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IMMOBILIZED D-AMINO ACID OXIDASE

MAKOTO NAOI, MISAKO NAOI and KUNIO YAGI

Institute of Biochemistry, Faculty of Medicine, University of Nagoya, Nagoya 466 (Japan)

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Summary

1. D-Amino acid oxidase (D-amino acid : oxygen oxidoreductase (deaminating), EC 1.4.3.3) apoenzyme, holoenzyme and the enzyme · benzoate complex were active and stable when immobilized to aminoalkyl or carboxyalkyl agarose *, or to cyanogen bromide-activated agarose. The immobilized enzyme · benzoate complex could be converted into the holo- and apoenzyme without being liberated from the agarose.

2. The apparent Michaelis constant and substrate specificity of the immobilized enzyme were similar to those of the free enzyme. The optimum pH of the reaction was shifted towards acidic side by 1.0—2.0 pH units from that of the free enzyme.

3. With increasing number of methylene groups of the 'spacer' from 3 to 5, molecular activity of the immobilized enzyme increased, while the apparent Michaelis constant decreased.

Introduction

To demonstrate direct influences of a microenvironmental parameter on enzymatic action, an artificial immobilization of the enzymes to membranes or to polymer particles has been proposed [1,2]. Immobilization of D-amino acid oxidase (D-amino acid : oxygen oxidoreductase (deaminating), EC 1.4.3.3) on solid surface was reported by Tu and McCormick [3], who insolubilized the enzyme by amide-linkage to ω-aminoalkyl agarose. Tosa, Sano and Chibata [4] immobilized the enzyme directly to the cyanogen bromide-activated polysaccharide particles. By using these techniques of immobilization, we studied on the properties of the immobilized holoenzyme and apoenzyme to obtain information on the effect of the changes in the charge and hydrophobicity of the

* Aminoalkyl or carboxyalkyl agarose means agarose activated with CNBr and reacted with an α,ω-diaminoalkane or ω-aminocarboxylic acid. CNBr-agarose means agarose activated with cyanogen bromide.

microenvironments of the protein moiety of this enzyme on its enzymatic activity.

Materials and Methods

D-amino acid oxidase · benzoate complex was prepared according to the method of Yagi et al. [5]. The holoenzyme of D-amino acid oxidase was prepared by replacing benzoate by D-alanine according to the method of Yagi et al. [5], and the apoenzyme was obtained from the holoenzyme according to the method of Massey and Curti [6]. Sepharose 4B was purchased from Pharmacia Fine Chemical Co., Uppsala, α,ω-aminoalkane and ω-aminocarboxyl acid from Nakarai Chemicals Co., Kyoto, and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride from Dojindo Lab., Kumamoto.

Preparation of ω-aminoalkyl or ω-carboxyalkyl agarose

ω-Aminoalkyl or ω-carboxyalkyl agarose was prepared by coupling α,ω-diaminoalkane or ω-aminocarboxyl acid with cyanogen bromide-activated (CNBr-) agarose (Sepharose 4B) according to the method of Axen, Porath and Ernback [7] with a slight modification. Agarose beads (10 ml) were suspended in 1.5 M sodium dibasic phosphate (pH 9.3) and 10 ml of 10% CNBr in dioxane was added. The reaction was carried out at 20° C and pH 11.0 for 12 min. After washed with cold water, 0.1 M sodium bicarbonate (pH 8.5) and 0.2 M sodium borate (pH 10.0), the activated beads were added to 0.1 M sodium borate (pH 10.0), containing 10 mmol of α,ω-diaminoalkane or ω-aminocarboxyl acid. The mixture was stirred at 5° C overnight, then the beads were washed with 0.2 M sodium borate (pH 10.0), water, 0.1 M acetic acid and finally again with water. The bound ligands were measured according to Naoi and Lee [8], and 20—25 μmol of aminoalkyl and 16—20 μmol of carboxyalkyl groups per ml were found to be coupled to the beads.

