Volume 308, number 1, 59–61 FEBS 11363 August 1992

Cooperative proton and calcium binding by sarcoplasmic reticulum ATPase

R. Bruce Martin

Chemistry Department, University of Virginia, Charlottesville, VA 22903, USA

Received 11 May 1992

The classic work on binding of calcium to CaATPase is analyzed by an objective non-linear least squares procedure of 74 data points over six pH values. Binding of two calciums to the basic form of the sites occurs with an equilibrium stability constant product of log K_1K_2 = 13.2. Owing to competition from protons, this value drops in acidic and neutral solutions, becoming, for example, 11.9 at pH 6.8. Binding of the two calciums is so strongly cooperative that its extent is difficult to estimate reliably; there is very little of the one calcium species. Two protons are also bound cooperatively to the calcium sites. In solutions of calcium free protein, at pH < 7.6 the predominant species holds two protons at the calcium sites, while at greater pH the dominant species bears no protons; there is very little of the intermediate one proton species. The analysis also reveals the likely presence of a small, less than statistical, amount of a ternary complex bearing one calcium and one proton.

CaATPase; Calcium; Cooperativity

1. INTRODUCTION

In a now classic paper, the calcium binding properties of sarcoplasmic reticulum ATPase studied at pH 6.8 revealed cooperativity in binding of two calcium ions [1]. Later this study was extended to results at five [2] and then six pH values from 5.5 to 8.5 and a tetramer model proposed to account for them [3]. This paper analyzes the same data by an objective, non-linear least squares technique and concludes that the data may be fitted without recourse to the tetramer model. Furthermore, the analysis suggests that proton, as well as calcium, binding is cooperative.

2. RESULTS

At a single pH the apparent binding equilibrium reactions and apparent (pH dependent) stability constants, K_1 and K_2, for binding of metal ion, M, to protein, P, to yield complexes PM and PM_2 may be expressed as:

$$P + M \rightarrow PM \qquad K_1 = [PM]/([P][M]) \qquad (1)$$

$$PM + M \rightarrow PM_2 \qquad K_2 = [PM_2]/([PM][M]) \qquad (2)$$

The average number of metal ions bound per protein molecule is given by:

$$\nu = \frac{K_1[M] + 2K_1K_2[M]^2}{1 + K_1[M] + K_1K_2[M]^2} \qquad (3)$$

At a given pH the ν vs. pCa data in the form of Eqn. 3 were analyzed by non-linear least squares [4] to give the best fit to the unknowns, K_1, and the product, K_1K_2, which is better known than either individual constant. The results for the apparent log K_1K_2 product (molar basis) at six pH values are as follows:

pH	5.5	6.0	6.4	6.8	7.5	8.5
log K_1K_2	8.9	9.8	10.9	11.9	12.7	13.1

In all cases the one standard deviation uncertainty is less than 0.1. Only for 13 points at pH 6.0 and 15 points at pH 6.8 did low scatter in the data permit refinement to a log K_1 value. The results are as follows: pH 6.0, log K_1 = 4.5 (2) and log K_2 = 5.3 (2); and pH 6.8, log K_1 = 5.1 (2) and log K_2 = 6.8 (2), where the number in parentheses represents one standard deviation in the last digit. At both pH values there is a pronounced cooperativity with K_2/K_1 = 6 and 50, respectively, compared to a statistical value of 1/4. (The alternative protocol with non-linear least squares fitting of Eqn. 3 rearranged to give [M] as a function of ν, K_1, and K_2 yields similar results.)

Fig. 1 shows a plot of the results for 74 points at six pH values and the fitted curves from the above constants for pH 6.0 and 6.8. The area of the points are sized to one sigma from the non-linear least squares analysis. The curves for the other four pH values conform to the form of Eqn. 3 and are placed on the pCa = −log [Ca] axis according to pCa = (1/2)log K_1K_2 at

Correspondence address: R.B. Martin, Chemistry Department, McCormick Road, University of Virginia, Charlottesville, VA 22903, USA. Fax: (1) (804) 924-3710.

