The use of dipeptide-p-nitranilides for the study of aminopeptidase specificity

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Summary. The use of dipeptide-p-nitranilides for the study of 2 placental aminopeptidases separated on Sephadex G200 helped in establishing some regular features of their specifities. The high-molecular (320,000 daltons) one prefers Phe in position P'_1 to Leu, whereas the lower-molecular aminopeptidase (145,000 daltons) prefers Leu. The high-molecular aminopeptidase splits very slowly the N-terminal Leu when Gly is in adjacent position. Leu-Gly-p-NA is therefore an inhibitor of this AP.

For the determination of aminopeptidase (AP) activity there are preferably p-nitranilides (-p-NA) of amino acids (Leu-, Ala-, CH_3-Cys-, etc.) used, mostly because of easy following of split p-nitraniline. The ability of AP to split the respective amino acid from the N-end of peptidic chain of natural substrate does not always correlate with the ability to split this amino acid from its p-nitranilide. So, for instance, we found that placental AP (mol.wt 320,000 daltons) though hydrolyzing β-naphtyl-amides and p-nitranilides of basic amino acids (Arg- and Lys-) fails to split N-end arginine of bradykinin. From the viewpoint of amino acid-p-NA hydrolysis some serum and tissue aminopeptidases seem to be strongly non-specific though this is actually not the case.

We have therefore searched for a regular dependence of AP specificity and employed dipeptide-p-NA. Starting from the assumption[1] of the reaction of substrate (NH_2-P_1‡P'_1-P'_2-P'_3-R_1) with enzyme (R_2-S_2-S_1–S'_1-S'_2-S'_3-R_3), we studied the role of amino acids of the substrate in position P_1 and P'_1 (when -p-NA in position P'_2 was situated) in the rate of complete splitting of dipeptide-p-NA (Leu-Leu-, Gly-Gly-, Gly-Leu-, Leu-Gly- and Gly-Phe-). The AP gradually degradates N-terminal amino acid from dipeptide-p-NA in the following pattern: A-B-p-NA $\xrightarrow{K_a}$ B-p-NA $\xrightarrow{K_b}$ p-NA. The course of hydrolysis primarily depends on the ratio enzyme/substrate (figure 1). The ability of used AP to split dipeptide-p-NA to dipeptide and p-NA (dipeptidase activity) was negligible (A-B-p-NA $\xrightarrow{K_{ab}}$ A-B + p-NA). This course was confirmed by semiquantitative determination of split amino acids by paper chromatography.

We used the above method for the study of specificities in 2 AP separated from NaCl eluate (1 M) of placenta on Sephadex G200 column (0.05 M phosphate + 0.15 M NaCl). When we compared the hydrolytic velocity of -p-NA used for the determination of both placental AP (Leu-, Ala-, CH_3-Cys-), we ascertained only small differences in their specificity[2]. Better differentiation we obtained by kinetic studies of Gly-Phe- and Gly-Leu-p-NA hydrolysis (figure 2). The high-molecular AP splits Gly-Phe-p-NA more quickly than Gly-Leu-p-NA, the lower-molecular one does the reverse. This AP tended to inactivate oxytocin, determined on isolated rat uterus[3], more readily. This phenomenon can be correlated with higher ability of this AP to split the linkage between cystein and tyrosine within oxytocin. Thus it requires Tyr in position P'_1 in oxytocin, Phe in Gly-Phe-p-NA or p-NA in amino acid-p-NA. From that point of view, the Leu-, Ala-, CH_3-Cys-p-NA appear to be suitable substrates for high-molecular placental AP too, but not for lower-molecular one.

We have further followed the splitting of Gly-Gly- and Leu-Gly-p-NA using high-molecular AP. Hydrolysis of these 2 substrates in comparison with that of Gly-p-NA was very low. That implies that the splitting of N-terminal amino acid is very slow in substrates with Gly in position P'_1 even when N-terminal amino acid is leucine. Consequently, Leu-Gly-p-NA possesses properties of an inhibitor (Vi/V = 0.27) if Leu-p-NA as substrate was used (0.05 M phosphate, pH 7.0, 2.5×10^{-5} M concentration of both substances, initial rate of hydrolysis).

