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PROMOTION OF TRYPSIN ACTIVITY BY AMMONIUM ION

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It has been shown that various alkyl ammonium inhibitors of trypsin (EC 3.4.21.4) are promoters of the enzyme active site acylation in the reactions with incomplete nonspecific substrates like N-acetyl-glycine and N-acetyl-L-alanine esters [1–4]; the binding constants of the effectors obtained from the promotion studies coincided with the values of their inhibition constants K_i in the reactions of the enzyme with its specific substrates, esters of N-acyl arginine or lysine. Such promotion effect has been explained as a replacement of the lacking positively charged amino acid side chain in incomplete substrates by alkyl ammonium cation [1]. Thus, the promoting effect of alkyl ammonium inhibitors on trypsin acylation might be based on the mechanism of the substrate-caused activation of trypsin in the acylation step [5, 6], which provides cationic lysine and arginine substrates with more than 1000-fold higher k_2 than their neutral analogues. In this activation, electrostatic interaction between cation and the carboxylic anion of Asp189 in the enzyme active site seems to be necessary. Most drastically, the importance of the plus–minus interaction for the activation finds support in the case of a point-mutated trypsin with Asp189 replaced by Ser189 where the activation of the enzyme in the reaction with arginine and lysine substrates is achieved by adding acetate anion into the reaction mixture [7].

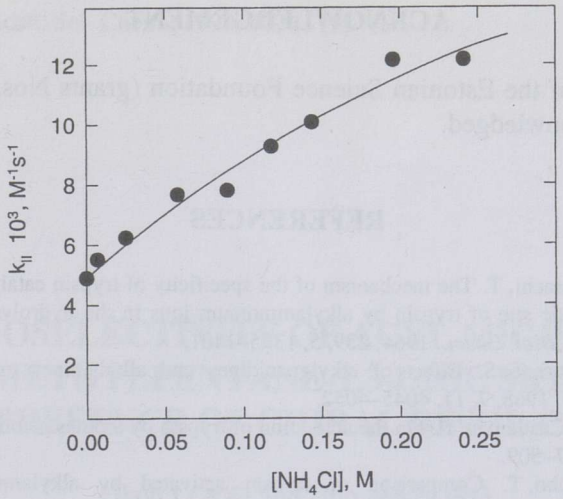
On the other hand, as monovalent metal cations of inorganic salts have been shown [8, 9] to be competitive inhibitors of tryptic hydrolysis of specific arginine and lysine substrates with rather high effectiveness (for Cs^+, $K_i = 0.12$ M, and for K^+ and Na^+, 0.28 and 0.72, respectively), we considered it interesting to check whether these cations could be promoters of the tryptic hydrolysis of incomplete substrates.

As can be seen in the Table, the most "inhibiting" cation, Cs^+, had practically no effect on the tryptic hydrolysis of N-acetyl-glycine methyl ester up to 0.43 M concentration that is 3.6 times above the K_i value of Cs^+ in the trypsin-catalyzed hydrolysis of specific N-benzoyl-arginine *p*-nitroanilide substrate. Similarly, there was no promotion in the case of K^+ and Na^+ (data not shown). Obviously, monovalent cations of inorganic salts, although forming ion pairs with the carboxylic anion of Asp189 in the substrate binding pocket left free by nonspecific substrates as discussed in [9], do not contribute in catalytically beneficial rearrangements in the activated complex of trypsin acylation.

Influence of CsCl and NH_4Cl on tryptic hydrolysis of N-acetyl-glycine methyl ester at 25 °C and pH 6.6 in 0.1 M KCl solution where k_{II}^0 was $4.89 \cdot 10^{-3}$ $M^{-1}s^{-1}$. Substrate hydrolysis was followed titrimetrically on a Radiometer pH-stat (TTT80/REC80/ABU80, Denmark); 0.01 M KOH was used as titrant

C_{SALT}, M	$k_{II}\,10^3$, $M^{-1}\,s^{-1}$	
	CsCl	NH_4Cl
0.007	–	5.517
0.025	–	6.238
0.044	4.306	–
0.058	–	7.665
0.085	4.217	–
0.090	–	7.806
0.118	–	9.269
0.144	–	10.08
0.163	4.487	–
0.196	–	12.15
0.236	4.571	–
0.241	–	12.16
0.300	4.656	–
0.427	4.581	–

But, surprisingly, another small cation, NH_4^+, occurred to be promoter of the tryptic hydrolysis of N-acetyl-glycine methyl ester. As can be seen in the Table, at 0.196 M concentration of NH_4Cl, which is close to the K_i value of 0.18 for ammonium cation inhibition in tryptic hydrolysis of N-benzoyl-arginine *p*-nitroanilide substrate [9], the rate of N-acetyl-glycine methyl ester hydrolysis has increased more than two times when compared with k_{II}^0 at no NH_4Cl added.



Influence of ammonium chloride on the tryptic hydrolysis of N-acetyl-glycine methyl ester. The line corresponds to $K_A = 0.503$ M and $k_{II}^A = 28.4\ 10^{-3}\ M^{-1}\ s^{-1}$.

The Figure shows the dependence of k_{II} upon the concentration of NH_4Cl. The curve-fitting by "Enzfitter" corresponds to the equation

$$k_{II} = (k_{II}^0\ K_A + k_{II}^A[A])/(K_A + [A])$$

in accordance with the following simplified reaction scheme for acylation promotion:

$$\begin{array}{l} \qquad\qquad k_{II}^0 \\ E + S \rightarrow E + P \\ + \\ A \\ \downarrow\uparrow\ K_A \quad k_{II}^A \\ EA + S \rightarrow EA + P \end{array}$$

where E is enzyme, S is substrate, P is the reaction product (N-acetyl-glycine), A is promoter, K_A is the dissociation constant of the enzyme-promoter complex, k_{II}^0 is the second-order rate constant of the substrate hydrolysis catalyzed by E, and k_{II}^A is the second-order rate constant of the substrate hydrolysis catalyzed by EA (the k_{II}^A/k_{II}^0 value of 5.8 has been calculated from the presented data).

The mechanism of the promoting effect of NH_4^+ remains to be elucidated in further studies. In comparison with monovalent metal cations, an obvious difference of NH_4^+ is its ability to contribute to the formation of hydrogen bonds. Ammonium cation can be used as an additional new instrument in studying the substrate-caused electrostatic activation in tryptic hydrolysis.

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