CATALYSIS BY ZYMOGENS: INCREASED REACTIVITY AT HIGH IONIC STRENGTH

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1. Introduction

Kinetic analysis of the intrinsic activity of trypsinogen and chymotrypsinogen has shown that a major cause for the diminished activity of these zymogens, as compared to the corresponding enzymes, is a deformation of the primary binding site [1–6]. The binding of inhibitors by trypsinogen is ~10^4-times less effective than by trypsin [2] while the maximum velocity of catalysis is only 10^2-times lower [6]. Attempts to increase the intrinsic catalytic activity of these zymogens without proteolytic activation have met with limited success. The addition of dipeptides corresponding to the amino-terminus of trypsin resulted in conformational changes of the *p*-guanidinobenzoyl derivative of trypsinogen similar to that obtained by tryptic activation [7,8]. However, this change requires the presence of the guanidinobenzoyl moiety and hence does not manifest itself directly as an increase in the catalytic activity of the zymogen ([7] and J. D. L.-E., unpublished observations). On the other hand the oxidation of Met-192 [3] results in a small but significant increase in the intrinsic activity of chymotrypsinogen by improving the binding of the substrate to the zymogen. In this study we show that high salt concentrations also produce significant increases in intrinsic catalytic activity of zymogens, by enhancing substrate binding.

Abbreviations: Boc-Ala-ONp, tertiary butyloxycarbonyl alanine *p*-nitrophenyl ester; NPGB, *p*-nitrophenyl, *p'*-guanidinobenzoate; DMF, dimethylformamide; Hepes, 4-(2-hydroxyethyl)-1-piperazine–ethanesulphonic acid; Pipes, piperazine–*N*–*N'*-bis (2-ethanesulphonic acid)

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2. Materials and methods

Once-recrystallized bovine trypsinogen, lyophilized bovine trypsin, 3 × crystallized chymotrypsinogen and bovine α-chymotrypsin were products of Worthington Biochem. Corp. Boc–Ala–ONp was obtained from Sigma Chem. Co. and NPGB from Cyclo Chem. Co. Met-192-oxidized chymotrypsinogen was prepared as in [3].

The kinetics of the hydrolyses of NPGB by chymotrypsinogen in 0.1 M Pipes or Hepes (pH 7.4) and of Boc–Ala–ONp in 0.1 M Pipes (pH 7.2) were determined as in [2,6].

3. Results

3.1. *Chymotrypsinogen*

A 10-fold increase in the second-order rate constant of the hydrolysis of Boc–Ala–ONp by chymotrypsinogen is observed in 4 M LiCl. With NPGB a similar enhancement occurs in the first-order rates of acylation (fig.1A). In this latter case, the steady-state velocity remains constant, but the pre-steady-state acylation rate increases. A plot of $1/b$ versus $1/S$ (fig.1B) fits the equation $1/b = 1/k_2 + K_s/k_2S$ and shows that the increased acylation rate is due mainly to an increase in the apparent binding strength ($1/K_s$) of NPGB to chymotrypsinogen. In this equation, b is the pseudo-first-order rate constant for the pre-steady-state reaction at a given concentration [S] of NPGB [3].

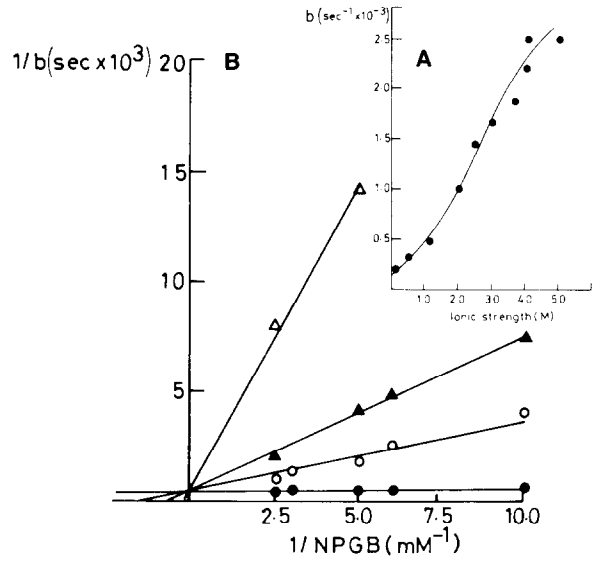

Fig.1(A) The effect of ionic strength on the apparent first-order rate constants of acylation (b) for the reacion of chymotrypsinogen with NPGB (0.4 mM). (B) The effect of ionic strength on the apparent first-order rate constant of acylation (b) for the reaction of chymotrypsinogen with NPGB. The final concentrations of NPGB were varied from 0.1–0.4 mM and the ionic strengths of 0.5 M (–△–), 1.0 M (–▲–), 2.0 M (–○–) and 4.0 M (–●–) were achieved by the addition of LiCl.

The rate of hydrolysis of Boc–Ala–ONp by chymotrypsinogen varies with different salts in a manner which can be approximately correlated with the Hofmeister series (table 1). However, at equal ionic strength, LiCl, NaCl, $CaCl_2$, or Na_2SO_4 all affect the hydrolysis of NPGB by chymotrypsinogen to a similar extent, while the rate of spontaneous hydrolysis is little affected.

Chymotrypsinogen in which Met-192 is oxidized also shows increased catalytic activity at high ionic strength but the effect is less noticeable. At low ionic strength (0.1 M) the modified zymogen has ~5-fold greater intrinsic activity than the native zymogen [3]. In 3 M salt the activities of both the native and modified zymogen towards NPGB are equivalent, while at higher salt concentrations the activity of the native zymogen is greater.

