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Effects of Carbodiimides as Coupling Agents on the Stability of Immobilized Aldolases

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ABSTRACT

Pig muscle aldolase was covalently attached to a polyacrylamide support possessing carboxylic functional groups activated by various water-soluble disubstituted carbodiimides. The stability and the degree of microheterogeneity of the immobilized aldolases were shown to depend on the structure of the carbodiimide used as coupling agent.

Index Entries: Stability of immobilized enzymes; aldolase, immobilized, pig muscle aldolase; water-soluble carbodiimides; carbodiimides in enzyme immobilization.

INTRODUCTION

Immobilization can improve the conformational stability of enzymes, which is one of the most important properties for practical application (*1*). The stability of the immobilized enzymes depends in part on the degree of microheterogeneity, which could arise from the multipoint attachment (*2*), the binding site heterogeneity (*3*), and the effect of the

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microenvironment. In this sense, microheterogeneity means the presence of a large number of closely related molecular subforms. The thermal inactivation model proposed by Kawamura et al. (4) took into account subunits deactivating at different rates. Agarwal (5) generalized the previous approach and described a denaturation model for the heterogeneous enzyme subpopulation possessing nonuniform initial activities and nonzero final activities. The statistical model presented by Malhotra and Sadana (6) includes the influence of microheterogeneity on enzyme inactivation and stability, and assumes the continuous distribution of properties in the population. Deactivation kinetics is described as a model of a heterogeneous population having a continuous distribution of the activation energy of deactivation, characterized by the mean activation energy (E_o) and the standard deviation of the activation energy (σ). In the case $\sigma = 0$, the population is homogeneous and the thermal inactivation exhibits first-order kinetics. The standard deviation indicates the degree of heterogeneity. Henley and Sadana (7) developed a graphical method for determination of these parameters from the experimental data. Using their approach, we have analyzed the complex process of thermal inactivation of an immobilized triosephosphate isomerase (8).

Carbodiimides are excellent reagents for the activation of carboxylic groups of supports under mild conditions (9–11). In a previous work, over 30 disubstituted carbodiimides were synthetized (12) and screened for enzyme immobilization (13). It was found that the degree of immobilization depended strongly on the structure of the carbodiimide used. In the present article, we demonstrate that the structure of the carbodiimide can influence the thermal stability and the microheterogeneity of the immobilized enzymes.

MATERIALS AND METHODS

Aldolase with a specific activity of 6.9 U/mg protein was isolated from pig skeletal muscle (14). One unit of enzyme activity was defined as the amount of enzyme that catalyses the splitting of one micromole of D-fructose 1,6-diphosphate/min at pH 7.5 and 25°C. The preparation was free from isoenzymes as assayed by PAGE (15,16). Carbodiimides were synthetized according to Jászay et al. (12). Akrilex C-100, a polyacrylamide bead (100–320 μm) polymer possessing carboxylic functional groups (4 meg/q dry wt), was a commercial product of Reanal Factory of Laboratory Chemicals (Budapest, Hungary). All other chemicals were reagent grade commercial preparations (Reanal).

Measurement of Protein

Protein determinations were performed according to the method of Lowry and coworkers (17). The amount of immobilized protein was cal-

culated as the difference between the amount of protein introduced into the reaction mixture and the amount of protein present in the filtrate and washing solutions after immobilization.

Assay of Aldolase Activity

The aldolase activity was determined in a coupled-enzyme reaction, using glyceraldehyde-3-phosphate dehydrogenase. The reaction mixture (3 mL) contained 0.8 m*M* D-fructose 1,6-diphosphate, 1.7 m*M* NAD^+, 0.8 m*M* Na_2HAsO_4 and 33 U of glyceraldehyde-3-phosphate dehydrogenase in 0.1*M* Tris/HCl buffer (pH 7.5). The reaction was started with the aldolase sample and NADH formation was registered at 340 nm. The temperature was 25°C.

In the case of the immobilized aldolases, 0.14 mg was suspended in the reaction mixture (3 mL). The suspension was stirred for an appropriate period of time (1–5 min) at 25°C. The immobilized enzyme was then filtered off quickly (a few seconds) and the absorbance of the filtrate was measured at 340 nm. The activity of the immobilized aldolase was calculated from the difference between the absorbance of the starting reaction mixture and that of the filtrate after the reaction.

Immobilization of Aldolase

The aldolase was covalently attached to a polyacrylamide bead polymer possessing carboxylic functional groups activated by water-soluble carbodiimides as previously described (*18*).

The general method of the immobilization was as follows: 1 g of Akrilex C xerogel was suspended and swollen in 50 mL of 0.1*M* potassium phosphate buffer (pH 7.0), and water-soluble carbodiimide in a stoichiometric quantity relative to the carboxylic functional groups located on the support, dissolved in 25 mL of cold (0°C) buffer, was added under continuous stirring and cooling in an ice bath. After 10 min, 25 mL of enzyme solution containing 0.48 g of protein was added and the pH was adjusted to 7.0. The mixture was incubated at 0–4°C during 48 h using two 6-h periods of agitation. The gel was filtered by suction and successively washed three times with 100 mL of buffer, three times with 100 mL of buffer containing 1*M* sodium chloride, again three times with 100 mL of buffer to remove unbound proteins, and finally, with a large volume of distilled water to remove the buffer ions. The gel was stored at 10°C until use.

