The Binding of Calcium by Chymotrypsinogen A

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Abstract

Chymotrypsinogen A and α-chymotrypsin possess a single strong binding site for calcium ions. From the pH-dependence of binding, it appears that the titration of a histidine or an α-amino group results in a loss of binding ability. The binding site is not in proximity to the catalytic site. The binding of calcium eliminates the self-association of the chymotrypsinogen monomer.

Introduction

It has been recognized for many years that Ca^{++} has an important influence upon the properties of trypsin, chymotrypsin, and their corresponding zymogens. In particular, the presence of Ca^{++} has a significant effect upon the activation of both zymogens, serving to increase the yield of active enzyme. The effect is especially dramatic in the case of trypsinogen, for which the extent of formation of inactive side-products is considerably decreased (1).

Desnuelle and Gabeloteau (2) have found that the presence of Ca^{++} during the activation of trypsinogen augments the rate of hydrolysis of the critical Lys (6)-Ileu (7) bond. DeLaage and Lazdunski (3) have reported the development of a pH-dependent difference spectrum below pH 6, which they attributed to a pH-dependent conformational isomerization. The presence of Ca^{++} blocked the structural transition and stabilized the form of the zymogen

prevailing at neutral pH. Ca^{++} also was found to stabilize trypsinogen against urea denaturation.

DeLaage, Abita and Lazdunski (4) have also found that Ca^{++} stabilizes chymotrypsinogen against urea denaturation and have concluded that Ca^{++} is preferentially bound by the native form. From the midpoint of the profile of denaturation rate versus Ca^{++} concentration, a value of $pK_{Ca^{++}}$ close to 3.0 was estimated.

Stroud, Kay and Dickerson (5) have proposed to interpret the above results in terms of a single strong binding site for Ca^{++}. They have discussed the possibility that the three carboxyls occurring at positions 72, 78 and 153, which exist in close spatial proximity, are involved in the binding site.

Despite its indirect nature, the evidence cited above is fairly compelling in establishing that some form of interaction exists between Ca^{++} and chymotrypsinogen, with significant structural consequences for the latter. It is the purpose of the present communication to extend and complete the above investigations through direct binding studies and to describe additional evidence as to the nature of the binding site.

Experimental: Materials

Crystalline chymotrypsinogen A and α-chymotrypsin, as well as the protease inhibitors ovomucoid and lima bean inhibitor, were purchased from the Worthington Biochemical Corporation. $^{45}Ca^{++}$ was obtained from the International Chemical and Nuclear Corporation.

Glass redistilled water and analytical grade reagents were used for the preparation of all solutions.

Experimental: Methods

The binding of Ca^{++} by chymotrypsinogen was measured by equilibrium dialysis using Ca^{++} labeled with $^{45}Ca^{++}$. Chymotrypsinogen solutions (1.5%) were placed within 1/4" diameter Visking dialysis sacs and equilibrated with $^{45}Ca^{++}$-labeled Ca^{++} solution with agitation for 48 hours at 8°. Radioactiv-

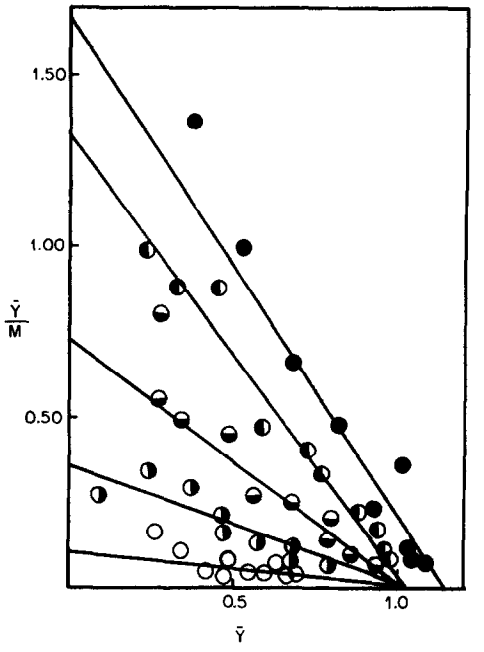

Fig. 1. Scatchard plots for the binding of Ca^{++} by chymotrypsinogen at a series of pH's. Here $\bar{Y}$ is the number of moles of Ca^{++} which is bound per mole of monomer and M is the molarity of free Ca^{++}. The temperature is 8°.