3.2. *Trypsinogen*

Like chymotrypsinogen the intrinsic catalytic activity of trypsinogen is modulated by high concentrations of salts (table 1). The hydrolysis of Boc–Ala–ONp by trypsinogen is increased 2.2-, 5.1- and 18.3-fold in 1 M, 2 M and 4 M NaCl, respectively while, under similar conditions, the hydrolysis of

Table 1
The effect of 3.2 M salt solutions on the activity of trypsinogen and chymotrypsinogen towards Boc–Ala–ONp[a]

Salt	Trypsinogen	Chymotrypsinogen
No salt	1.0	1.0
LiCl	9.8	6.0
NaCl	7.5	4.9
KCl	8.2	3.4
RbCl	4.3	3.0
CsCl	3.6	2.3
$CaCl_2$	6.3	4.9
$BaCl_2$	7.7	5.7
Na_2SO_4	5.5	2.7
NaBr	3.4	2.5
$NaNO_3$	2.6	2.5
$(CH_3)_4NBr$	0.2	0.7
NaI	–	0.2
NaSCN	0.1	0.1

[a] The assay conditions consisted of 1 ml 0.1 M Pipes (pH 7.2) containing 3.2 M salt; 10 μl zymogen (10 mg/ml); 10 μl Boc–Ala–ONp (10 mM in DMF). The data are presented as rate of $\Delta A^{1\,cm}_{400}$ compared to that without added salt. The concentration of $CaCl_2$, $BaCl_2$ and Na_2SO_4 was 1.07 M (i.e., equal in ionic strength to the other salts)

Table 2
The kinetic parameters of Boc–Ala–ONp hydrolysis by trypsinogen at varying ionic strength[a]

NaCl (M)[b]	V_{max}/K_m	K_m (10^{-3} M)	V_{max} (ΔA_{400}/min)
0 (6)	0.018 (±0.001)	3.8 (±3.7)	0.07 (±0.06)
1 (8)	0.04 (±0.002)	0.71 (±0.12)	0.028 (±0.004)
2 (8)	0.092 (±0.008)	0.35 (±0.08)	0.032 (±0.005)
4 (5)	0.33 (±0.07)	0.23 (±0.02)	0.075 (±0.006)

[a] The assay system consisted of 1.0 ml Hepes buffer (0.1 M, pH 7.6) containing 0, 1, 2 or 4 M NaCl; 50 μl trypsinogen (5 mg/ml); 20 μl substrate in DMF. The ΔA_{400} was recorded automatically

[b] Numbers in parentheses correspond to the number of points used in the linear regression analysis for the estimation of the parameters V_{max}, K_m and V_{max}/K_m by Lineweaver-Burk analysis. Each point was the average of at least 2 and usually 3 separate experiments

NPGB by chymotrypsinogen is increased 2.7-, 6.7- and 16.0-fold. As shown in table 2 the increases in the second-order rate constants of the hydrolysis of Boc–Ala–ONp by trypsinogen are accompanied by decreases in K_m while the V_{max} values remain approximately constant.

At low ionic strength (0.1 M) *p*-aminobenzamidine acts as a competitive inhibitor of the hydrolysis of Boc–Ala–ONp as well as that of NPGB [2], K_i ranging from $4–8 \times 10^{-2}$ M. However, in 2 M NaCl, *p*-aminobenzamidine has a complex effect, causing a 1.8-fold increase in k_{cat} accompanied by a 1.8-fold increase in K_m. This change has the characteristics of 'uncompetitive activation'.

4. Discussion

It has been suggested [9] that trypsinogen may be envisaged as an allosteric enzyme that can exist in more than one conformation. The modulation of the catalytic activity of trypsinogen (and of chymotrypsinogen) by changes of ionic strength is in accord with such a hypothesis. So also is the change in the nature of the interaction between trypsinogen and *p*-aminobenzamidine at low and high salt concentrations.

The increased hydrolysis of Boc–Ala–ONp by trypsinogen at high ionic strength suggests that this zymogen may, under these conditons, adopt a more 'enzyme-like' conformation. It is noteworthy that early attempts to recrystallize trypsinogen resulted in its activation [10]. These procedures employed high salt concentrations which, besides removing pancreatic trypsin inhibitor, may well enhance the autocatalytic activation observed [11]. Indeed, by using different crystallization media, trypsinogen is observed to occur in different conformational states [12–14].

A similar situation may exist with chymotrypsinogen. It is reasonable to suppose that the increased catalytic activity of Met-192-oxidized chymotrypsinogen [3] might be the result of some local perturbations of the protein structure. The increased activity at high salt concentrations may be due to a similar perturbation. However, the methionine–oxidized chymotrypsinogen is not as active at low ionic strength (0.1 M) as the native zymogen at high ionic strength (4 M) and, furthermore, the modified zymogen does not respond to increases in ionic strength to the same extent as does the native zymogen.

It is also possible that the increased catalytic activity of the zymogens in high salt is caused by a 'salting-out effect'. According to this interpretation the hydrophobic substrates would be forced out of the aqueous medium into a hydrophobic binding site on the zymogen, possibly the *p*-nitrophenyl binding site described [15]. The fact that no apparent change was observed [16] in the circular dichroism of chymotrypsinogen at high ionic strength suggests that high salt concentrations may indeed affect the substrate only. However, in this situation one might expect the effect of each salt to be similar regardless of the catalyst employed. This is not the case as can be seen in table 1. Furthermore, the present results do not parallel the general pattern observed for the hydrophobic 'salting out' of benzene by a variety of salts [17]. Finally, it would seem unlikely that a positively charged molecule such as NPGB would be affected by salts to the same extent as a neutral one (Boc–Ala–ONp) as was observed with the different zymogens. Consequently, it would seem more likely that the high salt concentrations induce better substrate binding by modifying the conformation of the zymogen although the possibility of additional effects upon the solubility of the substrate cannot be completely excluded.

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