Transient Removal of Proflavine Inhibition of Bovine $\beta$-Trypsin by the Bovine Basic Pancreatic Trypsin Inhibitor (Kunitz)

A CASE FOR "CHRONOSTERIC EFFECTS"*

(Received for publication, December 30, 1982)

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The formation of the bovine $\beta$-trypsin-bovine basic pancreatic trypsin inhibitor (Kunitz) (BPTI) complex was monitored, making use of three different signals: proflavine displacement, optical density changes in the ultraviolet region, and the loss of the catalytic activity. The rates of the reactions indicated by the three different signals were similar at neutral pH, but diverged at low pH. At pH 3.50, proflavine displacement precedes the optical density changes in the ultraviolet and the loss of enzyme activity by several orders of magnitude in time (Antonini, E., Ascenzi, P., Menegatti, E., and Guarneri, M. (1983) Biopolymers 22, 363-375). These data indicated that the bovine $\beta$-trypsin-BPTI complex formation is a multistage process and led to the prediction that, at pH 3.50, BPTI addition to the bovine $\beta$-trypsin-proflavine complex would remove proflavine inhibition and the enzyme would recover transiently its catalytic activity before being irreversibly inhibited by completion of BPTI binding. The kinetic evidences, here shown, verified this prediction, indicating that during the bovine $\beta$-trypsin-BPTI complex formation one transient intermediate occurs, which is not able to bind proflavine but may bind and hydrolyze the substrate. Thus, the observed peculiar catalytic behavior is in line with the proposed reaction mechanism for the bovine $\beta$-trypsin-BPTI complex formation, which postulates a sequence of distinct polar and apolar interactions at the contact area.

The reaction of serine proteases with proteinase protein inhibitors is a good example of protein-protein interaction and has received great attention, both from a structural and dynamic point of view (for a review see Ref. 1). Recently, it has been found that the kinetics of complex formation between bovine $\beta$-trypsin and BPTI may be described as a multistage process, this being especially evident at low pH (2). In this study, the reaction between bovine $\beta$-trypsin and BPTI was monitored employing three different signals: (i) the displacement of bound proflavine, (ii) the optical density changes in the ultraviolet region associated with complex formation, and (iii) the loss of the catalytic activity in the hydrolysis of $\text{ZLysONp}$.

With BPTI in excess over bovine $\beta$-trypsin, the time course of the reaction for all three signals, corresponded to a pseudo-first order process. The apparent reaction rate was proportional to BPTI concentration at low inhibitor concentration and, as the latter increased, it tended to a limiting value. This behavior was interpreted in terms of fast pre-equilibria followed by limiting first order processes according to the scheme

$$E + I \rightarrow (EI)_1 \rightarrow (EI)_2 \rightarrow K_i$$

where $(EI)_1$ is a labile, rapidly formed, complex; $(EI)_2$, a final complex; $K_i$, the pre-equilibrium constant; and $k_{-i}$, the limiting pseudo-first order rate constant, at infinite BPTI concentration.

The values of $K_i$, $k_{-i}$, and of the second order rate constant ($k_{on}=k_{+i}/K_i$) for the reactions indicated by the three signals were very similar at neutral pH, but diverged at low pH. At pH 3.50, the values of the kinetic parameters differ from one another by several orders of magnitude. In particular, proflavine displacement precedes optical density changes in the ultraviolet and the latter precedes the loss of the catalytic activity. Thus, at pH 3.50, the loss of the enzyme activity occurred much later than proflavine displacement. Since proflavine is a competitive inhibitor of bovine $\beta$-trypsin (Ref. 2 and references cited therein), it could be predicted, on the basis of these results, that on adding BPTI to the bovine $\beta$-trypsin-proflavine complex, the proflavine inhibition might be removed and the enzyme would temporarily recover its catalytic activity.

The prediction was indeed verified in the experiments reported in the present paper. The peculiar behavior of the system, whereby addition of a second inhibitor restores transiently the activity of an enzyme, suggests that effects of this kind might eventually be involved in the regulation of biological functions.

