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Reactivities of Various Amines in the Modifications of Acetic Acid and Aspartic Acid-101 of Lysozyme in the Carbodiimide Reaction

RYOTA KUROKI, NAOE KANESAKA, HIDENORI YAMADA and TAIJI IMOTO*

Faculty of Pharmaceutical Sciences, Kyushu University 62, Maidashi, Higashi-ku, Fukuoka, Fukuoka 812, Japan

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Reactivities of various amines in the modifications of acetic acid and Asp-101 of hen egg-white lysozyme in the carbodiimide (1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC)) reaction were investigated at pH 5.0 and room temperature. The reactivity of an amine towards EDC-activated Asp-101 was at least 50 times higher than that towards EDC-activated acetic acid. Such a high efficiency of modification of Asp-101 could not be explained only by the EDC-binding mechanism in which the EDC molecule binds to the active site cleft of lysozyme close to Asp-101 to activate Asp-101 selectively [R. Kuroki, H. Yamada, and T. Imoto, *J. Biochem.* (Tokyo), **99**, 1493 (1986)]. Therefore, in addition to the above mechanism, an amine-binding mechanism in which an amine molecule binds to lysozyme close to the EDC-activated Asp-101 residue so as to increase the effective concentration of the amine by more than 50 times is proposed.

Keywords——lysozyme; 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride; carboxylic acid amine modification; amine reactivity

Introduction

1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC), a water-soluble carbodiimide, has been widely used for the modification of carboxyl groups of proteins with amine nucleophiles.[1,2] Previously, we have reported the selective modification of Asp-101 in hen egg-white lysozyme with various amine nucleophiles by EDC reaction.[3,4] Asp-101 is located at the edge of the active site cleft of lysozyme and is involved in substrate binding.[5] We have shown that the EDC molecule binds to the active site cleft of lysozyme and activates Asp-101 specifically to lead the selective modification of this residue with an amine nucleophile.[6] We are interested in the alteration of lysozyme function by introduction of a new catalytic function into Asp-101. In order to establish whether any kind of amines can be used for this purpose or not, we examined the reactivities of various amines towards EDC-activated Asp-101 of lysozyme, and compared them with those against EDC-activated acetic acid.

Experimental

Materials——Five-times-recrystallized hen egg-white lysozyme was donated by QP Company (Japan). EDC was purchased from the Protein Research Foundation (Osaka, Japan). *N*-Ethylacetamide, *N*-(2-hydroxyethyl)acetamide, *N,N*-dimethylacetamide, *N,N*-diethylacetamide, ethylamine, 2-hydroxyethylamine, dimethylamine, diethylamine, and bis(2-hydroxyethyl)amine were obtained from Nakarai Chemicals Ltd. (Kyoto, Japan). *N,N*-Bis(2-hydroxyethyl)acetamide was prepared as follows. *N,N*-Bis(2-acetoxyethyl)acetamide (3 g, 13 mmol), synthesized by the method of Mann,[7] was dissolved in 26 ml of 1 N NaOH and stirred overnight at room temperature. The reaction mixture was applied to the column of Dowex 50W-X2 (H^+ form, 2 × 40 cm) and the column was eluted with water. The iodine-positive fraction was concentrated, and dried under vacuum, to give 1.06 g (52.5%) of *N,N*-bis(2-hydroxyethyl)acetamide as a colorless liquid. ^{1}H-NMR (D_2O): 2.27 (3H,s), 3.45—4.03 (8H,m). *Anal.* Calcd for $C_6H_{13}NO_3$: C, 48.97; H, 8.90; N, 9.52. Found: C, 48.22; H, 8.90; N, 9.25.

Simultaneous Reaction of Various Amines with EDC-Activated Acetic Acid under Competitive Conditions——Five kinds of amines (ethylamine, 2-hydroxyethylamine, dimethylamine, diethylamine, and bis(2-hydroxyethyl)amine; the final concentration of each amine is listed in Table I) and acetic acid (final concentration 1 M) were dissolved in water and the pH of the solution was adjusted to 5.0 with HCl. The volume of the solution was adjusted to 10 ml with water, then 30 mg of EDC (0.156 mmol or 15.6 mM) was added. The mixture was stirred at room temperature (22 °C) for 1 d and then the products were analyzed by high-performance liquid chromatography (HPLC) on a reversed-phase column.

