Ion Binding by α-Chymotrypsin*

F. Friedberg and S. Bose

ABSTRACT: The binding of Na^+, Ca^{2+}, Cl^-, and SO_4^{2-} by deionized α-chymotrypsin was investigated as a function of pH at 25° with the use of permselective membrane electrodes. The enzyme has an affinity for all these ions even close to the isoionic point but it holds more cations than anions. Graphs of $\bar{\nu}/c$ with respect to $\bar{\nu}$ give linear relationships for these four ions and apparent maximum number of binding sites as well as apparent association constants are deduced from such plots.

Information relevant to the effect of the organization of tertiary structure of proteins on the binding affinities toward inorganic ions should emerge from studies using well-characterized proteins. Previously, our laboratory reported on the combination of ATP creatine transferase (Floyd and Friedberg, 1966) and myoglobin (Friedberg and Emiola, 1968) with such ions. Here, we present data on the interaction of α-chymotrypsin with chloride, sulfate, sodium, and calcium.

Experimental Procedure

For these studies, α-chymotrypsin, three-times crystallized (Worthington), was utilized. Evidence has been presented for the existence of at least two active components in this preparation (Erlanger *et al.*, 1964). Enzyme concentrations were determined from absorbancy measurements at 280 mμ using a value of E_{mol}, of 5×10^4 (Dixon and Neurath, 1957). The molecular weight of the protein was taken as 25,000 daltons (Wilcox *et al.*, 1957). In our laboratory, the lyophilized preparation was deionized as follows. Protein (500 mg) dissolved in 25 ml of cold, twice-distilled water was dialyzed against the same type of water in the cold room for 6 hr, then, transferred onto a 40-cm column packed with well-washed ion-retardation resin AG 11A8 (Bio-Rad) and eluted at a flow rate of 0.3 ml/min with twice-distilled water.

A calculated amount of the solution of the salt under investigation was added to an aliquot of protein solution to give a concentration within the ionic strength desired. All solutions were prepared by weight. For Na^+ binding measurements, pH was adjusted by NaOH, for those of Ca^{2+} by $Ca(OH)_2$ for those of Cl^- by HCl, and for those of SO_4^{2-} by H_2SO_4 (also by weight). Binding measurements were made with the aid of permselective membranes according to the method originally described by Saroff and Healy (1959) under conditions given in a previous paper (Floyd and Friedberg, 1966). Experiments were performed at 25° (±1.5°) and pH was measured on a Beckman Model GS pH meter. Sodium and calcium chloride served as sources of Na^+, Ca^{2+}, and Cl^- ions, ammonium sulfate as that of SO_4^{2-} ions.

Results and Discussion

Table I and Figure 1 summarize the data for the pH dependence of the binding of Cl^- to α-chymotrypsin. Close to the isoionic point in the presence of 0.033 M salt (pH 6.61) about 1.4 chloride ions are attached to the enzyme. As the pH decreases there is a gradual increase in the number of ions bound which changes into an abrupt increase below pH 4. Thus, at pH 5.5 in the presence of approximately 0.032 M NaCl, 1.5 ions/chymotrypsin molecule are held and at pH 3, in the presence of approximately 0.036 M NaCl, 4 ions/protein molecule are ligated. Chymotrypsinogens A and B undergo a structural transition below approximately pH 4 (Delaage *et al.*, 1968) and this is probably also the case for α-chymotrypsin. Thus, one may assume that many more ions are bound when the protein is in a less compact conformation. An alternative explanation would be that the increase in the number of negative ions held is merely due to an increased positive charge on the protein rather than to the conformational change.

The application of the law of mass action gives the following relationship (Scatchard, 1949) $\bar{\nu}/c = Kn - K\bar{\nu}$, where $\bar{\nu}$ is the moles of ion bound per mole of protein, c is the concentration of free ion, n is the number of binding sites, and K is the apparent association constant. This equation neglects correction for possible electrostatic effects which might be induced by the attachment of the ion to the protein. The value of the apparent maximum number of binding sites (n) (Figure 2, intercept on $\bar{\nu}$ axis) is 7 for the Cl^- ions, and the apparent association constant is 7. (Figure 2, intercept on $\bar{\nu}/c$ axis divided by n.) From the plot of $\bar{\nu}/c$ with respect to $\bar{\nu}$ (Figure 2) one might assume that the binding sites are equivalent and independent.

Table II and Figure 1 show the experimental data for a study of the pH dependence of SO_4^{2-} ion binding by α-chymotrypsin. The shape of the curve $\bar{\nu}$ *vs.* pH for SO_4^{2-} ions is similar to that for Cl^- ions. Again, we observe binding capacity for this ion close to the isoionic point. About 0.5 sulfate ion/protein molecule are held in the presence of 0.01 M salt.

