Asian Journal of Chemistry Vol. 22, No. 2 (2010), 1029-1036

Interaction of Molybdenum With Trypsin and Pepsin by Dialysis Equilibrium Method

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The binding of molybdenum with trypsin and pepsin enzymes has been studied using dialysis equilibrium technique. The effect of pH and temperature on the anion binding behaviour has been explained in the light of protonation and deprotonation behaviour of the two enzymes. The differences in binding behaviour has been attributed to large differences in their cationic groups contents. The binding constants, *viz*. intrinsic association constants (K) and binding sites (n) were determined from Scatchard's plots. The linear nature of plots under similar conditions of pH and temperature in two cases is an indication of the involvement of a single one class of sites. The pH dependence of anion binding exhibited the involvement of cationic groups in the interaction. The nearly similar values of free energy and entropy changes for trypsin and pepsin is an index of their similar anion binding capacity. The enthalpy and entropy changes at pH 5.50 provided evidence for covalent linking between molybdenum and enzyme groups.

Key Words: Dialysis equilibrium, Trypsin, Pepsin.

INTRODUCTION

Owing to several biological and biochemical application of molybdenum ions, its interaction with different proteins was made by Malik and Arora[1,2], Arora and coworkers[3-5] and Singh *et al*.[6-9] employing polarographic, equilibrium dialysis *etc*. methods. In the present paper low molecular weight proteins *viz*. pepsin and trypsin, have been selected for molybdenum binding interactions. These are enzyme proteins and are well characterized with respect to their molecular weights *viz*. trypsin, 23,800 and pepsin, 34,500. The former occurs in pancreas, while the later in the stomach of the digestive tract. The two enzyme proteins widely differ in their basic amino acid residues, pepsin contains only four while trypsin contains approximately 22 basic amino acid residues. These two enzyme proteins also differ in their isoionic points *i.e*., trypsin has an isoionic point of pH 7.0 while pepsin below pH 3.0. Due to these difference, these were selected for oxomolybdenum binding studies. This paper describes the effect of temperature and pH on the binding interaction of molybdate ions to pepsin and trypsin.

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EXPERIMENTAL

Trypsin and pepsin were purchased from Sigma Chemical Products and their stock solutions were prepared in doubly distilled water. The concentration of protein solutions were determined by colorimetric biuret method using bovine serum albumin (BSA) as standard protein.

Sodium molybdate (E. Merck) was dissolved in double-distilled water and estimated gravimetrically by oxine method. Sodium acetate and acetic acid (BDH) were used for the preparation of acetate buffers while phosphate and carbonate buffers were prepared from reagent grade chemicals. Potassium chloride solution was used for the adjustment of ionic strengths.

Equilibrium dialysis measurements: Cellophane bags prepared from commercial sausage casing (23/32 inch in diameter) were filled with a 5.0 mL protein solution and sodium molybdate solutions of different known concentrations were taken in corning boiling tubes. All the bags were placed on a constant water thermostate adjusted at the desired temperature for a week in order to attain the equilibrium. The bags were removed and the external solutions were analyzed colorimetrically. Blanks were also run to determine the amount of molybdenum bound to the material of the dialysis bag. There were found to be negligible. For these measurements several sets were arranged for trypsin and pepsin, respectively, *i.e.*, 2.08×10^{-4} molar proteins inside the bag and varying concentration of sodium molybdate 'outside' the bag (total volume 5.0 mL 'outside' and 'inside'). Results are reported at 10, 20, 30 and 40 ºC at pH 5.50, while at all other pH values at 30 ºC only. The ionic strength was maintained at 0.15 M by the addition of requisite amount of 1.0 molar KCl solution in each of these experiments 'outside' and 'inside' the bags. The number of moles of molybdate anions bound to trypsin and pepsin was obtained by the following expression.

$$V_M = \frac{C_T - C_F}{[\text{Trypsin/pepsin}]} = \frac{C_B}{[\text{Trypsin/pepsin}]}$$

where C_T, C_F and C_B are the total, free and bound moles of molybdate, while [Trypsin/pepsin] is the total molar concentration of trypsin or pepsin. The values of V_M and C_F are used to calculate the binding parameters of these studies.

