Rate of hemoglobin oxidation by various oxidants under aerobic and anaerobic conditions. The rate of hemoglobin oxidation by ferricyanide, hydroxylamine, H_2O_2, β-naphthoquinone-4-sulfonate, and autoxidation was expressed as μM heme/min. The rate of hemoglobin oxidation by chlorate and nitrite was expressed as $t_{1/2}$ (half time), since the reaction proceeded sigmoidally

	Final concentrations of oxidants	Aerobic IHP (−)	IHP (+)	Anaerobic IHP (−)	Temperature (°C)
Ferricyanide	45 μM	20.0 μM/min	195.0 μM/min	very fast	25
		1.8 μM/min	22.0 μM/min	very fast	4
Hydroxylamine	900 μM	13.5 μM/min	22.0 μM/min	52 μM/min	25
Chlorate	110 mM	52 min	27 min	13 min	25
H_2O_2	140 μM	12.2 μM/min	43.8 μM/min	very fast	25
β-Naphthoquinone-4-sulfonate	900 μM	16.9 μM/min	146.0 μM/min	very fast	25
Autoxidation		1.8 μM/15 min	9.6 μM/15 min		38
Nitrite	900 μM	5.2 min	35 min	very slow	25

hemoglobin oxidation under anaerobic conditions was performed in a Thunberg-type quartz cell after replacement of air with Q gas (helium/isobutane, 99.05:0.95). The experiment of autoxidation was performed at 38 °C and the changes in absorbance were pursued at 578 nm.

Results and discussion. The mechanism of hemoglobin oxidation by various oxidants was studied under aerobic conditions with or without IHP, and under anaerobic conditions without IHP. The oxidation rate by ferricyanide, hydroxylamine, chlorate, H_2O_2, β-naphthoquinone-4-sulfonate and nitrite under these conditions is summarized in the table. The oxidation of hemoglobin by these oxidants was classified into 2 groups with regard to the reaction mechanism. Ferricyanide, hydroxylamine, chlorate, H_2O_2 and β-naphthoquinone-4-sulfonate belonged to the 1st group. In this group, the acceleration of the hemoglobin oxidation by these reagents was observed as hemoglobin was bound with IHP and deoxygenated. Since the structural change in oxyhemoglobin from the R to the T state occurs by deoxygenation[14] and is probably induced by the binding of IHP to oxyhemoglobin, it may be possible to say that the oxidation rate by these oxidants was accelerated as the fractions of the T state hemoglobin were increased. The oxidation of hemoglobin by ferricyanide was also accelerated as much as 12 times in the presence of IHP at 4 °C. Autoxidation of hemoglobin might also be involved in the 1st group as far as the reaction mechanism is concerned, since it is well known that autoxidation is considerably accelerated in accordance with the decrease in oxygen concentration[15].

The course of oxidation of hemoglobin by nitrite seems to be different from that by the oxidants stated above and belongs to another category. The rate of oxidation by this reagent was accordingly decreased as hemoglobin was liganded with IHP and when deoxygenated. These results suggest that the R state of hemoglobin is favored for the oxidation of this protein by nitrite in preference to the T state.

Although the differences in the reaction mechanism of hemoglobin oxidation by various species of oxidants under aerobic and anaerobic conditions have so far been considered due to the properties of oxidants, our results suggest that the differences in the reaction mechanism may be essentially due to hemoglobin itself, which is equilibrated between the R and the T state with its quaternary structure.

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Determinant of efficiency of a monomeric enzyme: Acceleration by site-specific molecules for trypsin

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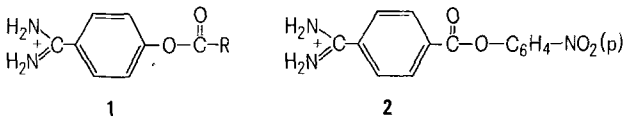
Summary. The interaction of a specific ligand at substrate binding site was shown to be responsible for the catalytic efficiency of trypsin. The reasoning of 'induced fit' theory was refined by kinetic analysis of characteristic properties of 'inverse' substrates.

It is generally believed that the endopeptidase, trypsin, and chymotrypsin[2] hydrolyze only those substrates which contain specificity-determining functional groups in their carbonyl side of the esteric or amide structure. However, in the preceding paper[3], we have reported that a certain class of compounds **1**, in which the arrangement of the site-specific group is of the 'inverse' type of the normal substrates of trypsin **2**[4] (fig. 1), e.g., a cationic center is included in the leaving group instead in the acyl moiety, are susceptible to the enzymatic hydrolysis as well. In this report we demonstrate that the site-specific cationic molecules enhance the efficiency of the deacylation process as a characteristics of 'neutral' acyl trypsin derived from the 'inverse substrates' providing possible novel examples of 'induced fit' concept.