Immobilization of D-amino acid oxidase

The enzyme · benzoate complex (1.5 μmol) was dialyzed against 0.01 M sodium pyrophosphate (pH 8.3) in the presence of 1 μM benzoate at 5° C overnight, and mixed with the washed aminoalkyl or carboxyalkyl agarose (4 ml). Then 200 mg of solid 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide was added and the pH of the mixture was maintained at 6.5 for 1 h with 1 M acetic acid. The reaction was allowed to continue for 24 h at 5° C. An equal quantity of carbodiimide was added and the reaction was continued for a further 24 h. The beads were washed with 0.1 M sodium pyrophosphate (pH 8.3) containing 1 M NaCl. To prepare the immobilized holoenzyme, the beads obtained above were suspended in 0.1 M sodium pyrophosphate (pH 8.3) and solid D-alanine was added to 0.1 M to replace benzoate [9]. The beads were then washed 3 times with 0.1 M pyrophosphate (pH 8.3). To prepare the immobilized apoenzyme, the above beads were suspended in 1 M KBr at 5° C overnight. The beads were washed 4 times with KBr and 3 times with pyrophosphate. The free holo- and apoenzyme were immobilized to agarose by the same procedure.

The enzyme was also immobilized to CNBr-agarose. The beads (7 ml) were added to 0.1 M sodium pyrophosphate (pH 8.3) containing 0.6 μmol of the

enzyme · benzoate complex, the holoenzyme or the apoenzyme. The mixture was stirred at 5° C overnight, and then the beads were washed 5 times with pyrophosphate. After immobilization, the remaining active sites were blocked by treating the beads with triethylamine.

The bound protein concentration was measured by the biuret method [10] or by the method of Lowry et al. [11] after alkaline hydrolysis [8]. The enzymatic activity was measured as reported previously [5].

Results

Yields of immobilized D-amino acid oxidase

The enzyme was amide-linked to ω-aminoalkyl agarose through carboxyl groups or to ω-carboxyalkyl agarose through ω-amino groups and guanidyl groups of the enzyme, respectively. The yields of the immobilized enzyme to aminohexyl, and to carboxycapryl agarose are shown in Table I. The yield of the immobilization of the holoenzyme and that of enzyme · benzoate complex were almost the same, and that of the apoenzyme was higher than the yields of the other two forms of the enzyme. Aminohexyl agarose was found to immobilize larger amounts of the enzyme than carboxycapryl agarose. In the case of aminoalkyl agarose, the yields increased with increasing number of methylene groups of the spacer as follows: aminopropyl agarose, 8%; aminobutyl, 14%; aminocapryl, 16%; aminohexyl, 38%.

TABLE I

YIELDS AND CATALYTIC PROPERTIES OF IMMOBILIZED ENZYME COUPLED TO AMINOHEXYL, CARBOXYCAPRYL OR CNBr-AGAROSE

Enzyme	Enzyme-binding ligand	Yield * (%)	Molecular activity **	K_m for D-alanine (mM)	K_i for benzoate (μM)
(I) Immobilized enzyme benzoate complex	aminohexyl	38	280 (78)	1.1	
	carboxycapryl	26	170 (46)	1.5	
	CNBr-	98	339 (91)	1.1	
(II) Immobilized holoenzyme prepared from (I)	aminohexyl	37	260 (70)	1.2	2.2
	carboxycapryl	26	170 (46)	1.5	2.5
	CNBr-	98	330 (89)	1.2	2.0
(III) Immobilized apoenzyme prepared from (II)	aminohexyl	37	229 (62)	1.2	
	carboxycapryl	25	127 (34)	2.3	
	CNBr-	97	300 (81)	1.2	
(IV) Immobilized holoenzyme prepared from free holoenzyme	aminohexyl	36	70 (19)	2.3	5.6
	carboxycapryl	29	36 (10)	6.1	7.7
	CNBr-	80	185 (50)	1.2	2.2
(V) Immobilized apoenzyme prepared from free apoenzyme	aminohexyl	54	37 (10)	43	
	carboxycapryl	57	23 (6)	50	
	CNBr-	67	34 (9)	1.4	
(VI) Native enzyme			370 (100)	1.3	3.7

* The yield was expressed as percentage of the amount of the coupled protein to the starting amount of enzyme.