RESULTS AND DISCUSSION

Equilibrium between a small ligand and a macromolecule separated by a cellophane membrane is a suitable method for the preparation of accurate isotherm. This method is not affected from the presence of other competitive ligands, since it involves a direct determination of a small ligand by appropriate method. If the dialysis membrane does not interact with any of the ligand, and if the Donnan equilibrium is negligable, the value of mole ligand bound per mole of protein (V_M) can be calculated directly. The results obtained with two enzyme proteins at different pH values and temperatures are recorded in the form of isotherms (Figs. 1-4). It is

evident from the respective indicated isotherm that the extent of binding increased with increasing concentration of the molybdate ions, but this increase is found more in case of trypsin than that of pepsin. In both cases the extent of binding is found to decrease with increasing temperature and pH.

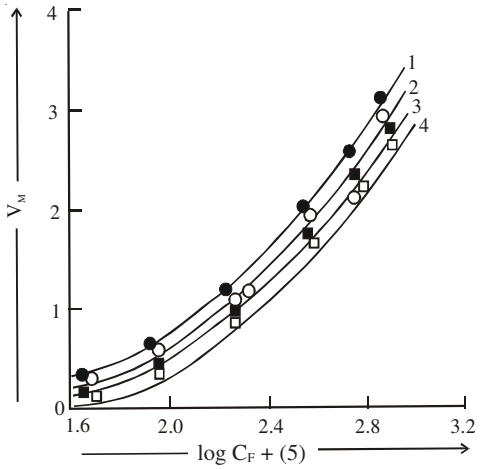

Fig. 1. V_M plotted against log free equilibrium Mo(VI) concentration V_M, at pH 0.15, m = 0.05 effect of temperature curves 1, 2, 3 & 4 corresponds to 10, 20, 30, 40 ºC, respectively

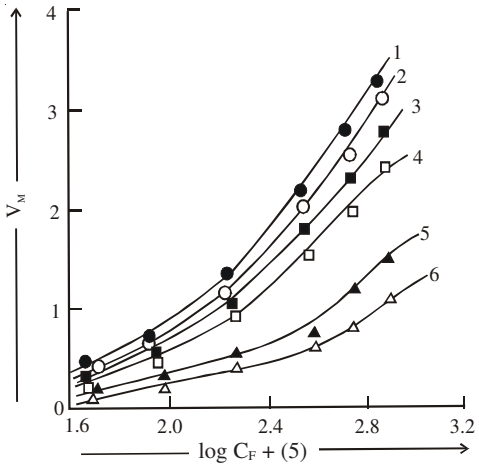

Fig. 2. V_M plotted against log free equilibrium Mo(VI) concentration effect of pH curves 1, 2, 3, 4, 5 and 6 corresponds to pH 3.00, 3.50, 5.50, 6.50, 7.50, respectively and 11.50 at 30 ºC

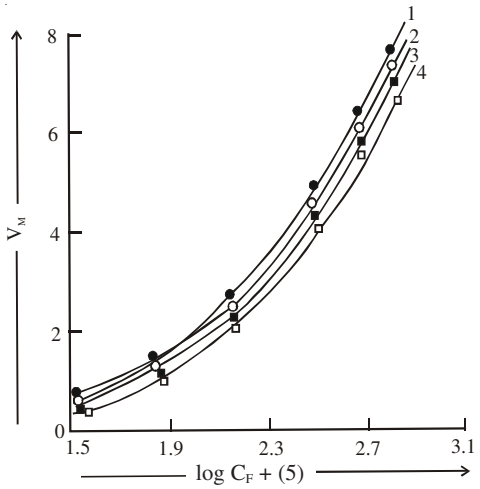

Fig. 3. Effect of temperature on logarithmic plots of Mo(VI)-Trypsin system. Curves 1, 2, 3 & 4 corresponds to 10º, 20º, 30º and 40 ºC, respectively