MATERIALS AND METHODS

Bovine trypsin, treated with diphenylcarbamyl chloride in order to abolish chymotryptic activity, was purchased from Sigma. Bovine $\beta$-trypsin was prepared as previously reported (3). Bovine $\beta$-trypsin preparations contained less than 5% of bovine $\alpha$-trypsin as judged from the kinetics of the reaction with p-nitrophenyl-p-guanidinoazetate (4).

BPTI was obtained from Lepetit (Milano, Italy) and further purified as previously reported (5, 6).

The homogeneity of bovine $\beta$-trypsin and BPTI was checked by polyacrylamide gel electrophoresis in 5% NaDodSO4, in the presence and absence of 1% mercaptoethanol (7). The preparations used contained less than 5% of protein contaminants.

The concentration of bovine $\beta$-trypsin and BPTI was determined spectrophotometrically using the following extinction coefficients at

$^{1}$ The abbreviations used are: BPTI, bovine basic pancreatic trypsin inhibitor (Kunitz); $\text{ZLysONp}$, N-$\alpha$-carbonyl-L-lysine-p-nitrophenyl ester; NaDodSO4, sodium dodecyl sulfate.
280 nm: bovine β-trypsin, $E_{1%}^{1} = 15.6$ (3); BPTI, $E_{1%}^{1} = 8.3$ (8).

ZLysONp, $p$-nitrophenyl-$p$-guanidinobenzoate, and proflavine were obtained from Sigma.

All the reagents were of analytical grade and used without further purification.

The hydrolysis of ZLysONp was monitored spectrophotometrically between 340 and 380 nm (8).

The experiments were performed at $21 \pm 0.5 ^\circ C$, using sodium acetate buffer, pH 3.50, ionic strength = 0.1 M. No differences were observed when the experiments were carried out in phosphate or citrate buffer at the same pH and ionic strength.

No effect of calcium, up to 1 x 10$^{-2}$ M calcium chloride, was observed.

The spectrophotometric measurements were carried out with a double beam spectrophotometer (Cary 219) equipped with a thermostated cell holder.

RESULTS

In a typical "activation" experiment, 20 µl of a bovine β-trypsin solution (final enzyme concentration 0.1 µM) were added to 2 ml of acetate buffer (pH 3.50, ionic strength = 0.1 M) containing 1 mM proflavine and 85 µM ZLysONp, and the enzymatic activity measured. After a few minutes, 4 µl of a buffered BPTI solution (acetate buffer, ionic strength = 0.1 M, pH 3.50; final BPTI concentration 20 µM) were added and the activity followed again. (As a consequence of the intrinsic absorption of free proflavine, at the wavelengths chosen to follow $p$-nitrophenol release, a corresponding concentration of the acridine dye was present in the reference cell).

Fig. 1 shows that, in the presence of 1 mM proflavine, the rate of hydrolysis of ZLysONp is about one fifth of that of the uninhibited enzyme. After the addition of BPTI, the rate of hydrolysis increases to a value corresponding to that observed in the absence of proflavine. It should be noticed that the activation stage shown in Fig. 1, on addition of BPTI, will be followed by the full inactivation of the enzyme due to the completion of the BPTI binding process.

The effect of varying ZLysONp concentration in experiments performed under conditions similar to that shown in Fig. 1 was investigated. From these experiments, values of $k_{cat} (= 0.82 \pm 0.20$ s$^{-1}$) and $K_m (= 100 \pm 20$ µM) obtained immediately after the addition of BPTI were found to be similar to those ($k_{cat} = 0.80 \pm 0.20$ s$^{-1}$; $K_m = 90 \pm 20$ µM) determined with the free enzyme.

The effect of varying the concentration of BPTI in experiments performed under conditions similar to that shown in Fig. 1 is reported in Fig. 2. The curve shown in Fig. 2 may be related to the affinity of bovine β-trypsin for BPTI at pH 3.50, in the early stages of the reaction.