The value of the apparent maximum number of binding sites for SO_4^{2-} is 5.4 (Figure 3), that is, slightly lower than that for the Cl^- ions and the apparent association constant is 16, that is, more than twice that for Cl^- ions. For this ion, a plot of $\bar{\nu}/c$ *vs.* $\bar{\nu}$ can also be considered to give a straight-line relationship, *i.e.*, here too one might assume that the binding sites are equivalent and independent.

Table III and Figure 4 present data for pH dependence of the binding of Na^+ to α-chymotrypsin. There are more cations

* From Howard University, Departments of Biochemistry and Chemistry, Washington, D. C. 20001. *Received November 25, 1968.* Supported in part by National Science Foundation Grant GB 6056.

TABLE I: Chloride Ion Binding to α-Chymotrypsin.

pH	α-Chymotrypsin Concn (M × 10⁴)	Anion Concn (M × 10³) Total	Free	$\bar{\nu}$ Anion
6.77	2.37	72.27	71.71	2.36
6.72	2.29	61.99	61.50	2.14
6.68	3.56	53.62	52.94	1.91
6.64	2.10	44.64	44.29	1.69
6.61	1.88	33.00	32.74	1.37
5.49	3.05	32.46	32.00	1.52
4.52	4.11	34.60	33.72	2.14
4.11	2.78	32.06	31.46	2.17
3.63	2.99	32.92	32.04	2.96
3.35	2.54	30.89	29.93	3.77
2.95	2.73	35.84	34.71	4.13

TABLE III: Sodium Ion Binding to α-Chymotrypsin.

pH	α-Chymotrypsin Concn (M × 10⁴)	Cation Concn (M × 10³) Total	Free	$\bar{\nu}$ Cation
6.69	1.63	32.45	31.81	3.93
6.71	1.94	42.90	42.00	4.66
6.73	2.75	62.94	61.42	5.52
6.77	2.18	52.58	51.45	5.20
6.80	2.42	74.82	73.30	6.28
6.81	2.38	75.83	74.22	6.75
7.32	2.42	32.60	31.36	5.12
7.72	2.33	36.10	34.75	5.79
8.00	2.16	35.30	33.99	6.07
8.33	2.34	37.42	35.93	6.37
9.82	2.46	31.26	29.56	6.91

than anions ligated to the protein close to the isoionic point: 3.9 Na^+ ions/mole of protein are bound in the presence of 0.032 M sodium chloride. As the pH increases, the number of sodium ions held increases almost linearly up to pH 8; thereafter, the binding capacity begins to level off. The apparent maximum number of binding sites for Na^+ may be deduced from Figure 5 as approximately 11 and the apparent association constant as 17.3. The relationship of $\bar{\nu}/c$ with respect to $\bar{\nu}$ is linear.

TABLE II: Sulfate Ion Binding to α-Chymotrypsin.

pH	α-Chymotrypsin Concn (M × 10⁴)	Anion Concn (M × 10³) Total	Free	$\bar{\nu}$ Anion
6.83	4.30	50.73	49.65	2.50
6.80	4.21	41.20	40.25	2.26
6.78	3.04	41.20	40.48	2.36
6.76	4.05	33.43	32.66	1.91
6.74	1.44	23.67	23.47	1.38
6.71	4.22	23.66	23.03	1.50
6.68	8.61	14.88	13.95	1.08
6.67	4.25	13.44	13.04	0.95
6.65	4.54	7.06	6.81	0.56
6.65	3.12	8.07	7.90	0.54
5.51	2.20	14.87	14.64	1.08
4.57	1.98	12.77	12.49	1.39
3.71	2.24	11.89	11.33	2.50
3.62	2.33	12.29	11.75	2.32
3.34	2.24	12.82	12.22	2.69
3.21	2.51	12.51	11.65	3.42
2.90	2.51	12.76	11.63	4.50
2.88	2.09	12.22	11.22	4.76
2.85	2.35	11.76	10.87	3.77
2.64	2.00	12.72	11.61	5.53
2.62	2.20	13.65	12.60	4.77

Because of the stabilizing effect of Ca^{2+} ions on chymotrypsin and on trypsin, the binding behavior of α-chymotrypsin toward this ion was also investigated. Some time ago it was reported that trypsin at pH 7.4 may hold about 35 moles of Ca^{2+}/1 × 10^5 g of enzyme and that the maximum number of ions is attached in the presence of 20 mM solution of the ion (Carr, 1953). Close to the isoionic point, 5.1 Ca^{2+} ions/mole of α-chymotrypsin are ligated when the salt concentration is 0.023 M (Figure 4) and the shape of the curve $\bar{\nu}$ *vs.* pH for Ca^{2+} is similar to that for Na^+ ions. From the graph $\bar{\nu}/c$ *vs.* $\bar{\nu}$, which is linear, we obtain as apparent maximum number of binding sites 17.7 and as apparent association constant 16.2 (Figure 5).