- ○ 0.01 M tris, 0.01 M KCl, pH 6.0
- ◐ pH 6.5
- ◒ pH 7.3
- ◑ pH 7.8
- ● pH 8.0

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ities were measured with a Nuclear Chicago scintillation counter using Aquasol (New England Nuclear) as a counting fluid.

Results

Stoichiometry and binding constants. Figure (1) shows Scatchard plots for the binding of Ca^{++} by chymotrypsinogen A over the pH interval 8 to 5 at 8°. For all conditions studied, the plots were linear within experimental uncertainty, extrapolating to one bound Ca^{++} per molecule at high Ca^{++} levels. The data were thus consistent with the presence of a single strongly binding site. Any additional sites must be of such low affinity as not to be readily detectible.

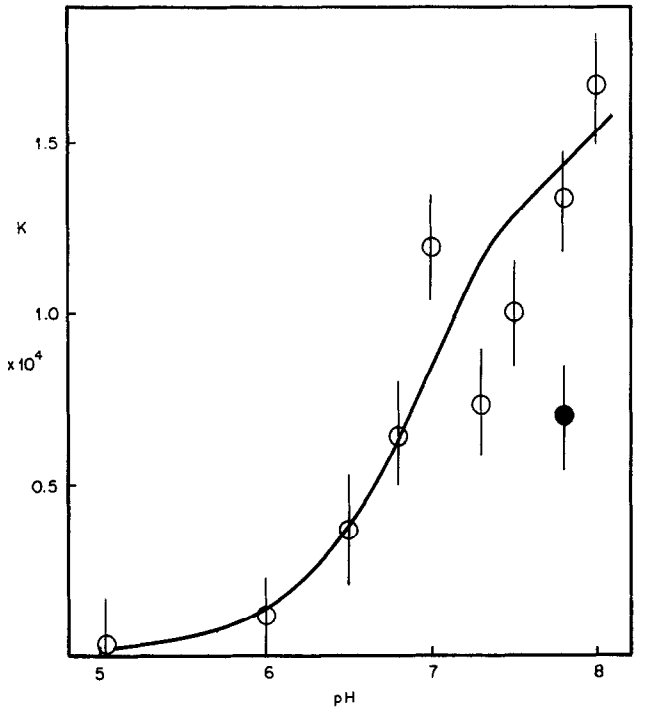

Fig. 2. The pH-dependence of the binding constant for Ca^{++} in 0.01 M tris, 0.01 M KCl, 8°.

○ 0.01 M tris, 0.01 M KCl

● 0.01 M tris, 0.3 M KCl

The equilibrium constant for binding by the single strong site was very pH-dependent, decreasing sharply over the pH range 8 to 5, the midpoint being close to pH 7 (Figure 2). At pH 5.0 there was no detectible binding.

For similar conditions the binding of Ca^{++} by α-chymotrypsin was essentially equivalent to the behavior of the zymogen (Figure 3). Moreover, the presence of the protease inhibitors ovomucoid and lima bean inhibitor in mole ratios of 1:1 or greater did not reduce the binding.

Effect upon self-association. Chymotrypsinogen has been shown to undergo self-association at alkaline pH and low ionic strength (6). Figure 4 shows the dependence of sedimentation coefficient ($S_{20°}$) upon total Ca^{++} concentration. The sedimentation coefficient decreases rapidly with increasing Ca^{++}

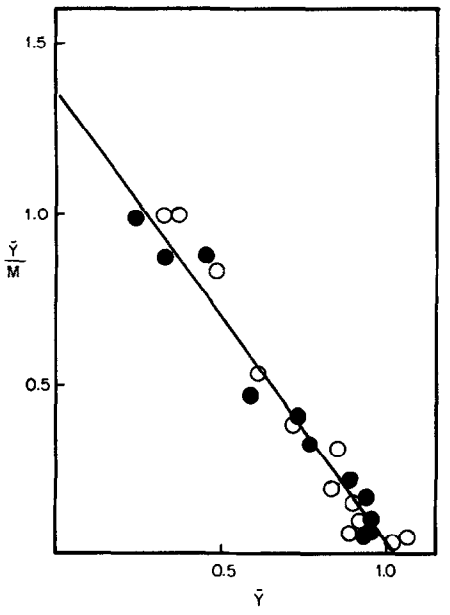

Fig. 3.

