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Enamine Formation of Various Amino Acids with Ethyl Acetoacetate in Aqueous Solution in Relation to Colonic Absorption of Cefmetazole

RIFA CHOH, HIDEKI INAGAKI, TOSHIAKI NISHIHATA,* and AKIRA KAMADA

Faculty of Pharmaceutical Sciences, Osaka University, 1–6 Yamadaoka, Suita, Osaka 565, Japan

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Amino acids such as DL-Ala, Gly and L-Leu form the enamine with ethyl acetoacetate even in aqueous solution, as determined by ultraviolet (UV)-spectrophotometry. Although the observed formation constant, K_{obs}, of the enamine from each amino acid showed a dependency on pH, the values of the intrinsic enamine formation constant, K, were rather similar for all the amino acids and showed no dependence on pH. A solution containing a mixture of ethyl acetoacetate and an amino acid increased the colonic absorption of cefmetazole significantly. The extent of the absorption enhancement depended on the pK_a of the amino group of the amino acid contained in the test solution.

Keywords——enamine; amino acid; ethyl acetoacetate; colonic absorption; cefmetazole

It has been reported[1,2] that enamine derivatives of amino acids such as phenylalanine and phenylglycine with ethyl acetoacetate enhance the rectal absorption of insulin. Since it has been demonstrated that a strong adjuvant action of enamines occurs in spite of rapid hydrolysis[3] in aqueous solution, it is of interest to clarify the behavior of enamines in aqueous solution. Recently, it was shown by ultraviolet (UV)-spectrophotometric and nuclear magnetic resonance (NMR) methods[4] that phenylalanine enamine of ethyl acetoacetate is easily formed even in aqueous solution, and the apparent enamine formation in the aqueous solution is pH-dependency. It has also been reported[5] that the colonic absorption of cefmetazole was significantly enhanced after administration in the form of an aqueous solution containing a mixture of phenylalanine and ethyl acetoacetate. These observations might indicate that the colonic absorption of cefmetazole is enhanced by the phenylalanine enamine of ethyl acetoacetate formed in the administered solution.

In the present paper work, we investigated the enamine formation of other amino acids as well as phenylalanine with ethyl acetoacetate in aqueous solution at various pHs. The effect of solutions of various amino acids and ethyl acetoacetate on rat colonic absorption of cefmetazole was also studied using an *in situ* loop method.

Experimental

Material——Glycine (Gly), DL-alanine (Ala), L-leucine (Leu), L-tryptophan (Try), DL-phenylalanine (Phe), and ethyl acetoacetate were purchased from Wako Pure Chemicals Co., Ltd. (Osaka, Japan). Sodium salts of the amino acids were routinely prepared by the neutralization method. Enamines of Gly, Ala, Leu, Try, and Phe with ethyl acetoacetate were synthesized according to the method described previously.[6] Sodium cefmetazole was supplied by Sankyo Co., Ltd. (Tokyo, Japan). Other reagents used were of analytical grade.

Studies of Enamine Formation and Hydrolysis——The UV-spectrophotometric method described previously[4] was employed for this study. Aqueous solutions containing 0.01 M sodium salt of an amino acid and 0.01 M ethyl acetoacetate at various pH value were prepared in 0.1 M sodium borate buffer and incubated at 37 °C. Aliquots of

sample solution (100 μl) were collected at designated time intervals during the incubation. Each sample solution was diluted more than 100-fold with methyl alcohol solution at below 10 °C and the absorbance was measured at 288 nm (UV-absorbance maxima of the enamines synthesized were around at 288 nm). Methyl alcohol did not affect the formation or hydrolysis of enamines below 10 °C. For the hydrolysis study of enamines, 0.01 M enamine in sodium borate buffer solution was employed. The decrease of UV absorbance at 288 nm was measured. Equilibrium was confirmed to have been reached in both method.

Animals——Wistar male rats, 220 to 240 g, were fasted for 16 h prior to experiments but water was given *ad lib.* During the experiment, rats were anesthetized with sodium pentobarbital (32 mg/kg, *i.p.*) and were placed on a hot plate at 38 °C.

Preparation of Administered Test Solution——An aqueous test solution containing 0.66 M sodium salt of an amino acid and 0.66 M ethyl acetoacetate (adjusted to pH 8.5 with HCl when necessary) was incubated at 37 °C for 2 h prior to administration to a rat. The preparation of drug test solution containing 10 mM sodium cefmetazole was carried out by dissolving the drug in the test solution immediately before the experiment.