It should be emphasized that the data presented in this paper for the binding of the various ions are for a limited range of salt concentration since reliable electromotive force measurements cannot be obtained at high or very low concentrations. Sites appear "equivalent and independent" in the restricted range of examination.

The addition of salt to the deionized chymotrypsin causes a slight increase in pH and one might argue that hydrogen ions

TABLE IV: Calcium Ion Binding to α-Chymotrypsin.

pH	α-Chymotrypsin Concn (M × 10⁴)	Cation Concn (M × 10³) Total	Free	$\bar{\nu}$ Cation
6.71	2.67	14.00	13.11	3.34
6.77	2.27	24.86	23.70	5.11
6.74	2.15	34.86	33.51	6.30
6.78	2.25	44.76	43.08	7.48
6.80	2.62	22.26	20.90	5.21
7.41	2.46	24.06	22.46	6.51
7.66	2.62	23.83	22.19	6.25
7.81	2.91	23.96	21.92	7.00
8.60	2.52	22.93	20.82	8.37

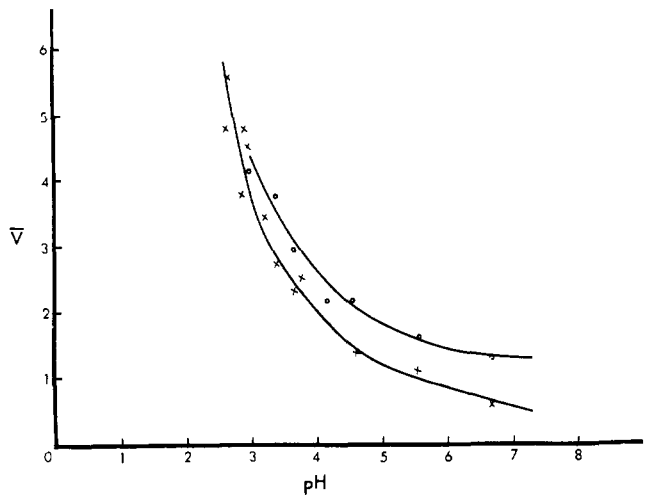

FIGURE 1: pH dependence upon binding of Cl^- and SO_4^{2-} to α-chymotrypsin; concentration approximately 0.03 M for Cl^- and 0.012 M for SO_4^{2-} O, Cl^-; ×, SO_4^{2-}.

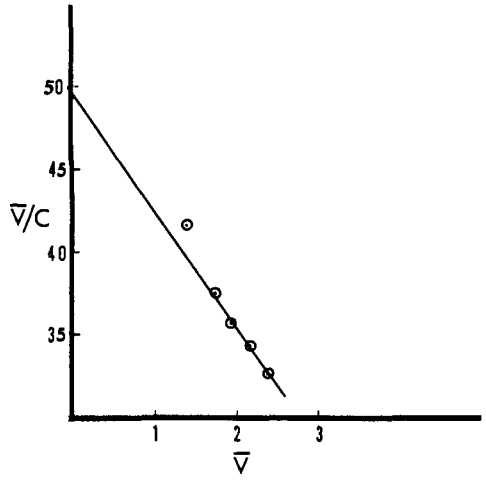

FIGURE 2: Plot of $\bar{v}_{Cl^-}/[Cl^-]$ with respect to $\bar{v}_{Cl^-}$ for α-chymotrypsin; pH 6.61–6.77.

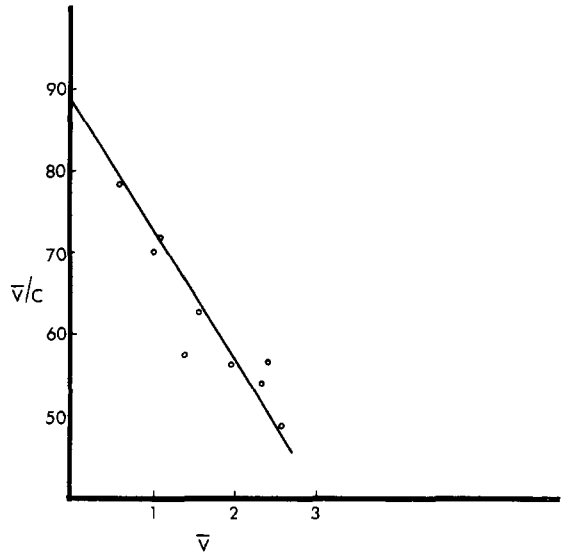

FIGURE 3: Plot of $\bar{v}_{SO_4^{2-}}/[SO_4^{2-}]$ with respect to $\bar{v}_{SO_4^{2-}}$ for α-chymotrypsin; pH 6.65–6.83.