Coupling of Lysozyme with Amine by EDC-Reaction——Lysozyme (20 mg; final concentration, 0.14 mM) and an amine (0.1 M) were dissolved in water and the pH of the solution was adjusted to 5.0 with HCl. The volume of the solution was adjusted to 10 ml, then 1.0 mg of EDC (5.2 μmol or 0.52 mM) was added with stirring at room temperature (22 °C). The pH of the solution was maintained at 5.0 for 2 h by addition of dilute HCl. Thereafter, the pH remained constant. After 1 d of stirring, the mixture was dialyzed against distilled water and then analyzed by cation-exchange HPLC.

Analytical Methods——HPLC was accomplished with a Hitachi 655 liquid chromatograph. Quantitative analyses of acetamide derivatives produced in the simultaneous reaction of various amines against EDC-activated acetic acid under competitive conditions were performed on a 0.4 × 30 cm-column of TSK gel ODS-120A (5 μm, Toyo Soda, Japan). An aliquot (20 μl) of the reaction mixture was injected and the column was eluted with a gradient formed from 40 ml of 0.1% concentrated HCl and 40 ml of 20% acetonitrile containing 0.1 % concentrated HCl at a flow rate of 0.8 ml/min. The elution of each acetamide was monitored by measuring the absorbance of the effluent at 210 nm. Yields of acetamide derivatives were determined from the peak areas, which were calibrated with authentic acetamides.

The yields of Asp-101 modified lysozyme with each amine was determined by ion-exchange HPLC on a 0.4 × 30 cm-column of TSK gel CM-2SW (5 μm, Toyo Soda), carboxylic cation-exchanger. Dialysate of the reaction mixture was injected, and the column was eluted with a gradient of 24 ml of 0.05 M phosphate buffer (pH 7) containing 0.1 M NaCl and 24 ml of the same buffer containing 0.76 M NaCl at a flow rate of 0.8 ml/min. The elution of protein was monitored by measuring the absorbance at 280 nm. Asp-101 modified lysozyme was eluted just after the native lysozyme peak in every case, as observed previously.[3] The yields of unreacted and Asp-101 modified lysozymes were calculated from the peak areas assuming that the molar extinction coefficient of lysozyme at 280 nm was not changed by the modification of Asp-101.

Results and Discussion

The reactivities of various amines [ethylamine, 2-hydroxyethylamine, dimethylamine, diethylamine and bis(2-hydroxyethyl)amine] towards two kinds of EDC-activated carboxylic acids, acetic acid and Asp-101 in lysozyme, were investigated at pH 5.0 and room temperature. The acetamide derivatives produced were all separated simultaneously by reversed-phase HPLC as shown in Fig. 1. Therefore, in the case of acetic acid, the reaction was carried out under competitive conditions. That is, acetic acid (1 M) was activated by 15.6 mM EDC and reacted with a mixture of various amines (total concentration of amines, 1 M) for 1 d. The concentration of each amine used is indicated in Table I. The reaction mixture was analyzed by reversed-phase HPLC and the yields of the respective derivatives were determined from the calibration lines of authentic samples. Two independent experiments gave almost the same results. The results are shown in Table I, where the pK_a value of each amine and its calculated relative reactivity are also shown. The yield of each acetamide derivative was extremely low. Under the conditions employed ([acetic acid] = 1 M, [EDC] = 15.6 mM, and pH 5), the direct decomposition of EDC with water could be neglected.[8] Therefore, all of the EDC (15.6 mM) was considered to have been converted to EDC-activated acetic acid. Nevertheless, the total yield of acetamide derivatives was only 23.5 μM, which corresponds to a yield of 0.15% on the basis of the amount of EDC used. These results indicated that the reactivities of amines towards EDC-activated acetic acid were extremely low compared with that of water, and consequently most of the EDC-activated acetic acid (99.85%) was decomposed by water.

As shown in Table I, the relative reactivities of the amines towards EDC-activated acetic acid were widely spread [from diethylamine (1) to dimethylamine (188)]. Difference in structure and /or pK_a of the amines may be responsible for the range of reactivities observed.