***In Situ* Loop Study**——This study was carried out according to the method described in our previous paper.[5] Briefly, the colon was exposed by midline abdominal incision, and a 3 cm loop of colon was prepared by ligation of both ends. The loop was removed 1 h after the administration of 0.5 ml of the test solution/kg, and components remaining in the loop were collected by washing with distilled water; the solution was adjusted to 25 ml with 0.01 N HCl. The absorption of cefmetazole was determined as the difference between the remaining amount and the administered amount in each loop. The colonic tissue weight (wet weight) used in this study was from 220 to 240 mg.

Analytical Method——Cefmetazole in the sample solutions in the absorption study was measured by the high performance liquid chromatographic method described previously.[6]

Results and Discussion

As shown in Fig. 1, the UV absorbance of the test solution containing 0.01 M ethyl acetoacetate and 0.01 M amino acid at 288 nm increased with incubation period at 37 °C, reaching a constant UV absorbance equal to that reached by a solution containing 0.01 M enamine. The observed formation constant, K_{obs}, for each enamine was determined at the constant UV absorbance thus obtained.

Enamine formation was determined by UV-spectrophotometry at 288 nm on the basis of the following findings: enamine synthesized from each amino acid and ethyl acetoacetate showed λ_{max} at 280 to 290 nm in methanol. The UV absorbances of each Ala, Leu, Gly or Phe (ε of each amino acid was less than 2 M^{-1} cm^{-1} at 288 nm) and ethyl acetoacetate ($\varepsilon = 18$ M^{-1} cm^{-1} at 288 nm) can be ignored in comparison to that of each enamine (ε is 2×10^4 M^{-1} cm^{-1} for enamine of Ala, 2.5×10^4 M^{-1} cm^{-1} for that of Leu, 2×10^4 M^{-1} cm^{-1} for that of Gly, and

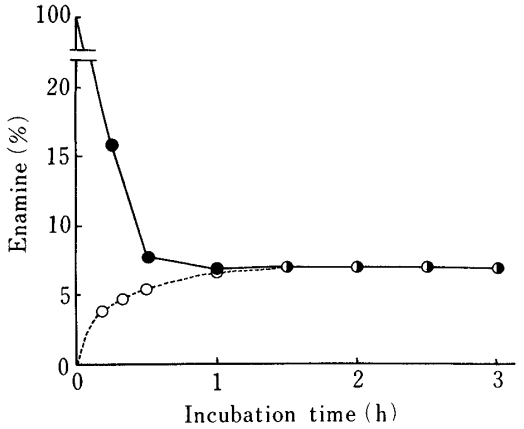

Fig. 1. Formation (○) and Hydrolysis (●) Profiles of Glycine Enamine of Ethyl Acetoacetate in Aqueous Solution as a Function of Time of Incubation at pH 9.0 and at 37 °C

Initial concentration of each component was 0.01 M.

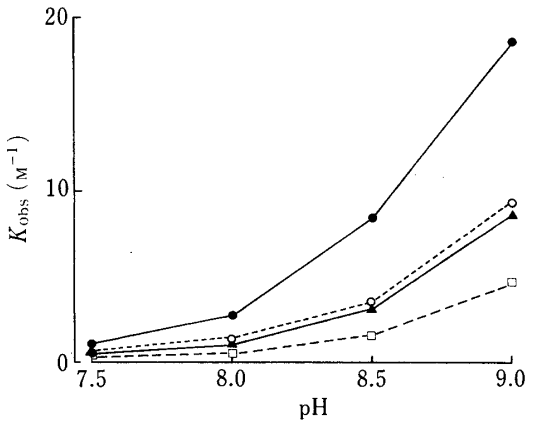

Fig. 2. Observed Formation Constants, K_{obs} of Enamine Formation of Gly (▲), Leu (○), Ala (□), and Phe (●) with Ethyl Acetoacetate at Various pHs and at 37 °C

K_{obs} was determined using 0.01 M amino acid and 0.01 M ethyl acetoacetate, or 0.01 M enamine.

$4 \times 10^4\,M^{-1}\,cm^{-1}$ for that of Phe at 288 nm). However, the UV-absorbance of Try at 288 nm in methanol ($\varepsilon = 4 \times 10^3\,M^{-1}\,cm^{-1}$) is not negligible compared to that of its enamine ($\varepsilon = 3.5 \times 10^4\,M^{-1}\,cm^{-1}$), and thus, we did not attempt to determine the K_{obs} for enamine formation of Try.