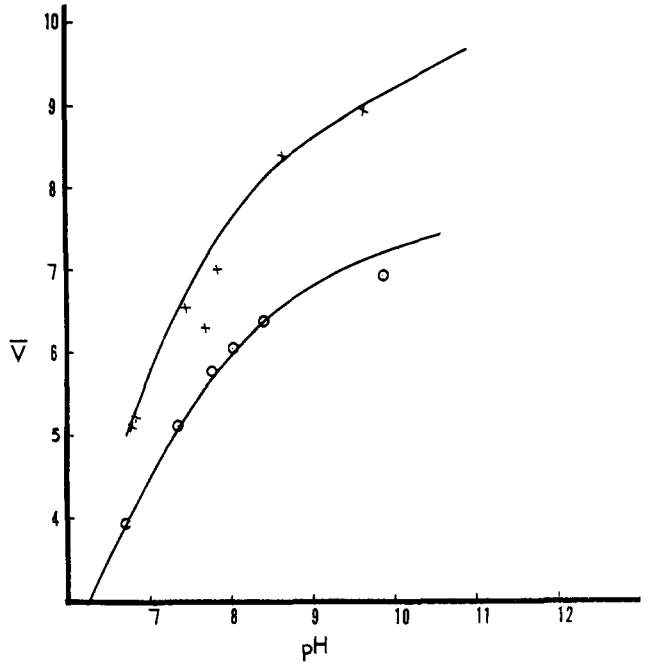

FIGURE 4: pH dependence upon binding of Na^+ and Ca^{2+} to α-chymotrypsin; concentration approximately 0.035 M for Na^+ and 0.02 M for Ca^{2+} O, Na^+; ×, Ca^{2+}.

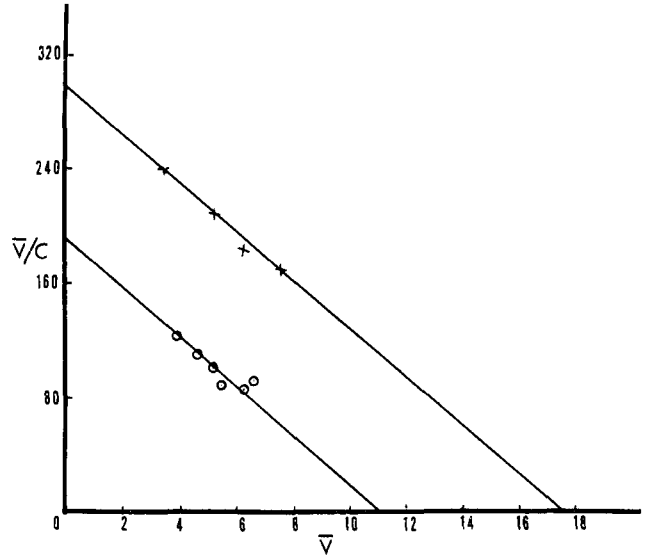

FIGURE 5: Plot of $\bar{v}_{Na^+}$ (or Ca^{2+})/[Na^+] (or [Ca^{2+}]) with respect to $\bar{v}_{Na^+}$ (or $\bar{v}_{Ca^{2+}}$) for α-chymotrypsin; pH 6.69–6.80 for Na^+ and pH 6.71–6.78 for Ca^{2+} O, Na^+; ×, Ca^{2+}.

are ligated to the protein to help neutralize an excess of anions bound by the protein (Scatchard and Black, 1949). This is inconsistent with the results reported here which indicate that cations are held more strongly by chymotrypsin than anions. Powell-Baker and Saroff (1965) show that addition of salt to isoionic β-lactoglobulin lowers the pH even though no binding of sodium occurs. They suggest that a fall in pH could result from a dissociation reaction involving aggregates. Ho and Waugh (1965) report that deionized α_S-casein binds potassium ions releasing a much smaller number of hydrogen ions in the process. The pH changes produced by the addition of KCl to

isoionic α_S-casein correspond to the liberation of a small fraction of one hydrogen ion per molecule even though several potassium ions are ligated. Hence, a rise in pH should not be taken as proof that more anions than cations are bound by chymotrypsin.

