Biochimica et Biophysica Acta, 481 (1977) 164—170

BBA 68069

ON THE RELATIVE LOCATION OF THE INHIBITOR- AND CALCIUM-BINDING SITES IN BOVINE TRYPSIN AS DETERMINED BY NUCLEAR MAGNETIC RESONANCE

POSSIBLE AMBIGUITIES IN PARAMAGNETIC PROBE MAPPING STUDIES

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(Received September 7th, 1976)

Summary

Possible pitfalls in mapping studies utilizing the nuclear relaxation rates induced by paramagnetic probes are pointed out. In cases in which a distance is sought between a paramagnetic ion and a small molecule (e.g. substrate, inhibitor, etc.), both bound non-covalently to a macromolecule, heterogeneity in the system with respect to the binding of either of them may result in ambiguous conclusions. It is shown that the trypsin-gadolinium (III)-inhibitor system is heterogeneous, as revealed in the dependence of the water and inhibitor proton line-widths upon both the Gd^{3+} and the enzyme concentrations and in the effects of added Ca^{2+} on the line-widths. The results imply that in published work (Abbott et al. (1975) Biochemistry 14, 4935) the distance from a weak rather than from the strong metal ion binding site of trypsin (EC 3.4.21.4) may have been determined.

Introduction

Recent attempts to locate the calcium-binding site on trypsin have resulted in a controversy. Abbott et al. [1] used an NMR approach to determine the relative position of competitive inhibitors of bovine trypsin (EC 3.4.21.4) and the paramagnetic gadolinium (III) ion believed to occupy the strong calcium

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binding site of the enzyme. For the interpretation of the results the fraction of bound Gd^{3+} was estimated from independent binding measurements and used to obtain intrinsic proton relaxation rates from the experimental data. The distance obtained, 10 Å, between the methyl protons of *p*-toluamidine and Gd^{3+}, along with the known position of the inhibitor [2] places the binding site of the metal ion at the side chains of Asp-194 and Ser-190 (using the chymotrypsin sequence numbering) [1]. This result should be contrasted with the crystallographic determination of the calcium site, which is composed of the carbonyl oxygens of Asn-72 and Val-75 and the carboxyl side chains of Glu-70 and Glu-80, and which is approx. 25 Å away from the possible location of the inhibitor protons [3]. Referring to the results of Abbott et al. [1] and taking their interpretation at face value it becomes clear that a Gd^{3+} ion situated 25 Å from the inhibitor protons will produce no observable effects on their relaxation rates.

The purpose of the present communication is to point out possible pitfalls in the NMR approach and to demonstrate that the application of this method to the trypsin-gadolinium (III)-inhibitor system may result in ambiguities.

Theoretical Background

Nuclear relaxation rates effected by paramagnetic ions are now often used to derive important structural information in systems of biochemical interest (compare for instance ref. 4). One of the main mechanisms responsible for these effects is the electron-nuclear dipolar interaction. The relaxation due to the dipolar interaction is related to the distance, r, between the observed nucleus and the paramagnetic center by the well known relation of the general form

$$1/T_{2M} = r^{-6} D f(\tau_C) \tag{1}$$

where $1/T_{2M}$ is the transverse relaxation rate of the nucleus in the paramagnetic environment, D is a constant for given nucleus and ion, and $f(\tau_C)$ is a function of the correlation time effectively modulating the interaction. In many cases a nucleus belonging to a small molecule (e.g. substrate, inhibitor, etc.) is observed and the effects of relatively low concentrations of a macromolecule containing a paramagnetic ion are monitored. The net increase in the relaxation rate under conditions of rapid ligand exchange between the bulk of the solution and the paramagnetic environment is given by

$$1/T_{2p} = P_M/T_{2M} \tag{2}$$

where $P_M = L_b/L_t$, L_t being the total ligand concentration and L_b that bound in the vicinity of the paramagnetic ion. Thus from relaxation measurements relative distances can be determined. For absolute distances estimates of P_M and $f(\tau_C)$ are needed. There are methods of obtaining both of them: P_M from studies of concentration dependence and $f(\tau_C)$ from studies of frequency dependence or from the ratio between longitudinal and transverse relaxation rates (for details compare ref. 4).

