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A STUDY OF THE ROLE OF HISTIDINE SIDE-CHAINS AT THE ACTIVE CENTRE OF AMYLOLYTIC ENZYMES*

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ABSTRACT

The role of histidine side-chains in reactions catalysed by porcine-pancreatic alpha-amylase, sweet-potato beta-amylase, and *Aspergillus niger* glucamylase has been studied by using diethyl pyrocarbonate, a specific protein reagent. Changes in the activity, binding affinity, and apparent kinetic parameters due to ethoxycarbonylation have been determined. For pancreas alpha-amylase, four of the eight histidine groups, for sweet-potato beta-amylase, six of the seven histidine groups, and for *A. niger* glucamylase, four of the six histidine groups were shown to be ethoxycarbonylated. Ethoxycarbonylation occurred as an apparent first-order reaction, with rate constants in the range 3.6–4.9 $\times 10^{-2}$min^{-1}. Ethoxycarbonylation of the histidine group at the active centre rapidly inactivated alpha-amylase, whereas the other three groups are not located in the active centre, although activity and substrate binding are only slightly affected by their modification. For beta-amylase and glucamylase, only slight or no change in activity could be detected on ethoxycarbonylation, whereas significant changes were observed in the binding affinity.

INTRODUCTION

The mode of action of amylolytic enzymes has been studied for many years, from physiological and industrial aspects, because of their effect on starch. The dimensions of the active centre of amylolytic enzymes and the number and location of substrate-binding sites have been investigated[1–5].

The function of the amino acid side-chains in the active centre of the enzyme can be studied by the application of specific protein reagents. In order to establish unambiguously whether the subsites present in the active centre play a role in the binding of the substrate and in the formation of products, or if they affect only the stability of the tertiary structure of the protein, the change in k_{-1}/k_{+1} occurring upon modification must be known. The change in binding affinity ($\Delta k_{-1}/k_{+1}$) due

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to modification can be determined by a relatively simple spectrophotometric method using cyclohexa-amylose bound to Sepharose 6B as a substrate analogue[6].

The role of histidine groups in amylolytic enzymes has been variously studied. It was concluded from p*K* data, mainly by reaction-kinetic methods (pH dependence of V_{max} and K_m), that the imidazole group of a histidine residue is catalytic for alpha-[7-9] and beta-amylases[10,11] of different origins. Similar conclusions were drawn from the results of photo-oxidative modification[7,10-14].

These results must be accepted with reservations, since the graphical determination of ionisation constants is rather inaccurate and the effect of charged neighbouring-groups cannot be considered unambiguously[15]. Also, the photo-oxidative method is not selective, since, in addition to the histidine residues, tryptophan, tyrosine, cysteine, and methionine residues are oxidised. Thus, inactivation cannot be ascribed unequivocally to the change in the imidazole group[13,16].

Ethoxycarbonylation by diethyl pyrocarbonate[17] (DEP) occurs at the imidazole group of histidine at pH 6.0, and the resulting *N*-ethoxycarbonylhistidine can be determined by spectrophotometry at 240 nm. The selectivity of the reagent can be verified by cleavage of ethoxycarbonyl groups by hydroxylamine[18].

Four of the eight histidine groups in pancreas alpha-amylase react[19] with DEP, with no observable change in the conformation of the protein. The enzyme is practically inactivated by the reaction of two of the histidine groups.

We now report on the reaction of DEP with various amylases, in relation to changes in enzyme activity and binding affinity.

MATERIALS AND METHODS

Crystalline enzymes were used and their content of inactive protein was determined by affinity chromatography[6].

Porcine-pancreatic alpha-amylase [EC 3.2.1.1, (1→4)-α-D-glucan 4-glucanohydrolase] from Reanal (Hungary) was further purified[20] by affinity chromatography (1 g of wet Sephadex 6B–cyclohexa-amylose (CHA) gel bound to 92 mg of alpha-amylase, which was eluted with M urea) and isolated by dialysis at pH 7.0 against mM $CaCl_2$. The activity[21] was 790 enzyme units/mg. The protein content was determined by spectrophotometry; a 1% solution had[22] E_{280}^{1cm} 24.0. The content of enzymically active protein was determined by affinity chromatography. A solution of enzyme (1–2 mg) was added to a column (0.5 × 3 cm) of Sepharose 6B–cyclohexa-amylose. Non-binding protein was eluted with the buffer used for the dissolution of enzyme, and enzyme was eluted with aqueous 1% CHA. The amount of active and inactive protein in each eluate was determined by u.v. spectrophotometry[20], and the content of active protein was 95.8%.

Sweet-potato, crystalline beta-amylase [EC 3.2.1.2, (1→4)-α-D-glucan maltohydrolase, obtained from Sigma] had an activity of 414 units/mg of protein[21] and a content of active protein of 67.8% (a 1% aqueous solution had[23] E_{280}^{1cm} 17.1).

A. niger glucamylase [EC 3.2.1.2, (1→4)-α-D-glucan glucohydrolase], prepared[24]

from Miles L100 enzyme, had an activity of 5.6 units/mg and a content of active protein of 40.6% (a 1% aqueous solution had[20] E_{280}^{1cm} 20).

An AGU is the amount of enzyme with which 1 g of D-glucose can be produced in 1 h at 60° and pH 4.2 from 50 ml of 4% aqueous soluble-starch[25].

Modification of the histidine groups of enzymes. — Modification was carried out[17] in 0.01M phosphate buffer (pH 6.0) in a pH-stat at 0°. Freshly prepared solution [20% (v/v) in ethanol] of DEP (6–10 molar excess) was added to an aqueous solution (2–3 mg/ml) of the enzyme. Aliquots were taken at intervals, the pH was adjusted (see Tables), and, after dilution, excess of reagent was removed by using a Technicon rapid dialyser. The degree of modification was determined from the change in absorbance at 240 nm (s 3200)[17].