Stability Tests on Immobilized Aldolases

The heat treatments were carried out in 0.05*M* Tris/HCl buffer (pH 7.5), with 1 mL mixtures at 50°C. The concentration of immobilized enzyme in the mixtures was 0.14 mg solid/mL. After appropriate periods of

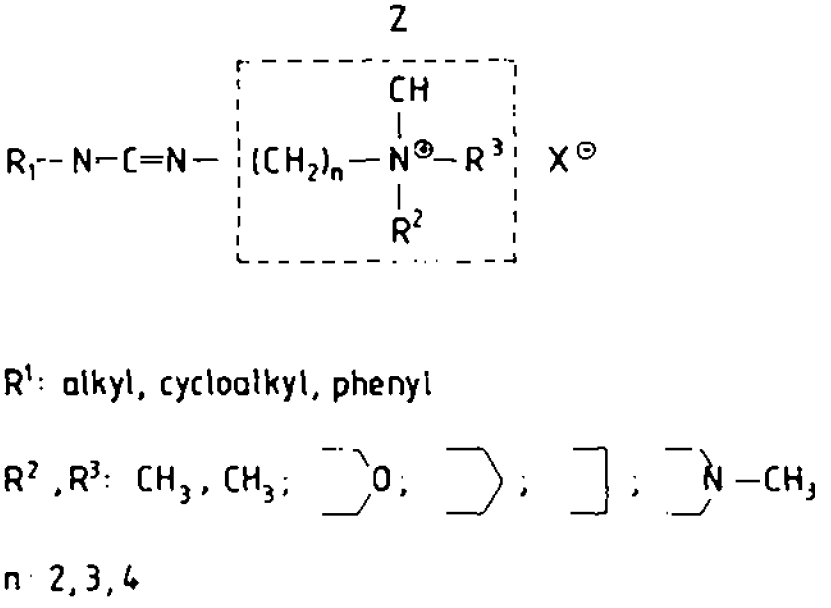


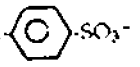
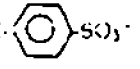
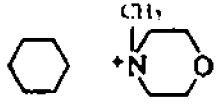
Fig. 1. General structure of carbodiimides.

incubation, the samples were rapidly cooled in an ice bath and the residual activities were assayed at 25°C.

RESULTS AND DISCUSSION

Pig muscle aldolase was covalently attached to a polyacrylamide support possessing carboxylic functional groups activated by water-soluble disubstituted carbodiimides. In accordance with earlier experience (*13*), the degree of immobilization depended strongly on the structure of the carbodiimide used (Fig. 1 and Table 1). The thermal stabilities of the immobilized aldolases produced by using different carbodiimides were compared. It was found that the stabilities of the immobilized aldolases were affected by the structure of the carbodiimide used as coupling agent for the immobilization (Fig. 2). For the preparation of the most stable immobilized aldolase, the carbodiimide was applied in which R^1 and R^2 = methyl, $n = 3$, and X = iodide. It appears that if substituent R^1 is larger, the stability of the immobilized enzyme is lower. This effect could be modified by the other substituents or the counterion. For the explanation of the phenomena, we presume that the structure of the O-acylisourea intermediate could sterically direct the attachment of protein molecules on the surface of support. The thermal stability profiles showed different extents of deviation from the exponential. This observation suggested the microheterogeneity of the immobilized aldolases. Since the soluble aldolase preparation was free from isoenzymes, the possibility of the presence of

Table 1
Effect of Carbodiimide Structure on the Immobilization of Aldolase

Symbols	Carbodiimide structure[a] R_1	R^2	R^3	n	X	Activity on dry wt basis (units g^{-1} solid)	Activity on protein basis (units g^{-1} protein)	(%)[b]	Activity bound (%)[c]	Activity loss (%)[b]	Protein bound (mg g^{-1} solid)
MAPC	CH_3	CH_3	CH_3	3	I^-	99.2	371.0	5.4	4.6	61.8	267.4
PAPC	CH_3-$(CH_2)_2$	CH_3	CH_3	2	H_3C-  -SO_3^-	108.2	872.4	12.7	5.0	64.9	123.6
BAPC	CH_3-$(CH_2)_3$	CH_3	CH_3	3	I^-	88.0	784.3	11.4	4.1	26.9	112.2
BAPCPB	CH_3-$(CH_2)_3$	CH_3	 CH_2	3	Br^-	84.8	834.6	12.1	3.9	50.2	101.6
CAEC		CH_3	CH_3	2	H_3C-  -SO_3^-	105.8	595.3	8.7	4.9	83.4	179.4
CAPC		CH_3	CH_3	3	H_3C-  -SO_3^-	83.2	696.6	10.1	3.6	78.3	119.9
CMPC				3	I^-	58.5	1198.8	17.4	2.7	82.6	48.8

[a] General formula of carbodiimides. R_1-N=C=N-$(CH_2)_n$-$\overset{CH_3}{\overset{|}{N^+}}$-$R^3$ X$^-$ (R^2)

[b] The activity of the soluble enzyme was taken as 100%.
[c] The total activity introduced into the coupling reaction mixture was taken as 100%.