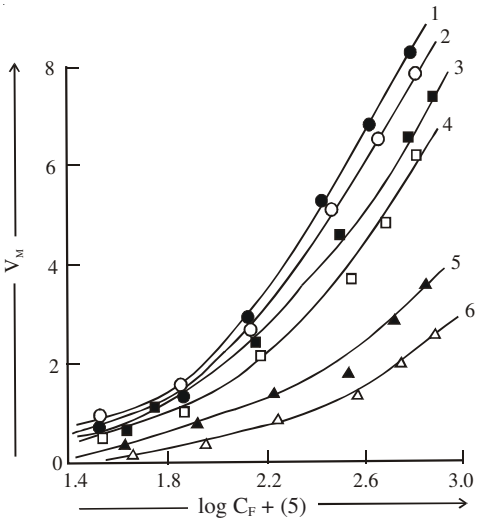

Fig. 4. Effect of pH on Mo(VI)-Trypsin binding curves 1, 2, 3, 4, 5 and 6 corresponds to pH values 3.00, 3.50 5.50, 6.50, 7.50 & 11.5, respectively at 30º C and μ = 0.15 M

The binding results were plotted in the form of logarithmic plots *i.e.*, V_M plotted against log of free equilibrium molybdenum concentration (log C_F). The general shape of the logarithmic plots are similar to those reported by Karush *et. al.*[10] in the binding of dye anions and by Scatchard *et al*.[11] in the binding of iodide ions with bovine serum albumin (BSA). If total number of binding sites (n) is known, the value of log C_F at which $V_M = n/2$ easily gives the value of log K. Since in the present cases no saturation limit were attained, the value of log K and the actual number of binding sites cannot be calculated from the logarithmic plots.

In order to make a quantitative interpretation of linking, it would be the expedient to determine the linking constants for the addition of only one molybdate anion to the one pepsin/trypsin molecule.

$$MoO_4^{2-} + Pr.NH_3^+ \rightleftharpoons PrNH_3^+ . MoO_4^{2-}$$

The equilibrium constant may then be expressed by

$$K_1 = \frac{[Pr\,NH_3^+ .MoO_4^{2-}]}{[MoO_4^{2-}][Pr.NH_3^+]}$$

The value of K_1 can be obtained by extra plotting a plot of V_M/C_F *vs.* C_F to C_F equal to zero. At this limiting value of C_F. We have

$$V_M/C_F = nK_{CF} = K_1$$
$$\text{At limit } C_F = 0$$

where, n is the number of equivalent sites on enzyme molecule and K_1 is the intrinsic association constant for the linking. For the convience the values of linking parameters were analyzed by the following equation of Scatchard[12-14].

$$V_M/C_F = nK - V_M.K$$

This equation is based on the assumption that there are 'n' binding sites with equal intrinsic association constants 'K' and that there is no interaction between the bound ions. Under these conditions plots of V_M/C_F *vs.* V_M will be linear and the values for n, K and $nK = K_1$, the intrinsic association constant for the first site, can be obtained from the slope and intercepts.

The results of the dialysis experiments were plotted as V_M/C_F *vs.* C_F. A linear relationship was found to exist between V_M/C_F *vs.* V_M and the extrapolation to V_M/C_F axis (nK) and the V_M axis (n). The values for the linking constants are given in Tables 1-4. The fact that the relationship between V_M/C_F and V_M was linear at all pH values and temperatures over the range of ligand concentration studied indicates the involvement of single one type of non-interacting site. However, if a second type of site exists it must have a much smaller intrinsic association constant than that observed for the primary binding sites (Tables 1-2). It is considered likely that free cationic groups on the respective enzyme protein molecule may be the primary molybdate anion binding sites.

