8$), the high pH data systematically deviate from the predicted equations. The calculated rate constants at

7°C and ionic strength 0.2 in $NaClO_4$ are: k_{21} = (9.8 ± 15) × 10^9 M^{-1} s^{-1} and k_{32} = (1.2 ± 0.3) × 10^3 s^{-1}, results which are within experimental error of those reported when the ionization of the ϵ-amino groups are ignored [1]. The observed relaxation process gives no information about the reaction rates for the ϵ-amino groups because these steps occur outside the experimental time range of this study.

Preliminary measurements have been carried out with BSA in the presence of Gd(III). Below pH 8.4 the data indicate that no complexation is observed, with the points superimposed upon equivalent BSA data in the absence of Gd. This is surprising because in the conformational study the presence of Ca promoted the change with the release of additional protons, and we would have expected similar results with the lanthanide [10]. However, measurements with Ca(II) and BSA in the same pH range were equivalent to those for the apo–BSA solutions. At pH's 8.5 and above, Gd(III) appears to bind to BSA. Since this is the region where Im^- starts to appear in significant concentrations, we conclude that the metal ion is binding to the ϵ-amino groups rather than the imidazole groups. When Ca is added to the BSA solutions in this pH region, the relaxation signal shifts beyond the time range, indicating that the behavior is similar to that of the Gd. Thus it appears from these results that Ca(II) and Gd(III) bind to the same sites in BSA under similar pH's, and our measurements indicate that the binding sites are the ϵ-amino groups. In addition, these results indicate that these metal ions are not involved in any existing BSA conformation change as has been predicted from the pH titration data.

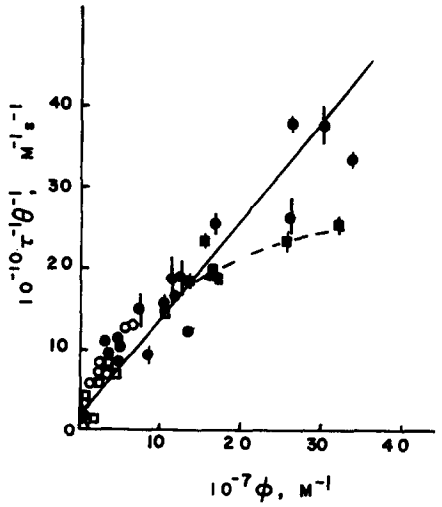

Fig. 1. Relaxation Data for BSA Solutions: (•) = apo–BSA; (▪) = Gd–BSA; (○) = apo–BSA (recalculated from reference [9]); (□) = Gd–BSA (recalculated from reference [9]).

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