There are, inter alia, two important conditions that must be simultaneously fulfilled in order to obtain unequivocal results in paramagnetic-probe-mapping

studies of systems in which both the metal ion probe and (in our case) the inhibitor are bound noncovalently to the macromolecule. (1) Only one binding site for the paramagnetic probe must be present. If there are additional binding sites (weak as they might be) in closer proximity to the inhibitor site of interest, the effect of binding to these sites on the observed relaxation may not be negligible due to the inverse sixth power dependence on the distance (cf. Eqn. 1). (2) The inhibitor (in our case) must bind only at one site. If there are additional binding sites, relaxation effects due to binding to those sites that are closer to the site of the probe may become important. Thus an almost absolute homogeneity of the system is required both with respect to the binding of the paramagnetic probe and regarding the binding of the inhibitor. Such homogeneity may be unattainable in practice, in particular with metal ions, since most of the free carboxyl side chains of proteins may serve as weak binding sites. It is usually feasible, however, to check for possible ambiguities that may arise due to the heterogeneity of the system by using the relaxation effects themselves as a monitor of the binding equilibria and comparing the results with independent determinations.

Experimental Section

Bovine trypsin was a product of Novo Industri A/S, Copenhagen, Denmark. For the experiments with acetamidine it was used as received. For the experiments with *p*-toluamidine it was dialyzed three times at pH 2.5 and then lyophilized. The inhibitors, acetamidine, a product of Fluka AG, Buchs Switzerland, and *p*-toluamidine, a product of Columbia Organic Chemicals Co., Columbia, S.C., were used without additional treatment. Solutions were made up in ^2H_2O containing 50 mM of 2-(*N*-morpholine)ethanesulfonic acid (MES) buffer at pH (meter reading) 6.0. Enzyme concentrations were determined spectrophotometrically from the absorbance at 280 nm using $\epsilon_{280} = 36\,600\ M^{-1} \cdot cm^{-1}$. Stock solutions of $GdCl_3$ were made up and standardized as previously described [5].

Proton magnetic resonance spectra were recorded on a Varian T-60 spectrometer operating at a probe temperature of 39°C. The line-width at half-height, Δ, was measured on the scales of 2 or 4 Hz/cm under conditions of slow sweep (0.2 or 0.4 Hz/s). The radio frequency power level was kept well below saturation. The line-width is related to the transverse relaxation time by $\Delta = 1/\pi T_2$. The experimental uncertainty in the Δ values is estimated to be ± 0.4 Hz.

Results

Acetamidine

A solution of 70 mM acetamidine containing 1 mM commercial trypsin was titrated with $GdCl_3$ and the line-width measured. In parallel a similar titration but in the absence of enzyme was also carried out. The results are presented in Fig. 1. The curve of $1/\pi/T_{2p}$ shown in Fig. 1 was obtained by subtracting the line-width measured in the absence of enzyme from that in its presence. Under the conditions of the experiment, i.e. constant inhibitor and enzyme concentrations with the inhibitor in great excess, the occupancy of the inhibitory sites

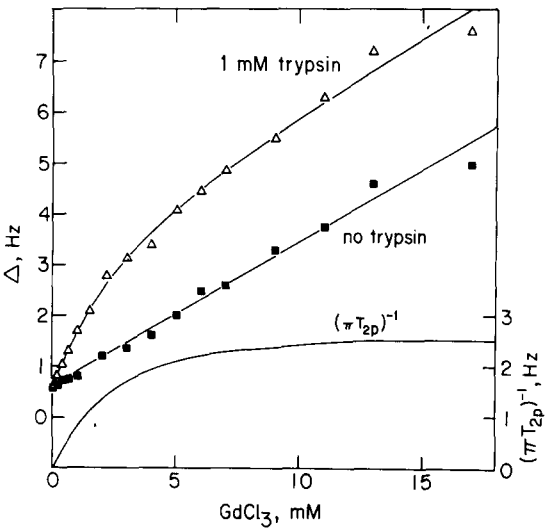

Fig. 1. The line-width of the acetamidine (70 mM) protons as a function of the $GdCl_3$ concentration in the absence and in the presence of 1 mM commercial bovine trypsin. The difference between the two curves is given as $1/\pi T_{2p}$.

may be considered to be constant. In this case the line-broadening ($1/\pi T_{2p}$) of the acetamidine protons reflects the binding of Gd^{3+} to the enzyme and L_b in Eqn. 2 is proportional to $[Gd^{3+}]_b$. An estimate of the dissociation constant of the trypsin-Gd^{3+} complex may be obtained from the metal ion concentration producing one-half of the maximum effect at which point $K_D = [Gd^{3+}]_{t,1/2} - 0.5[E]_t$. From the results in Fig. 1 we obtain $K_D = 1.2$ mM. This value is much larger than the value of 0.4 mM obtained by equilibrium dialysis [1] or than the even smaller values obtained by spectroscopic methods [5]. The line-broadening effected by the trypsin-Gd^{3+} complex was found to be abolished by the addition of $LaCl_3$, but not by the addition of $CaCl_2$.