References

Carr, C. W. (1953), *Arch. Biochem. Biophys. 46*, 424.
Delaage, M., Abita, J. P., and Lazdunski, M. (1968), *European J. Biochem. 5*, 285.
Dixon, G. M., and Neurath, H. (1957), *J. Biol. Chem. 225*, 1059.
Erlanger, B. F., Cooper, A. G., and Bendich, A. J. (1964), *Biochemistry 3*, 1880.
Floyd, B. F., and Friedberg, F. (1966), *J. Biol. Chem. 241*, 5533.
Friedberg, F., and Emiola, L. O. (1968), *Biochemistry 7*, 2183.
Ho, C., and Waugh, F. (1965), *J. Am. Chem. Soc. 87*, 110.
Powell-Baker, H., and Saroff, H. A. (1965), *Biochemistry 4*, 1670.
Saroff, H. A., and Healy, J. W. (1959), *J. Phys. Chem. 63*, 1178.
Scatchard, G. (1949), *Ann. N. Y. Acad. Sci. 51*, 550.
Scatchard, G., and Black, E. S. (1949), *J. Phys. Colloid Chem. 53*, 88.
Wilcox, P. E., Kraut, J., Wade, R. D., and Neurath, H. (1957), *Biochim. Biophys. Acta 24*, 72.

The Oxygenation of Hemoglobin in the Presence of 2,3-Diphosphoglycerate. Effect of Temperature, pH, Ionic Strength, and Hemoglobin Concentration*

Ruth E. Benesch, Reinhold Benesch,† and Chi Ing Yu

ABSTRACT: The interaction of 2,3-diphosphoglycerate with hemoglobin is markedly influenced by environmental factors. The strength and extent of binding is inversely proportional to pH, as would be expected for electrostatic interaction with a polyanion. The effect of 2,3-diphosphoglycerate in lowering the oxygen affinity of hemoglobin can be regarded as a special salt effect, since neutral salts act similarly, albeit in concentrations 1000 times greater than 2,3-diphosphoglycerate. This molecule is thus capable of regulating oxygen affinity at the millimolar concentrations in which it occurs in the erythrocyte without disturbing the osmotic equilibrium. Both oxygen and 2,3-diphosphoglycerate binding by hemoglobin are exothermic.

Since oxygenation involves displacement of 2,3-diphosphoglycerate, the temperature dependence of the oxygenation reaction is lowered in the presence of the phosphate cofactor, permitting correct oxygen release over a wider range of temperature. The reaction of 2,3-diphosphoglycerate with deoxyhemoglobin is accompanied by a decrease in entropy, which, together with other evidence, bears out the previously proposed model for the interaction of 2,3-diphosphoglycerate with hemoglobin.

We first demonstrated in 1967 that D-2,3-diphosphoglycerate and certain other organic phosphates of the human red cell substantially decrease the oxygen affinity of hemoglobin (Benesch and Benesch, 1967). This effect was quickly confirmed in other laboratories (Chanutin and Curnish, 1967; Tyuma and Shimizu, 1969). It subsequently became clear that D-2,3-diphosphoglycerate only affects the over-all affinity for the ligand but has little if any influence on the other allosteric properties of hemoglobin, *i.e.*, the cooperativity of the oxygen binding and the Bohr effect (Benesch *et al.*, 1968b). The general subject of intracellular organic phosphates as regulators of oxygen release by hemoglobin has been reviewed recently (Benesch and Benesch, 1969).

This report deals with the effect of temperature, pH, ionic strength, and the hemoglobin concentration on the reciprocal binding of D-2,3-diphosphoglycerate and oxygen by hemoglobin in buffered solutions. Information on the energetics of the reaction between D-2,3-diphosphoglycerate and hemoglobin could be expected to lead to a better understanding of the mechanism of the interaction as well as to certain extrapolations on the influence of D-2,3-diphosphoglycerate on oxygen release by hemoglobin in the red cell.

* From the Department of Biochemistry, College of Physicians & Surgeons of Columbia University. *Received February 5, 1969.* This work was supported by U. S. Public Health Service Grants HE-05791 and HE-06787 from the National Heart Institute and Grant GB-4530 from the National Science Foundation and by the American Heart Association.

† Recipient of a U. S. Public Health Research career award from the National Heart Institute.

Experimental Section

Hemoglobin was prepared from the blood of healthy donors at weekly intervals as described previously (Benesch *et al.*, 1968b). It was "stripped," *i.e.*, rendered phosphate free by dialysis against 8 l. of 0.1 M NaCl flowing at the rate of 500 ml/hr at 4°. The dialyzing membrane was two-dimensionally