TABLE I. Relative Reactivities of Various Amines towards EDC-Activated Acetic Acid under Competitive Conditions at pH 5.0 and Room Temperature[a]

Amine (pK_a)	Concentration (M)	Yield of corresponding acetamide		Relative reactivity
		(μM)	(%)[b]	
Ethylamine (10.63[c])	0.10	3.2	0.020	20
2-Hydroxyethylamine (9.50[c])	0.10	10.8	0.069	69
Dimethylamine (10.64[c])	0.02	5.9	0.038	188
Diethylamine (10.98[c])	0.70	1.1	0.007	1
Bis(2-hydroxyethyl)amine (8.88[d])	0.08	2.5	0.016	20

a) 1 M acetic acid and 15.6 mM EDC. Details are given in the text. *b*) Based on EDC. *c*) H. K. Hall, Jr., *J. Am. Chem. Soc.*, **79**, 5441 (1957). *d*) N. F. Hall and M. R. Sprinkle, *J. Am. Chem. Soc.*, **54**, 3469 (1932).

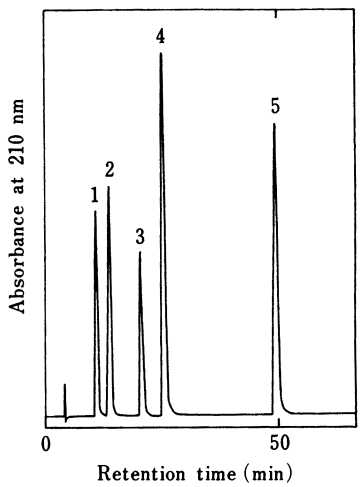

Fig. 1. Reversed-Phase HPLC of Acetamide Derivatives

A mixture of authentic acetamides was injected into a 4 × 300 mm column of TSK gel ODS-120A (5 μm; Toyo Soda, Japan). The column was eluted with a gradient formed from 40 ml of distilled water and 40 ml of 20% acetonitrile containing 0.1% concentrated HCl at a flow rate of 0.8 ml/min. 1, *N*-(2-Hydroxyethyl)acetamide; 2, *N*,*N*-bis(2-hydroxyethyl)-acetamide; 3, *N*-ethylacetamide; 4, *N*,*N*-dimethylacetamide; 5, *N*,*N*-diethylacetamide.

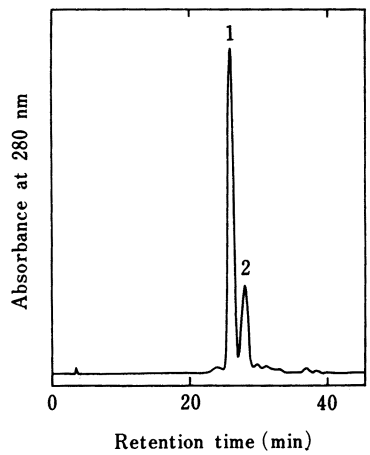

Fig. 2. Ion-Exchange HPLC of the Reaction Mixture of Lysozyme with Ethylamine Catalyzed by EDC on TSK Gel CM-2SW

The column (0.4 × 30 cm) was eluted with a gradient of 24 ml of 0.05 M phosphate buffer containing 0.1 M NaCl and 24 ml of the same buffer containing 0.76 M NaCl at a flow rate of 0.8 ml/min. 1, unreacted lysozyme; 2, Asp-101 modified lysozyme with ethylamine.

In the case of lysozyme, the reaction under competitive conditions could not be utilized because there was no effective method to separate the Asp-101 modified lysozymes from each other simultaneously. However, each Asp-101 modified lysozyme was well separated from unreacted native lysozyme by ion-exchange HPLC. Therefore, the coupling reaction of lysozyme (0.14 mM) with each amine (0.1 M) by EDC (0.52 mM) was carried out separately. The chromatographic pattern obtained from ethylamine is shown in Fig. 2 as a representative example. Asp-101 modified lysozyme, a main product, appeared clearly as a single peak and other by-products were not obvious. For all other amines, the situations were almost the same except for the yields of the respective Asp-101 modified lysozymes. These observations