p-Toluamidine

A solution containing 50 mM *p*-toluamidine and 1 mM $GdCl_3$ was titrated with a solution containing treated bovine trypsin and the same concentrations of inhibitor and Gd^{3+}. The line-widths of the residual water protons (H^2HO) and of the methyl group of *p*-toluamidine were measured. The results are shown in Fig. 2. It is seen that addition of the enzyme results in broadening of both lines. The broadening of the water line is due to the enhanced relaxation rate of the water protons in the vicinity of the bound Gd^{3+} ion relative to that of the free aquo-ion and may serve to monitor the binding of Gd^{3+} [6]. In this case L_b in Eqn. 2 is proportional to $[Gd^{3+}]_b$. The broadening of the inhibitor protons should reflect the binding of Gd^{3+} in a similar way. For both the water and inhibitor the ratio between the initial slope and the maximum observed broadening is a function of the Gd^{3+}-enzyme complex. Mere inspection of the curves in Fig. 2 reveals however that this ratio is different for the water and inhibitor protons. In particular the broadening of the inhibitor line proceeds with a much smaller initial slope, whereas the water line-broadening exhibits a

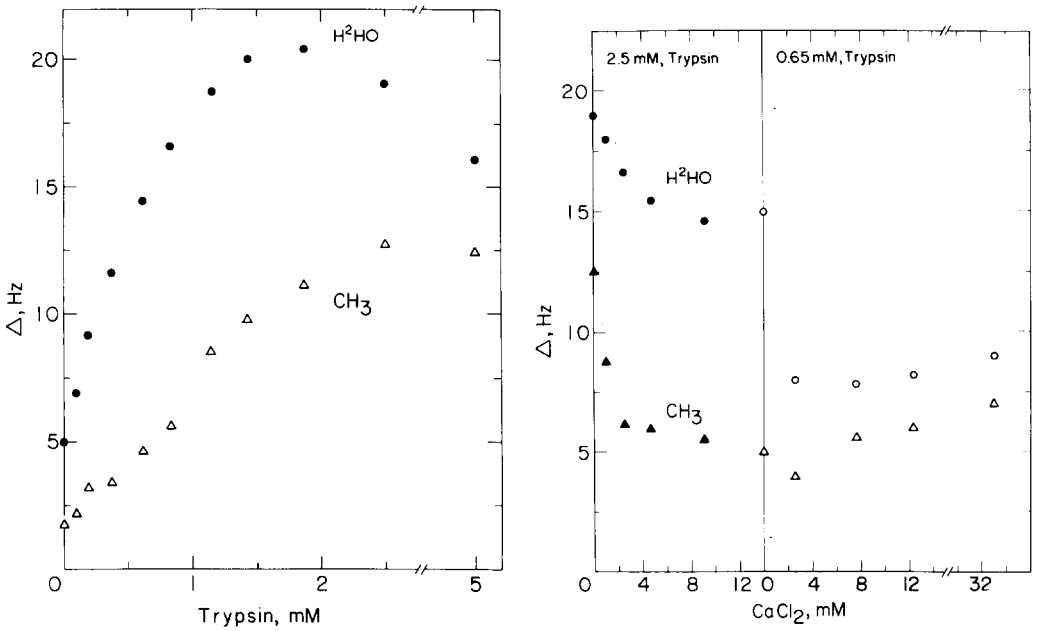

Fig. 2, Line-widths of the residual water (H^2HO) protons and of the methyl of *p*-toluamidine (50 mM) in solutions containing 1 mM $GdCl_3$ as a function of the concentration of bovine trypsin.

Fig. 3. Line-widths of the residual water (H^2HO) protons (circles) and of the methyl (triangles) of *p*-toluamidine (50 mM) in solutions containing 1 mM $GdCl_3$ and 2.5 mM (left, filled symbols) or 0.65 mM (right, open symbols) bovine trypsin as a function of the concentration of added $CaCl_2$.

pronounced maximum. Thus, the line-widths of the water and inhibitor protons seem to reflect in different ways more than one binding equilibrium, i.e., the binding of Gd^{3+} appears to be heterogenous.