TABLE-1
BINDING CONSTANTS OF Mo(VI)-TRYPSIN SYSTEM AT DIFFERENT TEMPERATURES (pH 5.50)

Temp. (ºC)	n	K	log K	ΔG^0 Cal/mol	ΔS^0 Cal/degree/mol
10	13	0.235×10^4	2.2577	–2943.0	7.7
20	12	0.225×10^4	2.2648	–3056.4	7.9
30	11	0.210×10^4	2.2810	–3183.4	8.0
40	9.5	0.185×10^4	2.3118	–3332.8	8.2

TABLE-2
BINDING CONSTANTS OF Mo(VI)-TRYPSIN SYSTEM AT DIFFERENT pH (TEMP. 30 ºC)

pH	n	K	log K	ΔG^0 Cal/mol
3.000	15.0	0.26×10^4	2.2405	–3127.04
3.500	13.0	0.22×10^4	2.2095	–3085.16
5.500	11.0	0.21×10^4	2.2810	–3183.40
6.500	10.5	0.17×10^4	2.2304	–3113.08
7.500	6.00	0.10×10^4	2.2201	–3099.12
11.50	4.00	0.05×10^4	2.0969	–2931.61

TABLE-3
BINDING CONSTANTS OF Mo(VI) – PEPSIN SYSTEM AT DIFFERENT pH (TEMP. 30 ^{0}C)

pH	n	K	log K	ΔG^0 Cal/mol
3.0	6.10	0.080×10^4	2.1239	–2959.52
3.5	5.25	0.070×10^4	2.1461	–3001.40
5.5	4.50	0.067×10^4	2.1703	–3029.32
6.5	3.75	0.062×10^4	2.1903	–3057.24
7.5	2.00	0.036×10^4	2.2553	–3054.90
11.5	1.50	0.020×10^4	2.1239	–2959.52

TABLE-4
BINDING CONSTANTS OF Mo(VI)-PEPSIN SYSTEM AT DIFFERENT TEMPERATURES (pH 5.50).

Temp. (ºC)	n	K	log K	ΔG^0 Cal/mol	ΔS^0 Cal/degree/mol
10	5.5	0.078×10^4	2.1523	–2802.52	+8.0
20	5.0	0.027×10^4	2.1584	–2909.84	+8.1
30	4.5	0.067×10^4	2.1703	–3029.32	+8.2
40	4.0	0.062×10^4	2.1903	–3157.27	+8.4

The molybdenum binding by pepsin and trypsin is observed to be modified by pH and temperatures. The binding regularly decreases from pH 3.0 to 11.5 at 30 ºC, while at pH 5.50, it decreases with a rise in temperature, *viz*. from 10 to 40 ºC. The value of binding sites decreases with rising pH and temperature in both cases, but

the values of log K are nearly constant at all pH values. With rising temperature the log K values slightly changes and may be considered nearly constant. The decreasing number of binding sites with rising pH is in accordance to Scatchard *et al.*[12-14] who believed that binding of anions by the enzyme increased with the binding of enzymes.

Thermodynamic parameters: These parameters of combining site are determined from the following equations:

$$\Delta G^0 = -2.303\ RT \log K$$

$$\Delta H^0 = -\frac{2.303RT_1T_2(\log K_1 - \log K_2)}{(T_2 - T_1)}$$

$$\Delta S^0 = \frac{\Delta H^0 - \Delta G^0}{T}$$

where, ΔG^0, ΔH^0 and ΔS^0 are the standard free energy change, enthalpy change and entropy change, respectively and K, T and R are the intrinsic association constant, absolute temperature and gas constant, respectively.

