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# EFFECT OF A RESTRICTED REACTION FIELD (RRF) ON ENZYMATIC REACTIONS

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The effect of a restricted reaction field (RRF) on activities of cytosolic and microsomal aminopeptidase was investigated. Activity of cytosolic enzyme increased with decrease in the space width of the RRF, while activity of microsomal enzyme was found to be sensitive to the nature of the surface of the RRF.

Comparative studies between reactions in a restricted space and those in a bulk space have been performed intensively by this author. In the previous paper, it has been demonstrated that the space width of the restricted reaction field (RRF) affects the reaction rates of both enzymatic and non-enzymatic reactions.[1] Most of important reactions in biological systems proceed in a very limited space which is enclosed by cell walls or organelles. Environment in which enzymes function may be divided into three classes; that is, in a cytosol, at the vicinity of a membrane, and in a membrane. If the physical environment itself plays an important role on the enzymatic actions in vivo, reactions with enzymes belonging to the different classes of the environment can be expected to be affected by the RRF in different manners.

This communication reports the effect of the space width and the nature of the surface providing an RRF on the rate of the reaction(1) with Aminopeptidase(cytosol) (EC.3.4.11.1, AP-cytosol) and Aminopeptidase(microsomal) (EC.3.4.11.2, AP-microsomal), which function in the cytosol and at the vicinity of membranes respectively. The vessel for restricted reactions consisted of two

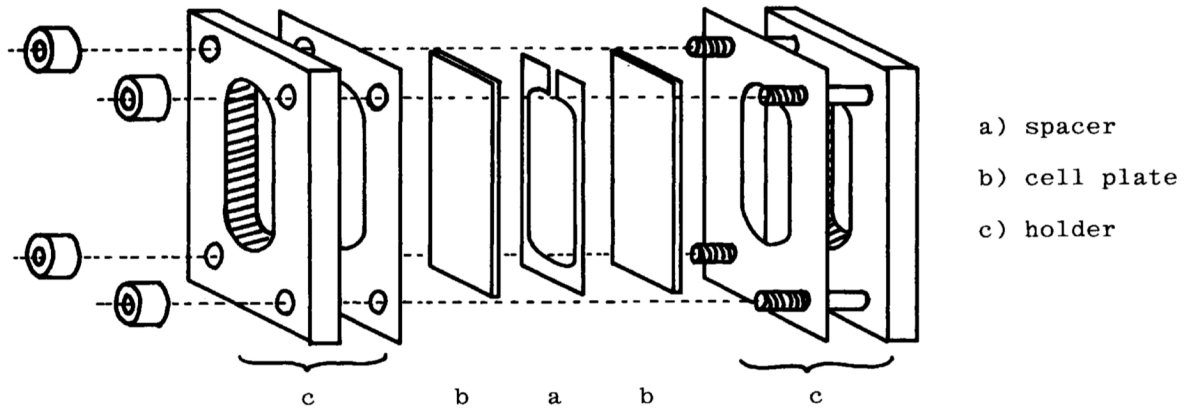


Fig. 1. Vessel for restricted reaction.

$$\underset{\text{(Leu-PNA)}}{\text{L-Leucine p-nitroanilide}} + H_2O \xrightarrow{\text{enzyme}} \text{L-Leucine} + \underset{\text{(PNA)}}{\text{p-nitroaniline}} \quad (1)$$

pieces of plates (12.5 mm x 45 mm x 1.25 mm), a spacer, and a holder, as shown in Fig 1. The space width between two plates was controlled by the spacer of which thickness was measured by the micrometer with an accuracy of ±2 μm from Japan Micrometer MFG.Co., LTD. Three different cell plates were used: quartz (N-Q) and quartz treated with 3-glycidoxypropyltrimethoxysilane[2] ($HO\text{-}CH_2\text{-}CH(OH)\text{-}CH_2\text{-}O\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}Si\text{-}$, 3GP-Q) and octadecyltrichlorosilane ($CH_3\text{-}(CH_2)_{17}\text{-}Si\text{-}$; Oc-Q). N-Q and 3GP-Q have hydrophilic surface, whereas Oc-Q has a hydrophobic surface.

Reaction rates were determined spectrophotometrically and the reaction vessel (Fig.1) containing the reaction mixture was used directly for a spectrophotometer. Control reactions (reactions in an unrestricted vessel) were carried out in flasks with a stopper and parts of the mixture were diluted with solvent in a

Table 1. Effect of an RRF on AP-cytosol activity

| Ex. No. | Surface | ln r | | | Amount of enzyme soln.[b] (μl) |
|---|---|---|---|---|---|
| | | 10 μm | 50 μm | 100 μm | |
| 1 | Oc | 1.50 | 0.92 | 0.21 | 200 |
| 2 | | 1.39 | 1.03 | 0.01 | 200 |
| 3 | | 1.55 | 0.96 | 0.14 | 200 |
| 4 | 3GP | 0.90[a] | 0.41 | 0.14 | 200 |
| 5 | N | 1.20[a] | 0.69 | 0.30 | 200 |
| 6 | Oc | -0.12 | -0.63 | | 40 |
| 7 | Oc | -0.26 | -0.92 | | 20 |