During the course of the tryptic hydrolysis of these substrates, ES complex formation and acylation proceed rapid-

ly, whereas the subsequent deacylation is a slow step. For example, the acylation and deacylation rate constants for **1a** were determined to be 17.0 sec^{-1} and 9.26×10^{-3} sec^{-1}, respectively[3]. The deacylation represents the stage of hydrolytic cleavage of the acyl enzyme intermediate in which hydroxyl group of Ser-183 serves as a leaving group. The behaviour of the 'inverse substrates' is dinstinct from that of the normal type substrates particularly in the deacylation process, because the acyl moiety introduced specifically to the catalytic functional group can no longer have specific interaction with the enzyme binding site. The exceedingly small value of deacylation rate may be thus explained.



1 **2**

a: R = CH_3–
b: R = CH_3CH_2–
c: R = $CH_3CH_2CH_2$–

Fig. 1.

In the presence of benzamidines, overall reaction rate of **1a** has been found to be markedly accelerated, inspite of the fact that the benzamidines are competitive inhibitors in the acylation step. The extent of activation caused by the presence of p-hydroxybenzamidine hydrochloride (1.8 mM) is about 10-fold for **1a** and 4-fold for **1b**. In figure 2 effects of p-hydroxybenzamidine on overall reaction rate (deacylation rate) of **1a**, **1b** and **1c** are shown. The acceleration was observed only when **1a** and **1b** were used as substrates. The higher homologue did not show any rate acceleration in the presence of p-hydroxybenzamidine. In the experiment using **1a** as substrate, only positively charged compounds were found to be the potent activator such as acetoamidine, benzamidine, p-hydroxybenzamidine and benzylamine, all known as competitive inhibitor of trypsin. β-Naphthamidine, however, was not effective as activator in spite of the fact that it is one of the most potent inhibitor[5].

All these observations may be explained by the cooperative effects of the positively charged molecules which could coexist with the introduced acyl group within the active site of trypsin interacting with the binding site. A small acyl

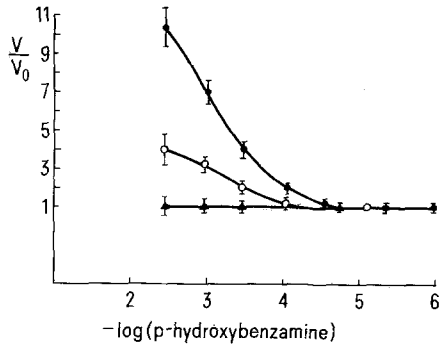


Fig. 2. Effects of p-hydroxybenzamidine concentration on the rate of tryptic hydrolysis of p-acyloxybenzamidines at pH 8.0, 25 °C 0.1 M KCl, 0.02 M $CaCl_2$. ●, p-acetoxybenzamidine 9.60×10^{-6} M; ○, p-propioxybenzamidine 9.60×10^{-6} M; ▲, p-butyloxybenzamidine 9.60×10^{-6} M. Each point represents the average of 3 separate determinations ± SE.

moiety could well be accomodated in the active site cleft together with a small amidinium molecule just like the acyl enzyme derived from normal substrate, while larger cationic species such as β-naphthamidine are too bulky to cooperate with the acyl group in the cleft. Thus this rate enhancement can be rationalized by considering the analogous mechanism suggested by Inagami et al. with nonspecific substrates-amines-trypsin pairs[6], i.e., the presence of site specific group induces conformational transition and promotes efficiency of deacylation as if it would simulate normal substrates. This elegant explanation envisions the formation of ternary complex with the neutral substrate and a small charged molecule within the active site. However, larger alkylamines, such as pentylamine, which were supposed to interfere spatially with a substrate (N-acetylglycine ethyl ester), also exhibited rate enhancement[7,8]. Based on these facts, the presence of 'auxiliary' binding site[9,10] or 'activating' site[7] in the enzyme was proposed. Further studies of the activation mechanism operative in appropriate p-amidinophenyl esters cationic compounds systems are expected to answer this question, since by virtue of the 'inverse substrate' method these effects of the cations could be separately analyzed in every acylation and deacylation processes[10].

Structural dynamism of enzyme was assumed to be an important but as yet largely unknown determinant for the catalytic rate. The above rate acceleration at the particular deacylation step, separately observed by applying the 'inverse' concept, suggests a new aspect for such dynamic properties of the enzyme even involving the turn-over process subsequent to the acylation[10,11]. A reasoning of 'induced-fit' theory[14] could be more refined by its further application to the individual deacylation stage. Allosteric enzymes can control their activity through the conformational transition initiated by the interaction at regulator site[15]. In monomeric enzymes such as trypsin and chymotrypsin, by contrast, conformational transition solely produced by the interaction with a specific ligand at the active site receptor seems to be of main physiological importance.

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