FIG. 2. Dependence of the initial velocity for the bovine β-trypsin-catalyzed hydrolysis of ZLysONp on BPTI concentration in the presence of 1 mM proflavine (C). $\bullet$ indicates the value of the initial velocity for the bovine β-trypsin-catalyzed hydrolysis of ZLysONp in the absence of BPTI. The continuous line through the experimental data is the best fit calculated for an apparent equilibrium constant of 1 x 10$^{-2}$ M$^{-1}$. The data were obtained about 1 min after the addition of BPTI. The reaction was followed for 3 to 5 min. Other experimental conditions are as in Fig. 1.

In another series of experiments, performed under conditions similar to that shown in Fig. 1, BPTI was added to bovine β-trypsin, in the presence of 1 mM proflavine, and the activity of the enzyme was measured (upon addition of the substrate in a small volume, 50 µl) at different times after the addition of BPTI to the bovine β-trypsin/proflavine solution. As shown in Fig. 3, the catalytic activity of the bovine β-trypsin/proflavine mixture is about one fifth of that of bovine β-trypsin alone. Immediately after the addition of BPTI to the mixture, the activity increases to a value corresponding to that of free bovine β-trypsin, confirming the results shown in Fig. 1. As the time of incubation of bovine β-trypsin with BPTI increases, the catalytic activity of the proflavine/bovine β-trypsin/BPTI mixture decreases and reaches a value near to zero in about 60 min. The half-time of this process is similar to the half time of inactivation of bovine β-trypsin by BPTI (at the same BPTI concentration) in the absence of proflavine (2).

DISCUSSION

The results here described show, in accordance with expectations, that, on addition of BPTI to the proflavine-inhibited
bovine β-trypsin, there is a transient full restoration of the catalytic activity of the enzyme on the hydrolysis of ZLysONp, both in terms of $k_{cat}$ and $K_m$. This stage is then followed by the irreversible inactivation of bovine β-trypsin due to the completion of the BPTI binding process.

Although it is obviously impossible to explain kinetic data in terms of static three-dimensional structures, the results described here are in keeping with what is currently known on the tertiary structures of bovine β-trypsin, of BPTI, and of the complex (9). As it is well known from the crystallographic analyses, the interaction of BPTI with bovine β-trypsin is mediated by eight hydrogen bonds at sites $P_3$ through $P'_5$, by a salt linkage at the bottom of the specificity pocket and by a large number of van der Waals interactions (approximately 200) (10). In consideration of the extended contact area between bovine β-trypsin and BPTI, a plausible mechanism for the enzyme-inhibitor reaction is that of a sequential formation of hydrogen bonds, polar and apolar interactions (2). According to this hypothesis, an incipient (proflavine displaced) bovine β-trypsin-BPTI complex, in which the enzyme-inhibitor reaction is not fully completed, can be reasonably visualized. In this transient state, the steric hindrance of BPTI in the strict neighborhood of the catalytic triad may be limited, and productive binding of ZLysONp may be permitted.

The situation visualized here is in some respects analogous to that observed in the inhibition of proteases by the α2-macroglobulin (1, 11); in this case, the final complexed proteases are inactive towards macromolecular substrates, but are fully active towards low molecular weight substrates.

In conclusion, this paper describes a curious, apparently paradoxical, situation where the addition of a second inhibitor to an inhibited enzyme restores (even though transiently) the catalytic activity. From a purely phenomenological point of view, and to the best of our knowledge, such behavior has never been described before. Although the system studied here is not physiological, it is tempting to suggest that the effects arising from this kind of behavior might have some role in the regulation of biological functions involving macromolecular interactions. Since these effects involve transient changes in the reactivity of sites, dependent on the interaction with specific effectors, they may be called “chronosteric effects”.

**Acknowledgments**—We thank Prof. M. Brunori and Prof. Q. H. Gibson for helpful discussions.

**REFERENCES**

Transient removal of proflavine inhibition of bovine beta-trypsin by the bovine basic pancreatic trypsin inhibitor (Kunitz). A case for "chronosteric effects".
E Antonini, P Ascenzi, M Bolognesi, E Menegatti and M Guarneri


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