$\nu = 1$ with a cooperative steepness of $K_2/K_1 > 30$. For all curves the placement on the pCa axis is determined by the K_1K_2 product and the steepness by the K_2/K_1 ratio. The slope of the ν vs. pCa curves at $\nu = 1$ is given by $m = 2\ln(10)/(2+s) = 4.605/(2+s)$, where $s^2 = K_1/K_2$. The limiting slope at high cooperativity ($s \rightarrow 0$) is $\ln(10) = 2.30$; the slope is already 2.0 at $K_2/K_1 = 10$.

The acceptable fit of the curves to the form of Eqn. 3 over the entire range of $0 < \nu < 2$ values suggests that at all six pH values Eqn. 3 describes the data within the scatter. Primarily on the basis of steep slopes of greater than 2.3 at $\nu = 1$, a tetramer model was proposed for the same data [3]. However, the acceptable computer fit of the curves in Fig. 1 over the entire range of ν suggests that, owing to scatter, the slopes drawn by eye [3] were overestimated, and thus the tetramer model is unnecessary. (Owing to a different basis, slopes in [3] need to be multiplied by $2\ln(10) = 4.6$ to scale up to those in this paper.) Only for pH 8.5 are the points steeper than accounted for by the form of Eqn. 3, but these points exhibit the greatest scatter, and there are only three points with $\nu < 1.3$.

The strong pH dependence of the data points and curves in Fig. 1 encourages construction of models to test quantitatively the form of that dependence. Instead of apparent stability constants that vary with pH, as shown in the above, we now define Eqns. 1 and 2 to apply to metal ion binding to basic calcium sites in the protein. In addition to metal ion binding these basic sites may also bind to protons according to:

$$P + H^+ \rightarrow PH \qquad K_{a1} = [PH]/([P][H]) \qquad (4)$$

$$PH + H^+ \rightarrow PH_2 \qquad K_{a2} = [PH_2]/([PH][H]) \qquad (5)$$

A mixed species with a proton at one site and calcium at the other may also form:

$$P + H^+ + M \rightarrow PHM \qquad K_m = [PHM]/([P][H][M]) \qquad (6)$$

The average number of metal ions per protein molecule is now given by:

$$\nu = \frac{(K_1 + K_m[H])[M] + 2K_1K_2[M]^2}{1+[H]K_{a1}+[H]^2K_{a1}K_{a2} + (K_1 + K_m[H])[M]+K_1K_2[M]^2} \qquad (7)$$

The last equation was used in a non-linear least squares analysis that simultaneously considered both pH and pCa as independent variables to fit ν for 74 points at six pH values. The excellent results are $\log K_1K_2 = 13.2(1)$, $\log K_{a1}K_{a2} = 15.2(1)$, and $\log K_m = 13.5(4)$, with one sigma = 0.10 in ν. Reliable constants could not be obtained for either K_1 or K_{a1}, indicating that the system is cooperative for *both* calcium ions and protons.

Additional equilibria provide only marginal or no

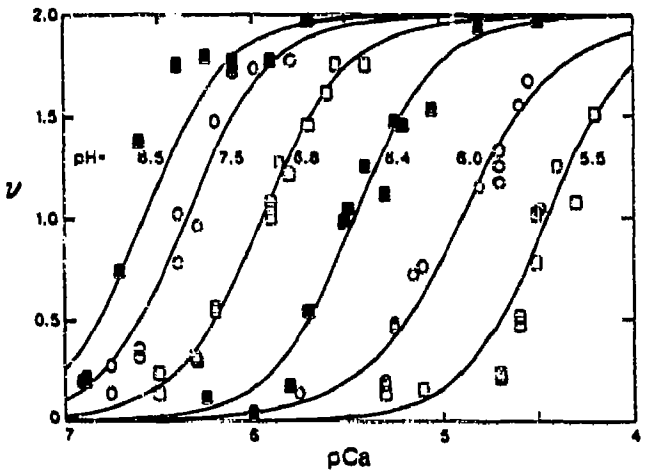

Fig. 1. The average number of calcium ions bound per molecule of ATPase at six pH values. Curves are six non-linear least squares fits to the points at each pH according to Eqn. 3.