As shown in Fig. 2, K_{obs} for enamine formation from each amino acid in aqueous solution increased with increase of the pH of the solution, and enamine formation in the aqueous solution was in the following order: Phe > Gly ≧ Leu > Ala. Since it has been reported[4] that only the amino-unionized form of Phe reacts with ethyl acetoacetate to form the enamine, the intrinsic formation constant, K, of the enamine of each amino acid was determined from K_{obs} by using the following equation as described in our previous report.[4]

$$\log(K/K_{obsd} - 1) = pK_a - pH \qquad (1)$$

The K value calculated from Eq. 1 for the enamine formation of each amino acid are

TABLE I. Intrinsic Formation Constant,[a] K, of Enamine of Various Amino Acids with Ethyl Acetoacetate in Aqueous Solution

Amino acid	K				
	(pH) 7.5	8.0	8.5	9.0	Mean
Gly	43.61	46.30	45.36	45.32	45.15
Leu	40.29	42.84	43.32	42.52	42.24
Ala	42.37	38.81	42.52	39.61	40.83
Phe	54.27	50.36	55.96	51.89	53.12

Initial concentrations of amino acid and ethyl acetoacetate or enamine were 0.01 M and the test solution was incubated at 37 °C. *a*) Intrinsic formation constant was determined by using the following equation (described in the text). $\log(K/K_{obs} - 1) = pK_a - pH$

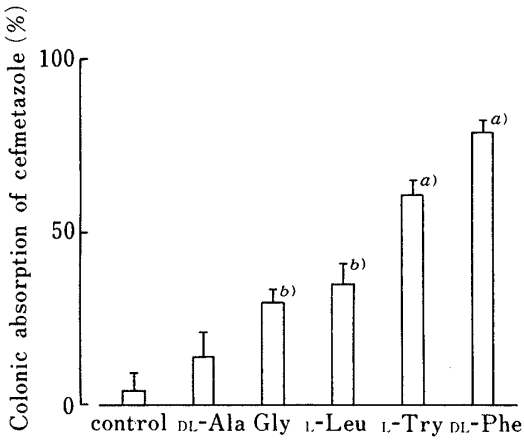

Fig. 3. Rat Colonic Absorption of Cefmetazole in the Presence of a Mixture of 0.66 M Sodium Salt of Amino Acid and 0.66 M Ethyl Acetoacetate in the Administered Solution

Absorption of cefmetazole was determined by measuring the loss of cefmetazole from the loop 1 h after the administration (see in text). The test solution containing 10 mM sodium cefmetazole was administered at a volume of 0.5 ml/kg. Each value represents the mean + S.D. ($n > 4$).

a) $p < 0.001$ *versus* control; *b*) $p < 0.005$ *versus* control.

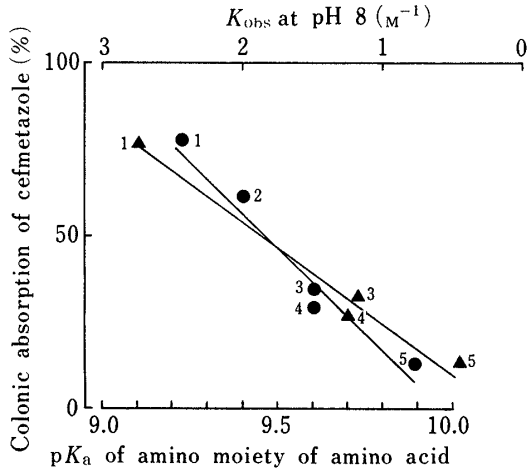

Fig. 4. Relationship of the Extent of Cefmetazole Absorption Enhancement Obtained from Fig. 3 with the pK_a (from the Merck Index) (●) of the Amino Group of the Amino Acid and with the K_{obs} (▲) of Enamine Formation Obtained from Fig. 2

1, Phe; 2, Try; 3, Leu; 4, Gly; 5, Ala.

summarized in Table I.

As can be seen in Table I, the K values of enamine formation were independent of the kind of amino acid used and also of the pH of the solution, suggesting that K_{obs} of each enamine at various pH values is dependent on the fraction of amino acid having an unionized amino group, which is related to the pK_a. Since it was found that various amino acids can form enamines with ethyl acetoacetate even in aqueous solution, we further studied whether each amino acid when administered with ethyl acetoacetate affects the rate colonic absorption of cefmetazole. As shown in Fig. 3, the test solution containing enamine formed in the solution caused increased colonic absorption of cefmetazole, which is normally poorly absorbed. The enhancing action by enamines was in the following order; Phe > Try > Leu ≧ Gly > Ala.

As shown in Fig. 4, it seems clear that the extent of enhancement of colonic absorption of cefmetazole was dependent on the pK_a of the amino group of the amino acid in the test solution, as was K_{obs} of enamine formation of each amino acid with ethyl acetoacetate in the aqueous solution. These findings indicate that the pK_a value of the amino group apparently influences the adjuvant efficacy of test solution containing a mixture of ethyl acetoacetate and various amino acids. Since it is considered that the hydrophobicity of Try and Phe (each containing a phenyl group) influences the affinity of the enamines for colonic tissue, the high affinity of their enamines compared to enamines of other amino acids test may be important in their strong adjuvant action.

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