In order to characterize in more detail placental AP and to eliminate the participation of some other peptidases in this effect, we have followed the AP and NH_2-dipeptidyl hydrolase activity on fractions obtained by polyacrylamide gel electrophoresis. The fractions from Sephadex G200 were densified with Ficoll at +2 °C and processed on discontinuous electrophoresis in 7.5% polyacrylamide

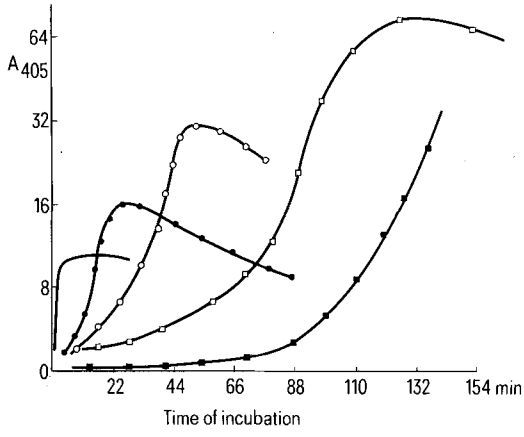

Fig. 1. Splitting rate of Gly-Phe-p-NA by placental AP (mol.wt 320,000 daltons) depending on substrate concentration. Concentration of the enzyme was identical throughout, substrate concentration was (in mM): 0.125 (———), 0.25 (-●-●-●-), 0.5 (-○-○-○-), 1.0 (-□-□-□-) and 2.0 (-■-■-■-), phosphate buffer 0.05 M, pH 7.0. A_{405} = absorbance et 405 nm (× f) per 1 min at 37 °C, at determined spectrophotometrically (Vitatron) with digital display $\left(\frac{A_t - A_o}{t}\right)$.

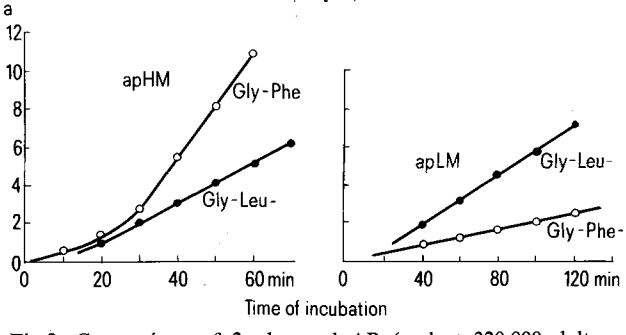

Fig. 2. Comparison of 2 placental AP (mol.wt 320,000 daltons, apHM, and 145,000 daltons, apLM, respectively) separated on Sephadex G200 (0.05 M phosphate + 1 M NaCl, pH 7.8), to split Gly-Phe-p-NA and Gly-Leu-p-NA at pH 7.0 (0.05 M phosphate). a = alteration of absorbance at 405 nm (× f) per 1 min at 37 °C (amount of released p-NA).

gel, with 3 mA current for tube[4]. For the determination of AP activity zone, the gel was squeezed out of the tubes following electrophoresis, and divided into 8 segments 6 mm each. The corresponding segments from more tubes were pooled, homogenized in phosphate buffer pH 7.0 (0.05 M) and the activity was followed in the eluates (substrate Leu-, Ala-, CH_3-Cys-, Gly-Phe- and Gly-Leu-p-NA). The fraction containing higher-molecular AP was divided in 4 proteinic zones with AP activity localized on a single site near the start. In the fraction with lower-molecular AP divided in 7 proteinic zones, the AP activity migrated 18–25 mm in separation gel. In these fractions, free from the NH_2-dipeptidyl hydrolase activity, the course of stepwise hydrolysis Gly-Phe- and Gly-Leu-p-NA was the same as shown in figure 2. Thus we can exclude the possibility that in this effect there participate some peptidases other than those studied.