** The reaction was carried out at 20°C. The immobilized or the native enzyme (1—2 μM in final concentration) was added to 0.1 M sodium pyrophosphate (pH 8.3) containing D-alanine of graduated concentrations and FAD (10 μM). Total volume was 3 ml. In parentheses, the ratio of molecular activity of the immobilized enzyme to that of the native enzyme was represented in terms of percentage.

The yields were higher in the immobilization of the enzyme to CNBr-agarose than in its coupling to aminoalkyl or carboxyalkyl agarose.

The immobilized enzyme · benzoate complex was turned into the holoenzyme by the addition of D-alanine. It was noted that upon addition of D-alanine, the yellow beads turned white. However, the purple intermediate, which is commonly observed in the reaction of the free enzyme with neutral amino acids [12], was not observed. The immobilized holoenzyme was further turned into the apoenzyme. The yields of the immobilized enzyme · benzoate complex, holoenzyme and apoenzyme were identical, indicating that the enzyme was not liberated from the agarose during the preparation.

Kinetic studies on immobilized D-amino acid oxidase

The immobilized enzyme-benzoate complex was found to have about 78% of the original activity for aminohexyl agarose and 46% for carboxycapryl agarose (Table I). The apparent Michaelis constant was evaluated with a Lineweaver-Burk plot, assuming that conventional rate equations were applicable to the suspension assays. As shown in Table I, the value for D-alanine obtained with the immobilized enzyme was similar to that with the native enzyme. The immobilized holo- and apoenzyme prepared from the immobilized enzyme · benzoate complex had K_m values for D-alanine similar to that of the native enzyme. The holoenzyme thus obtained had a dissociation constant for benzoate similar to that of the native enzyme. The activities of the immobilized holoenzyme and apoenzyme prepared from the immobilized enzyme · benzoate complex were reduced only slightly during the preparation procedure.

Upon immobilization of the free holo- and apoenzyme to aminohexyl or carboxycapryl agarose, the activity was reduced considerably, and the apparent K_m value for D-alanine increased remarkably in the case of the apoenzyme.

The enzyme immobilized directly to CNBr-agarose was found to have K_m and K_i values similar to those obtained with the native enzyme.

The pH profile of the activity is shown in Fig. 1. The optimum pH for the oxidation of D-alanine was found to be pH 9.0—9.2 for the oxidase bound to aminoalkyl agarose and pH 9.5 for that bound to carboxyalkyl agarose, while the optimum pH for the free enzyme was 11. The enzyme immobilized to CNBr-agarose had the optimum pH of 10.5.

The activity of the immobilized enzyme was restricted to D-forms of amino acids and hydroxy acids (Table II). The relative rates of oxidation of a series of D-amino acids were not much influenced by the nature of the ligands, viz. amino or carboxyl group. However, neutral hydrophobic amino acids such as D-valine, norvaline and leucine were oxidized faster by the enzyme immobilized to aminohexyl or carboxycapryl agarose than by the free enzyme.

Effects of number of methylene groups of the spacer on the enzymatic activity

The oxidation of D-amino acids was affected by the length of the spacer as shown in Table III. The activity of the enzyme immobilized to aminoalkyl agarose increased with increasing number of methylene groups from 3 to 5. The enzyme immobilized to aminocapryl agarose had the highest activity with the substrates examined and that immobilized to CNBr-agarose had a considerably high activity with D-alanine, but a low activity with D-norvaline or D-norleu-