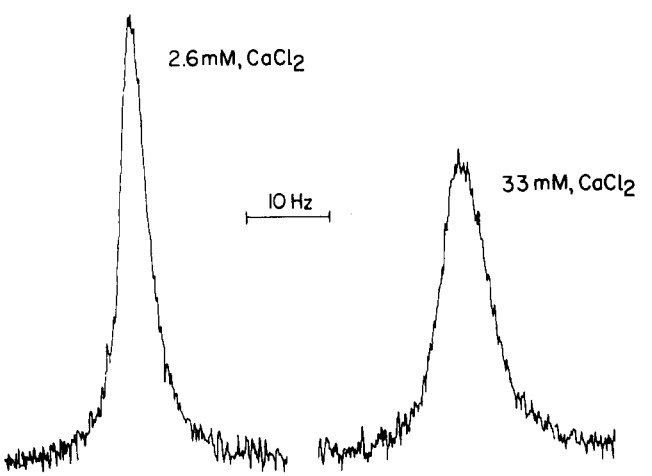

Fig. 4. The proton magnetic resonance spectral lines of the methyl of *p*-toluamidine (50 mM) in solutions containing 1 mM $GdCl_3$, 0.65 mM bovine trypsin and 2.6 mM (left) or 33 mM (right) $CaCl_2$. Both lines were recorded under the same instrumental settings.

Effects of added Ca^{2+}

The effects of added $CaCl_2$ at constant concentrations of $GdCl_3$ (1 mM) and *p*-toluamidine (50 mM) were investigated at two enzyme concentrations: 2.5 and 0.65 mM. The results are shown in Fig. 3. Again different behavior is observed for the water and inhibitor line-widths. At the higher enzyme concentration the water line-width decreases by about 25% whereas the methyl line narrows by more than 50% in the Ca^{2+} concentration range studied. The heterogeneity of the system is more clearly revealed at the lower enzyme concentration where the initial addition of Ca^{2+} causes a line narrowing, but subsequent additions lead to line-broadenings. This effect is dramatically demonstrated by the methyl lines shown in Fig. 4. An increase in the Ca^{2+} concentration leads to a conspicuous decrease in the peak height and an increase in the line-width.

Discussion

The results presented here clearly demonstrate the magnetic as well as the chemical heterogeneity in the trypsin-Gd^{3+}-inhibitor system. It might be of interest to point out here that the heterogeneity in lanthanide binding is also revealed in the concentration dependence of the circularly polarized luminescence of Tb^{3+} (Epstein, M., Reuben, J. and Levitzki, A., unpublished). A qualitative rationalization of the observed trends in the data involves the assumption of two binding sites for Gd^{3+}. Binding to the stronger among the sites results in smaller broadenings as evidenced by the decrease in line-widths observed at higher enzyme concentrations, in particular for the water protons. This appears to be the site that can accommodate Ca^{2+}, as evidenced by the effects of Ca^{2+} on the line-widths. Addition of Ca^{2+} competes with Gd^{3+} for the strong site leading to an effective increase in the concentration of free Gd^{3+}, which in turn results in higher occupancy of the weak stie. The latter is the one responsible for the larger broadenings when occupied by Gd^{3+}. The difference between the enhancements in the water relaxation rate produced by the binding of Gd^{3+} to the two sites may arise from a difference in the hydration states and correlation times of the bound Gd^{3+} ions. The effects observed in the line-width of the inhibitor seem to reflect heterogeneity in the binding of the inhibitor itself. It is possible that in addition to the inhibitory site there is an additional binding site in relatively close proximity to the strong metal ion binding site.

The above discussion suggests that, because of the heterogeneity in the trypsin-Gd^{3+}-inhibitor system, attempts to pinpoint the strong calcium binding site using nuclear relaxation measurements may lead to ambiguous results. From the experimental data and concentration ranges in the work of Abbott et al. [1] it seems that predominantly the effects of the weak metal ion binding site have been observed and that these authors may in fact have correctly located this weak site. Gomez et al. [7] have studied the acceleration by lanthanides of the trypsin catalyzed conversion of trypsinogen. Optimal activity was attained at metal ion concentrations of 0.08—0.5 mM. However at higher lanthanide concentrations inhibition was observed, which was found to be due to the inhibition of the trypsin activity. It seems now that this inhibitory effect could

result from the weak lanthanide binding at the carboxyl of Asp-194 and the hydroxyl of Ser-190, the latter being essential for enzyme specificity and activity.

Acknowledgements

We wish to thank Professor Alexander Levitzki for his interest in this work. J.R.'s participation was supported in part by a grant from the United States-Israel Binational Science Foundation.

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