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Changes in the activities of protein kinase modulators in the cerebellum of mice due to ethanol, caffeine, or phenobarbital administration[1]

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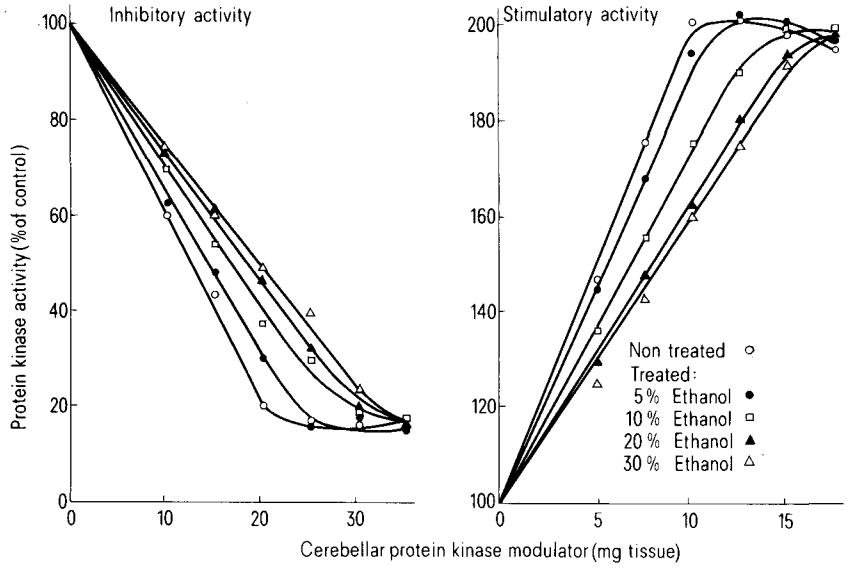
Summary. Decreased activities of both the inhibitory modulator of adenosine 3′:5′-monophosphate (cAMP)-dependent protein kinase (A-PK) as well as the stimulatory modulator of guanosine 3′:5′-monophosphate (cGMP)-dependent protein kinase (G-PK) from the mouse cerebellum were noted due to the administration of excessive doses of ethanol, caffeine, and phenobarbital for up to 28 days. The dose-dependence of the inhibition of A-PK or the stimulation of G-PK was observed as a function of the amount of protein kinase modulators in the cerebellum of mice receiving different doses of ethanol.

Recently, we have reported that the inhibitory modulator of A-PK and the stimulatory modulator of G-PK are separated proteins which exist as a mixture in crude protein kinase modulator preparation obtained from mammalian tissues[2-5]. The inhibitory modulator appears to be the same as the protein inhibitor of Walsh et al.[6]. It was reported that the level of inhibitory modulator in rabbit heart decreased due to starvation and alloxan treatment[7]. Skala et al. showed that inhibitory modulator in brown rat adipose tissue was highest perinatally and that it declined 10 days after birth[8]. Moreover, it was found that in diabetic rats the inhibitory and stimulatory activities of the modulators from the pancreas were higher, whereas those from the fat were lower[9]. We were able to show the suppression of modulators in the liver of mice due to ethanol, caffeine, or phenobarbital administration in recent studies[10], but there still remains to be explored the possible relation between the physiological and toxicological effects of these drugs on the 2 activities of the modulators in the mouse cerebellum.

Materials and methods. 8 groups of young adult male ICR mice (mean b.wt, 19.1±2.0 g) were used. The drinking water contained 5% sucrose and one of the following drugs: ethanol (5%, 10%, 20% and 30%, v/v) caffeine (0.5 mg/ml), phenobarbital (1.0 mg/ml), and vitamin C (1.0 mg/ml) respectively. Each group contained 15 - 25 mice, and the duration of drug administration was 2 or 4 weeks. The drugs were omitted from the drinking water for the control mice. After the animals were killed by decapitation, the cerebellum was removed immediately and homogenized with 5 vol. of ice cold 5 mM potassium phosphate buffer,



The inhibition of A-PK and the stimulation of G-PK as a function of the amount of protein kinase modulators from the cerebellum of mice treated with different doses of ethanol for four weeks. Each point shown represents mean of the values obtained with triplicate samples.