The ΔH^0 at pH 5.50 for trypsin and pepsin are found to be 736 and 516 cal/mol, respectively. Klotz and Urquhart[15] found values of ΔH^0 to be 2100 and 2000 cal/mol for methyl orange and azosulphathiazole, while Arora *et al.*[16,17] reported ΔH^0 values of 368.4, 328.8 and 1372.8 cal/mole for HSA, BSA and BPT, respectively. The smaller values of enthalpy changes are in agreement with the heat of binding of chloride ions to proteins[18]. The nearly similar values of free energy and entropy changes for trypsin and pespsin are an indication of their similar anion linking capacity. It is seen that small entropy change associated with binding[19] may be due to the balancing of an over all entropy loss on binding with an entropy gain due to breakage of some hydrogen bonds between positive and negative polar side chain groups.

Effect of pH: The linking constants are found to be pH dependent. These indicate that molybdenum binding decreases with rising pH and greately reduced at pH 11.50, since at this pH all the imidazolium and amino groups undergo deprotonation leaving only the guanidinium groups protonated which can bind the molybate ions. The extensive increase of negative charge on respective enzyme protein molecule may produce repulsion between the anionic macromolecule and the molybdate anions. The progressive decrease of extent of linking with rising pH may also be explained due to depolymerization of complex molybdenum species to simple dinuclear molybdate species.

The large difference in the number of binding sites for trypsin and pepsin is due to the presence of different number of cationic groups in them. For example trypsin possesses 22 anion binding sites, while pepsin only 4 such sites. It appears that all these 4 sites of pepsin becomes available for linking to the molybdate ions, while only half of the total sites of trypsin interacted with molybdate ions. The

involvement of lesser sites in interaction from trypsin may also be assumed by the fact that each two groups may be present in close vicinity which may react with a single divalent molybdate ion. On this ground both trypsin and pepsin may be suggested to exhibit 1:1 stoichiometry of combination with molybdate ion.

The uniformity of logarithm of intrinsic association constants (log K) in both cases supports that all the binding sites are equivalent and independent in the pH range investigated. The significance of the uniformity of log K at all pH values is that a single type of site is reacting with molybdate ion. The appearance of different sites is therefore, not responsible for the increased binding which then be due to the increased availability of the same class of sites. The increasing value of 'n' as the pH decreases is an indication of this. The all over linking results are in agreement that the available sites are pH dependent, yet they have the same intrinsic association constants. The enthalpy and entropy changes at pH 5.50 for interaction could furnish a general nature of oxometal ion-protein linkage. According to some workers[20] in case of divalent metal-amino acid complexes, the negative enthalpy changes established covalent linking between metal atom and the amino groups of amino acids. In molybdenum-protein interaction the magnitude of enthalpy change indicates a covalent linking between molybdenum atom and the nitrogen containing side chain groups on the protein molecule. On the other hand the entropy values indicated the replacement of water molecules from the solvated charged sites and the interacting oxomolybdenum ion. In all, the sign of enthalpy and entropy provided evidence for a reaction similar to that suggested by Craig *et al.*[21] in chloroaurate-ovalbumin reaction.

Koltz[22] suggested that the positive entropy changes observed for the association of various anions with serum albumin may arise from the release of water molecules as a result of neutralization of charged hydrated sites on both the enzymes and the interacting anions. Although, the results presented for the binding of two enzymes at all pH-values follow the order of reactivity: trypsin > pepsin, yet both at all pH-values revealed identical sites. The binding behaviour of this divalent molybdenum ion is analogous to the well known binding behaviour of vanadate[23-25], chromate in[26], dye anion[27,28], phenolate ion[29] and detergent ions[30-32] by proteins. In all these anion protein interactions, the charged nitrogen locii of amino acids are assumed to be the points of attachment on the protein molecule. This view would find support that unlike other metal cations the binding of molybdate ion decreases with increase in pH. It also seems that owing to the deprotonation of carboxylic groups and cationic sites, the two protein arised complicated nature between cationic site-anion combination.

ACKNOWLEDGEMENT

One of the authors (Ritu Rani) is thankful to UGC, New Delhi for providing financial assistance in form of JRF.

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(*Received*: 28 November 2008; *Accepted*: 12 October 2009) AJC-7942