improvements in the simultaneous fit of all 74 points. Addition of a third proton according to $PH_2 + H^+ \rightarrow PH_3$ with $K_{a3} = [PH_3]/([PH_2][H])$ refines to $\log K_{a3} = 5.7(1)$ with a marginal improvement in fit. Results in the most acidic solutions, pH 5.5 and 6.0, have the greatest influence on the value of this constant. In neutral solutions this third site is deprotonated. Thus, looked at from the acidic side, the triprotonated species deprotonates with successive pK_a values of 5.7, >8.2, and <7.0, the last two values summing to 15.2 and the second being greater than the third, consistent with strong but quantitatively unspecifiable cooperativity. Addition of a PH_2M species offers no improvement in fit.

3. DISCUSSION

This computer analysis confirms cooperative calcium binding to sarcoplasmic reticulum ATPase. Furthermore, the objective non-linear least squares approach shows that cooperativity is very strong with $K_2/K_1 \geq 30$ for all curves except that at pH 6.0, for which a lower value was obtained. However, precise determination of such strong cooperativity is difficult. Once $K_2/K_1 > 16$, the precise value makes little difference in handling equations and plotting the results. For a given product, K_1K_2, all curves with $K_2/K_1 > 30$ fall within one line-width of the curves shown in Fig. 1. Thus the conclusion of the original analysis [1] for pH 6.8, that $K_2/K_1 = 400$, also falls on the pH 6.8 curve of Fig. 1.

The non-linear least squares analysis also shows that the binding of two protons at the calcium sites is so strongly cooperative that its extent cannot be specified. The sum of these two pK_a values is 15.2, and the first of the two is at least 1.2 log units greater than the second. Thus for calcium-free species, the two proton form predominates at pH < 7.6, and the no proton form predom-

inates above this pH. There is only a minute amount of the intermediate one proton form.

The fitting of all 74 points over six pH values indicates that for binding of two calciums to the basic sites of CaATPase the equilibrium product constant, $\log K_1K_2 = 13.2$. Because of competition from proton binding in acidic and neutral solutions, the last value drops to the values given in the text for several pH values.

The analysis reveals the likely formation of a mixed PHM complex with one calcium ion and one proton. At a given pH the maximum mole percentage (protein basis) of this complex rises to about only 10% at the pCa where $\nu = 1$ and the mole percentages of the species bearing two calcium and no calcium are equal. The stability constant for the mixed PHM complex is relatively uncertain, because of its low occurrence. The overall stability constant, $\log K_m = 13.5$ for the ternary PHM complex is substantially less than the value of 14.5 (a factor of 10) expected statistically based upon the stabilities of the binary PH_2 and PM_2 complexes. Thus, to a good approximation, only species bearing two or no calcium need be considered. The PHM species may serve as an intermediate in passing reversibly from PH_2 to PM_2.

We conclude that over the 5.5–8 pH range, the CaATPase system is adequately described by Eqns. 1, 2, 4 and 5, with a small contribution from Eqn. 6. The associated equilibrium constants are macroconstants, and as such do not indicate the details of reactions occurring at a single site. Presumably, the cooperativity for both calcium and protons is explained by a pronounced rearrangement after the addition of the first cation that provides an even more compelling environment for the second cation.

Acknowledgements: This analysis would not have been possible without the willingness of Giuseppe Inesi to furnish the data, for which I am grateful.

REFERENCES

[1] Inesi, G., Kurzmack, M., Coan, C. and Lewis, D.E. (1980) J. Biol. Chem. 255, 3025–3031.
[2] Watanabe, T., Lewis, D., Nakamoto, R., Kurzmack, M., Fronticelli, C. and Inesi, G. (1981) Biochemistry 20, 6617-6625.
[3] Hill, T.L. and Inesi, G. (1982) Proc. Natl. Acad. Sci. USA 79, 3978–3982.
[4] Bevington, P.R. (1969) Data Reduction and Error Analysis for the Physical Sciences, McGraw-Hill, New York.