Table 2
Effect of the Structure of the Carbodiimide Used for the Coupling on the Microheterogeneity of the Immobilized Aldolases

Notations of carbodiimudes	E_o (kJ/mol)	σ (kJ/mol)	k ($*10^{-5}$ s^{-1})	SQ
MAPC	115.9	12.8	0.05	0.0449
PAPC	103.5	1.4	12.29	0.0436
BAPC	103.6	4.5	11.84	0.0540
BAPCPB	106.5	3.5	4.02	0.0252
CAEC	104.7	2.5	7.86	0.0563
CAPC	103.5	5.5	11.84	0.0460
CMPC	102.7	1.3	16.56	0.0386

E_o, mean activation energy for deactivation;
σ, standard deviation of activation energy,
k, deactivation rate constant

enzyme molecules with different affinities toward the activated groups of the support could be excluded. Therefore, it was presumed that the carbodiimide structure could influence the degree of microheterogeneity too. The approach previously described (6-8) was used to analyze the experimental data. The results are summarized in Table 2. The deactivation kinetics was described as a model of a heterogeneous population with a continuous distribution of the activation energy for deactivation. The distribution was characterized by the mean activation energy (E_o) and the standard deviation of the activation energy (σ). In the case of $\sigma = 0$, the population is homogeneous and the thermal inactivation exhibits first-order kinetics. The value of the standard deviation indicates the degree of heterogeneity. Similarly, as for the thermal stability, the size of the substituents in the carbodiimide structure seems to be the most important factor concerning the microheterogeneity of immobilized aldolases. Small substituents could make the activated carboxyl groups of the support available for the enzyme molecules. Multipoint attachment might lead to an enhanced stability and a higher degree of conformational heterogeneity. It is proposed that this finding should be taken into account in the selection of the most appropriate carbodiimide for activation of the support.

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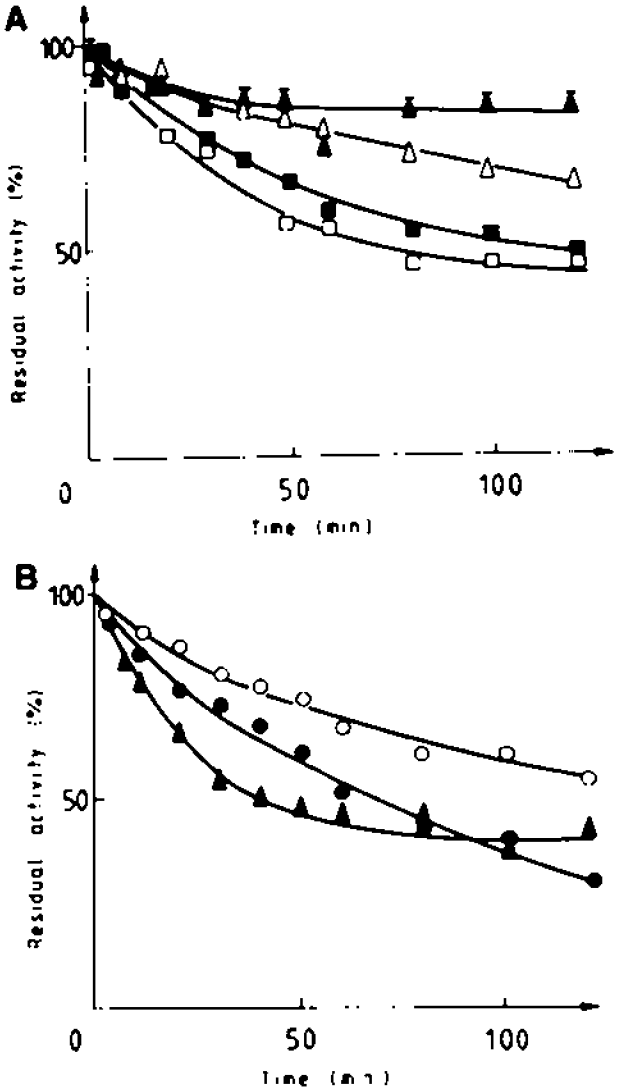

Fig. 2. Effect of the structure of the carbodiimide used for the coupling on the thermal stability of immobilized aldolases. Experiments were performed in 0.05*M* Tris/HCl buffer (pH 7.5) at 50°C. Enzyme concentration used: 0.14 mg solid/mL. Symbols: ▲, MAPC; ■, PAPC; □, BAPC; △, BAPCPB; ○, CAEC; ▲, CAPC; ●, CMPC. The starting activity was taken as 100%.

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