TABLE II. Yield of Asp-101 Modified Lysozyme and Recovery of Unreacted Lysozyme in the Coupling Reaction of Lysozyme (0.14 mM) with Various Amines (0.1 M) by EDC (0.52 mM) at pH 5.0 and Room Temperature for 1 d

Amine	Recovery of unreacted lysozyme (%)[a]	Yield of Asp-101 modified lysozyme		
		(%)[a]	(%)[b]	Relative yield
Ethylamine	78	20	5.4	7
2-Hydroxyethylamine	62	38	10.2	13
Dimethylamine	61	36	9.7	12
Diethylamine	96	3	0.8	1
Bis(2-hydroxyethyl)amine	95	4	1.1	1.3

a) Based on lysozyme. *b*) Based on EDC.

indicated that among 10 carboxyls[9] in lysozyme, only Asp-101 was selectively modified with an amine in the carbodiimide reaction, as observed previously.[3] The yield of Asp-101 modified lysozyme based on lysozyme used was determined from the relative peak area of Asp-101 modified lysozyme to the total peak area. The results are summarized in Table II. In Table II, the relative yields of Asp-101 modified lysozymes for each amine are also shown. If the relative yields of the Asp-101 modified lysozymes were assumed to be the same as the relative reactivities of the respective amines towards EDC-activated Asp-101, the results indicated that the relative reactivities of amines against EDC-activated Asp-101 were not so widely spread [from diethylamine (1) to 2-hydroxyethylamine (13)] as those against EDC-activated acetic acid.

In order to compare the reactivity of an amine towards EDC-activated Asp-101 with that towards EDC-activated acetic acid, the yields of respective Asp-101 modified lysozymes based on EDC may be more convenient than those based on lysozyme. Therefore, they are also shown in Table II. As shown above, in the case of acetic acid, the total yield of acetamide derivatives was only 0.15% based on EDC at 1 M concentration of total amine. On the other hand, in the case of lysozyme, the yield of Asp-101 modified lysozyme was in the range of 0.8-10.2% based on EDC at 0.1 M concentration of an amine. Since water should compete with an amine in the reaction with EDC-activated carboxylic acid, these results indicated that the amines used in this study reacted with EDC-activated Asp-101 at least 50 times more effectively than with EDC-activated acetic acid. Since the concentration of acetic acid used (1 M) was more than 7000 times higher than that of lysozyme (0.14 mM), this value is considered to be an underestimate.

In order to explain such high reactivity of an amine towards EDC-activated Asp-101, we must consider some mechanism to enhance the effective concentration of an amine in the case of lysozyme. Thus, we propose that an amine molecule could bind to the active site cleft of EDC-activated lysozyme close to the activated Asp-101 residue. This mechanism would increase the effective concentration of amine and also explains the observations that the relative reactivities of amines against EDC-activated Asp-101 (Table II) were considerably different from those against EDC-activated acetic acid (Table 1). Namely, the yields of Asp-101 modified lysozymes would be affected by differences in the binding ability of amines to EDC-activated lysozyme and by differences in the orientation of the amines in the complex.

Previously,[6] we have shown that the EDC molecule binds to the saccharide binding site of the lysozyme molecule close to Asp-101 and selectively activates Asp-101 among 10 carboxyls[9] in lysozyme. Thus, the present results and the results reported previously[3,6] would indicate that the extremely high reactivity of Asp-101 of lysozyme in the modification using EDC and an amine nucleophile is not only due to the specific activation of Asp-101 with EDC

by the EDC-binding mechanism but also due to the effective attack of an amine molecule on EDC-activated Asp-101 through the amine-binding mechanism.

The modification of Asp-101 of lysozyme with amines in the EDC reaction was so effective that we need not use a large excess of EDC over lysozyme in this reaction. This may be a great advantage in the modification of Asp-101 of lysozyme, because many side reactions have been reported on using an excess of EDC.[2] Therefore, this reaction would be very useful when the conversion of lysozyme to some new functional enzyme is attempted by the introduction of an amine with substituent(s) showing a new catalytic ability into Asp-101. As there seems to be a tendency that primary amines are more reactive than secondary amines (Table II), primary amines possessing functional substituents would be more suitable for this purpose.

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