Substrate solution consisted of 5 mM† Leu-PNA, 30 mM phosphate, 25 mM tris and 0.5 mM $MgCl_2$ at pH 7.96 and at 24 °C. Reactions were started by addition of the indicated amount of AP-cytosol preparation (enzyme soln.)[b] to 5 ml of substrate solution at 24 °C. The spectrum changed from that of Leu-PNA ($\varepsilon^{312}$=12600 $mol^{-1}$ $cm^2$) to that of PNA ($\varepsilon^{380}$=13200 $mol^{-1}cm^2$) with isosbestic points at 259 nm and 342 nm during the reaction. The reaction rate was expressed in μM/min. Vc was 5.2-6.5 μM/min in ex.No.1-5, 1.8 μM/min in ex.No.6, and 0.8 μM/min in ex.No.7. In the control reaction, 15 μl of reaction mixture was diluted to 3 ml with 50 mM tris (pH 7.04, containing 1 mM $MgCl_2$) in a conventional cell with a 1 cm light path for spectrophotometric measurements.

a) The reaction rate was measured as in the control reaction.

b) AP-cytosol was purchased from Sigma Co. (Type III-CP from porcine kidney: 1.9 mg Protein/ml, Chromatographically purified suspension in 2.9 M $(NH_4)_2SO_4$, 0.1 M Tris, and 5 mM $MgCl_2$ solution at pH 8 ) and used without further purification.

† 1 M=1 mol $dm^{-3}$.

conventional cell for spectrophotometry.

The effect of an RRF on the activity was evaluated as ln r = ln (Vr/Vc), where Vr and Vc are the reaction rates in restricted and unrestricted reaction vessels respectively.

The effect of an RRF on AP-cytosol activity was examined and results are listed in Table 1. Some results confirming the reproducibility of the data are also included in Table 1 (ex.No.1-3). When a large amount of enzyme was used (ex.No.1-5), ln r values were positive in all cases, regardless of the nature of the surface of the RRF, and the value increased with decrease in the space width. These results indicate that the space width of the RRF affects the enzymatic activity.

Since the ratio of the amount of enzyme to the surface area is less in the restricted reaction vessel than in an unrestricted one, adsorption of the enzyme to the surface of the vessel could be one of possible factors causing the difference in enzymatic activity. This possibility was examined by determining the affinities of AP-cytosol to 3GP and alkyl residues by liquid chromatography. TSK-GEL-GSWP (7.5 mm IDx7.5 cm) from Toyo Soda Manuf. Co. and Ultrapore$^{TM}$ RPSC (4.6 mm IDx7.5 cm) from Beckman were used as columns with surface 3GP and alkyl residues respectively. Protein was eluted with 0.05 M tris (pH 7.4) or 0.1 M phosphate (pH 7.0) at 0.5 ml/min. No affinity of AP-cytosol to GSWP was observed. On chromatography on RPSC, no enzyme was eluted. AP-cytosol exhibited no affinity to 3GP but very high affinity to the alkyl substituent. Thus adsorption of the enzyme to the surface of the vessel has a negligible affection on the effect of the space width of the RRF in the 3GP-Q vessel, but must be taken into account in the Oc-Q vessel. As shown in Table 1, when a small amount of enzyme was used (ex.No.6 and 7), ln r had negative values and increased with decrease in the space width. Thus both the space width and the nature of the surface of the RRF affect the enzymatic activity in the Oc-Q vessel. In this vessel, adsorption of the enzyme to the Oc-surface may contribute to the effect of the RRF.

On the other hand, results on AP-microsomal are listed in Table 2. The space width of the RRF had no effect in a 3GP-Q vessel, but a very strong effect in an Oc-Q vessel. Since a surfactant can be expected to prevent adsorption of enzyme to the hydrophobic surface by inclusion of the enzyme in its micells and by changing the Oc-Q surface from a hydrophobic to a hydrophilic surface by formation of a double layer, the activity of the enzyme in RRF was examined in the presence of the surfactant, Triton X-100. Table 2 shows that the enzymatic activity was not affected by the surfactant in a 3GP-Q vessel but was affected in an Oc-Q vessel. Thus, the disappearance of AP-microsomal activity could be ascribed to the interaction of the enzyme to the Oc-surface.

From the above results, it is apparent that activity of AP-cytosol which functions in the cytosol was mainly affected by the space width of the RRF but that of AP-microsomal which functions at the vicinity of membrane was sensitive to the nature of the surface of the RRF. These findings suggest that it is necessary for understanding the enzymaic action *in vivo* to take into account the environment itself, in which enzymes function.

Table 2. Effect of an RRF on AP-microsomal[a] activity

| Surface | Width of space (μm) | ln r | |
|---|---|---|---|
| | | Triton X-100 | |
| | | - | +[b] |
| 3GP | 10 | 0.13 | 0.13 |
| | 50 | -0.03 | -- |
| Oc | 10 | no reaction | 0.00 |
| | 50 | no reaction | -- |
| Vc[c] | | 52 | 48 |

The substrate solution was the same as that for Table 1. The reaction was started by addition of 20 μl of AP-microsomal preparation to 5 ml of substrate solution at 24 °C.

a) AP-microsomal preparation was purchased from Sigma Co. (Type IV-S from porcine kidney: 1 mg Protein/ml, suspension in 3.5 M $(NH_4)_2SO_4$ and 10 mM $MgCl_2$ solution at pH 7.7 ) and used without further purification.

b) 0.2 μl of Triton X-100 was added to 5 ml of substrate solution.

c) The reaction rate was expressed in μM/min.

Three dimensional structural changes of enzyme and substrate(e.g., orientation), induced by the restriction of the space width and changes in water structure itself and hydration modes of the substrate and the enzymes can be considered as factors to contribute to the enhancement of the reaction rate which was caused by the effect of the space width of the RRF. Further investigations to elucidate the respective contribution of factors to the effect of the RRF are in progress.

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