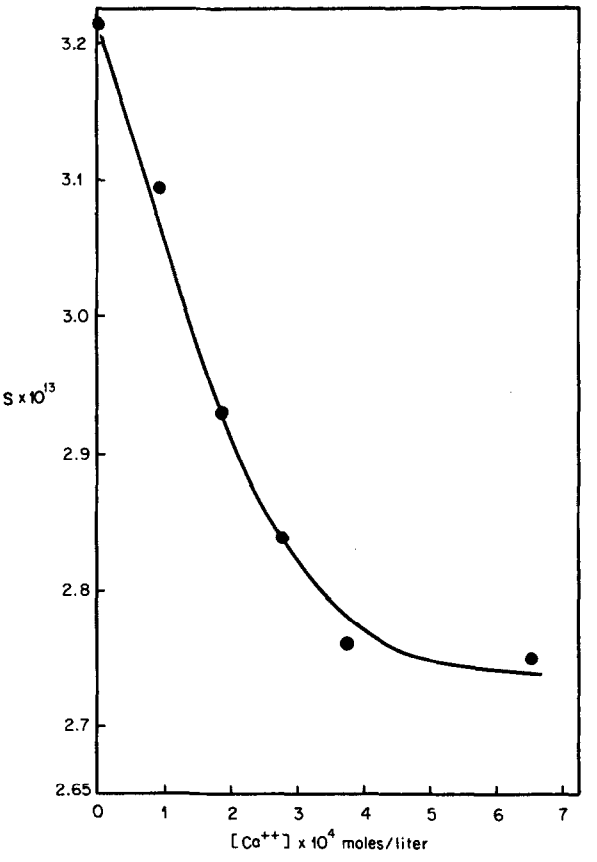

Fig. 4.

Fig. 3. A comparison of the Scatchard plots for the binding of Ca^{++} by chymotrypsinogen (●) and by α-chymotrypsin (○) in 0.01 M tris, 0.01 M KCl, pH 7.8.

Fig. 4. The dependence of the sedimentation coefficient (s_{20}) of chymotrypsinogen (8 mg/ml) in 0.01 M tris, pH 8.0, upon the molarity of Ca^{++}.

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level, approaching the value for the monomer (2.6×10^{-13}) at high Ca^{++} levels.

It is evident that the binding of Ca^{++} by chymotrypsinogen serves to block its self-association.

Discussion

The binding of Ca^{++} by chymotrypsinogen appeared to occur at a single

site. The binding affinity is quite high, corresponding to a value of ΔF of -5.4 kcal.

The binding site remains intact in chymotrypsin. Since prior combination with the relatively bulky protease inhibitors does not appear to prevent binding, it may be inferred that the binding site is not in proximity to the catalytic site, but occurs in some other portion of the molecule.

A point of particular interest is the pronounced pH dependence of the binding constant, from which it appears that the protonation of a group with pK $\sim$ 7 is sufficient to block binding, with the implication that this group is an element of, or is in proximity to, the binding site.

The nature and location of the site remain conjectural. The finding of Abita and Lazdunski (7) that chemical modification of 13 of the 14 carboxyls of chymotrypsinogen abolished the ability of Ca^{++} to protect against urea denaturation suggests that the binding site contains one or more carboxylate groups and supports the proposal of Stroud, Kay and Dickerson (5) that the carboxylates at positions 72, 78 and 153 are involved in the site.

The titration data of Marini and Wunsch (8) have been interpreted to indicate that the only groups titrated between pH 5.5 and 8.5 are the two histidines and the N-terminal α-amino group. In view of the results with chymotrypsin, His-57 is unlikely to be involved since it is an element of the catalytic site. The remaining two groups, His-40 and the α-amino group, are not greatly distant from the above three carboxyls in the crystalline state and may be in even closer proximity in solution. The alternative explanation exists that titration of either group induces a conformational change which indirectly alters the properties of the binding site. However, there is no direct evidence for a conformational change in this pH region.

The abolition of the self-association of chymotrypsinogen in the presence of Ca^{++} raises the possibility that the Ca^{++}-binding site may be involved in the zone of contact of two monomer units. If the electrostatic interaction

of oppositely charged zones of the two surfaces is an element in the stabilization of the complex species (9), then one explanation of the action of Ca^{++} might be in terms of charge neutralization, although other, more specific, mechanisms cannot be excluded at the present time.

References

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