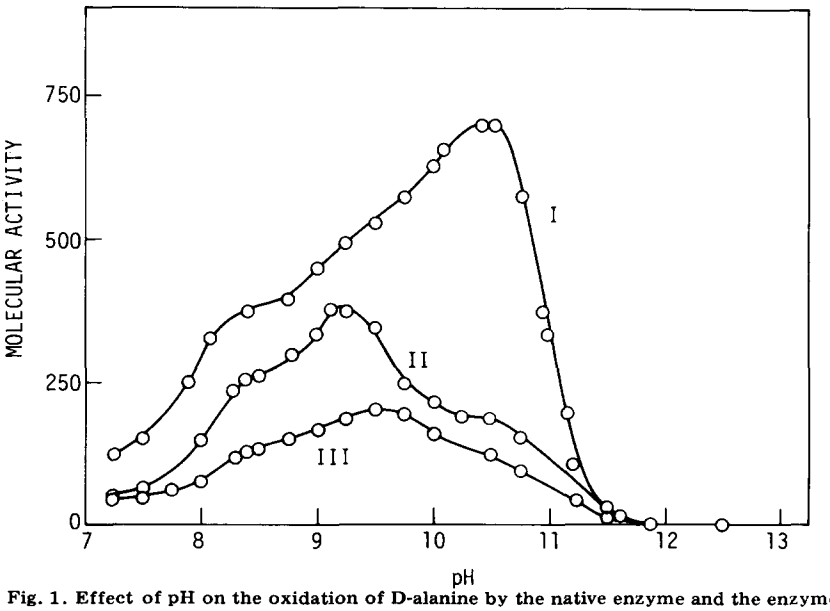

Fig. 1. Effect of pH on the oxidation of D-alanine by the native enzyme and the enzyme bound to aminohexyl or to carboxycapryl agarose. D-alanine (1 M in final concentration) was dissolved in 3 ml of 0.1 M sodium pyrophosphate (pH 7—9) or 0.2 M sodium borate (pH 9—12). The enzyme (1—2 μM in final concentration) and FAD (10 μM) were added to the substrate solution. The reaction was followed by the oxygen uptake, measured with an oxygen electrode at 20°C. Curve I was measured by using the native enzyme, Curve II by using the enzyme immobilized to aminohexyl agarose and Curve III by using that to carboxycapryl agarose.

TABLE II

SUBSTRATE SPECIFICITY OF IMMOBILIZED ENZYME

The reaction was carried out at 20°C. The native or the immobilized enzyme (1—2 μM in final concentration) was added to 0.1 M sodium pyrophosphate (pH 8.3) containing substrate (0.1 M) and FAD (10 μM). Total volume was 3 ml.

Amino acid	Molecular activity		
	Native enzyme	Enzyme bound to	
		Aminohexyl agarose	Carboxycapryl agarose
D-Alanine	370 (100)	280 (100)	170 (100)
D-Valine	233 (63)	293 (105)	146 (86)
D-Norvaline	60 (16)	185 (66)	156 (92)
D-Leucine	83 (22)	153 (55)	115 (68)
D-Isoleucine	330 (89)	248 (89)	226 (133)
D-Norleucine	210 (57)	313 (112)	250 (147)
D-Proline	1532 (414)	751 (268)	429 (252)
D-Phenylalanine	664 (180)	401 (143)	272 (160)
D-Tryptophan	150 (41)	387 (138)	210 (124)
D-Methionine	578 (156)	420 (150)	344 (202)
Glycine	21 (6)	25 (9)	24 (14)
D-Serine	315 (85)	258 (92)	220 (129)
D-Threonine	59 (16)	76 (27)	76 (45)
D-Cysteine	447 (121)	342 (122)	292 (172)
D-Cystine	5 (1)	23 (8)	9 (5)
D-Tyrosine	816 (221)	660 (236)	320 (188)
D-Lysine	62 (17)	47 (17)	31 (18)
D-Arginine	184 (50)	210 (75)	142 (84)
D-Histidine	433 (117)	325 (116)	310 (182)
D-Aspartic acid	15 (4)	11 (4)	5 (3)
D-Glutamic acid	21 (6)	0 (0)	0 (0)
D-Lactic acid	3.5 (1)	3 (1)	2 (1)
L-Alanine	0	0	0
L-Methionine	0	0	0
L-Lactic acid	0	0	0

TABLE III

EFFECTS OF CHAIN LENGTH OF SPACER ON THE OXIDATION OF AMINO ACIDS

The reaction conditions were the same as those of Table II, except that the substrates of graduated concentrations were used.

Enzyme bound to	Substrate					
	D-Alanine		D-Norvaline		D-Norleucine	
	V	K_m (mM)	V	K_m (mM)	V	K_m (mM)
Aminopropyl agarose	100	7.1	120	4.6	209	2.2
Aminobutyl agarose	125	1.2	167	3.7	278	1.7
Aminocapryl agarose	350	0.7	240	1.5	417	0.7
Aminohexyl agarose	280	1.1	185	0.7	313	0.4
CNBr-agarose	339	1.1	30	0.9	80	0.2
Native enzyme	370	1.3	60	0.9	210	0.4

cine. The length of the spacer also affected the K_m value. The K_m value decreased with the increase in the length of the spacer from 3 to 5.

Stability of the immobilized enzyme

The immobilized holoenzyme and apoenzyme prepared from the immobilized enzyme · benzoate complex were far more stable than those of the free forms (Table IV). The difference in stability is most obvious in the case of the apoenzyme.

The increased stability of the immobilized enzyme was also demonstrated by the effect of urea on the enzymatic activity. The denaturation of the enzyme immobilized to aminohexyl agarose and the soluble enzyme by urea is shown in Table V. In both cases of the immobilized holoenzyme and enzyme · benzoate complex, most of the specific activity remained for 3 h, while in the soluble enzyme it decreased with incubation time.

TABLE IV

STABILITY OF FREE AND IMMOBILIZED ENZYME

The immobilized holoenzyme and apoenzyme were prepared from the enzyme · benzoate complex immobilized to amino hexyl agarose. The enzymes were stored at 5°C for various days indicated. The reaction conditions were the same as those of Table II, except that D-alanine was used as substrate. The enzymatic activity is expressed in terms of percentage of the initial time.

Days of storage	Enzymatic activity				
	Free		Immobilized		
	Holoenzyme	Apoenzyme	Enzyme · benzoate complex	Holoenzyme	Apoenzyme
0	100	100	100	100	100
7	80	53	100	98	100
14	65	43	95	83	95
21	60	40	76	72	81

TABLE V

TIME COURSE OF DENATURATION OF FREE AND IMMOBILIZED ENZYME

The immobilized holoenzyme was prepared from the enzyme · benzoate complex immobilized to aminohexyl agarose. The reaction conditions were the same as those of Table II, except that D-alanine was used as substrate and before measurement of the enzymatic activity, the enzymes were incubated in 0.1 M sodium pyrophosphate (pH 8.3) containing 2 M urea at room temperature for various times indicated. The enzymatic activity was expressed in terms of percentage to that of the initial time.

Incubation time (h)	Enzymatic activity			
	Free		Immobilized	
	Enzyme · benzoate complex	Holoenzyme	Enzyme · benzoate complex	Holoenzyme
0	100	100	100	100
0.5	100	92	100	100
1	100	70	99	96
1.5	80	65	99	90
2	70	63	98	85
2.5	60	50	98	83
3	60	49	98	83

Discussion

Upon immobilization, the holoenzyme and the apoenzyme reduced their enzymatic activity, while the enzyme · benzoate complex maintained considerable activity, and the immobilized holoenzyme and apoenzyme prepared from the enzyme · benzoate complex retained a similar activity. This indicates that the substrate binding site and the enzyme conformation are protected by the binding of benzoate [13]; in other words, the disorder of the enzyme conformation or the blocking of the amino acid residues necessary for the substrate binding occurs in the cases of the holoenzyme and the apoenzyme.

It is noted that the property of the catalytic activity changed upon immobilization. Remarkable changes were observed on the optimum pH, the apparent Michaelis constant and the maximum velocity. In addition, the purple intermediate was not observed with the immobilized enzyme when reacted with D-alanine. These results indicate that the change in microenvironments of the enzyme including that of the binding sites for the substrate occurs.

The fact that the immobilized enzyme was more stable than the free enzyme, especially in the case of the apoenzyme, indicates that the enzyme conformation is stabilized by immobilization.

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