The effect of ethoxycarbonylation was checked by treatment with 0.2M hydroxylamine at pH 7.0 and 20° for 1 h. For each enzyme, 93–95% of the original activity was regenerated and the absorbance at 240 nm due to ethoxycarbonylation disappeared.

The degree of inactivation of a modified enzyme was determined by using a mixture of properly diluted enzyme (1 ml), 1% soluble starch (Merck) in buffer [1 ml; 0.01M phosphate (pH 7) containing 0.1mM $CaCl_2$ for alpha-amylase, 0.01M acetate (pH 4.8) for beta-amylase, and 0.01M acetate (pH 4.2) for glucamylase]. The mixture was incubated at 20° for 3 min, and the content of reducing sugar was determined by photometry at 540 nm[21].

Determination of the change in binding affinity. — The change in k_{-1}/k_{+1} due to modification was determined by affinity chromatography[6] using cyclohexaamylose bound to Sepharose 6B. The affinity gel (10–50 mg, Y) was added to an enzyme solution (2 ml) of known absorbance (E_0), and the mixture was stirred at 17° until equilibrium was attained (45 min). The mixture was stored for 15 min and, after sedimentation of the gel grains, the absorbance (E_m) was measured. The amount of enzyme bound to the gel can then be obtained by using the expression $100 \times (E_0 - E_m)/E_0XY$, where X is the active ratio of the enzyme (enzymic protein content divided by the total protein content: for alpha-amylase, 0.958; beta-amylase, 0.678; and glucamylase, 0.406).

Knowing the affinity of the original enzyme, the change of binding due to modification can be determined.

Determination of reaction kinetic constants. — For the determination of K_m, V_{max}, rate constant k_2, and classical Michaelis–Menten kinetics, the conventional reaction-kinetic method was performed at 25° with 0.01–0.1% solutions of soluble starch as the substrate, and enzyme concentrations and reaction periods that kept the degree of hydrolysis to <5%.

Measurements were performed[20] with a Technicon autoanalyser. For constant reaction periods (4.3 min), the substrate concentration was periodically changed in the constant enzyme flow. The reaction was stopped by the introduction of 0.3M NaOH, and reducing sugar was determined by the ferricyanide method (420 nm).

First-order rate constants and V_0 values were calculated from the yield of product. From the data derived, K_m and V_m were determined graphically by the

Lineweaver–Burk method. Rate constants k_2 were calculated from the quotient V_{max}/E_t.

RESULTS

For the three amylolytic enzymes studied, changes in activity, binding affinity, and degree of modification due to ethoxycarbonylation are summarised in Tables I–III.

Under the chosen experimental conditions, only the exposed histidine groups could be modified by the DEP method[17].

TABLE I

CHANGES IN THE ACTIVITY, BINDING AFFINITY, AND NUMBER OF MODIFIED HISTIDINE GROUPS DURING ETHOXYCARBONYLATION OF PORCINE-PANCREATIC ALPHA-AMYLASE[a]

Time of modification (min)	*Number of modified histidine groups*	*Remaining activity (%)*	*Remaining binding-affinity (%)*
0	0	100	100
2	0.3	89.1	[b]
5	0.6	70.0	99.0
10	1.2	58.2	96.1
15	2.0	49.5	89.3
20	2.1	37.2	[b]
30	2.6	29.2	83.0
40	3.1	22.8	[b]
50	3.3	18.0	[b]
70	3.7	12.6	68.2
90	3.8	8.8	[b]

[a]Reaction conditions: enzyme concentration, 48 μM; 0.01M phosphate buffer (pH 6.0) and 0.1mM $CaCl_2$; 54 mol of DEP per mol of enzyme; 0°. After sampling, the pH value of the enzyme solution was adjusted to 7.0. [b]Not determined.

TABLE II

CHANGES IN THE ACTIVITY, BINDING AFFINITY, AND NUMBER OF MODIFIED HISTIDINE GROUPS DURING ETHOXYCARBONYLATION OF SWEET-POTATO BETA-AMYLASE[a]

Time of modification (min)	*Number of modified histidine groups*	*Remaining activity (%)*	*Remaining binding-affinity (%)*
0	0	100	100
5	1.5	97.8	[b]
10	2.2	95.5	74.2
15	3.3	92.1	[b]
20	3.9	90.3	48.5
30	4.6	86.2	39.4
40	5.2	82.1	[b]
50	5.5	79.6	18.2
60	5.9	74.5	[b]

[a]Reaction conditions: enzyme concentration, 46 μM; 0.01M phosphate buffer (pH 6.0); 42 mol of DEP per mol of enzyme; 0°. After sampling, the pH value of the enzyme solution was adjusted to 4.8. [b]Not determined.

TABLE III

CHANGES IN THE ACTIVITY, BINDING AFFINITY, AND NUMBER OF MODIFIED HISTIDINE GROUPS DURING ETHOXYCARBONYLATION OF *A. niger* GLUCAMYLASE[a]

Time of modification (min)	*Number of modified histidine groups*	*Remaining activity (%)*	*Remaining binding-affinity (%)*
0	0	100	100
5	0.8	100	[b]
10	1.2	99	92.3
20	2.1	100	79.1
30	2.6	100	[b]
40	3.0	99	[b]
50	3.4	100	75.9
60	3.6	99	[b]
70	3.7	98	[b]
80	3.8	99	62.3

[a]Reaction conditions: enzyme concentration, 31 μM; 0.01M phosphate buffer (pH 6.0); 60 mol of DEP per mol of enzyme; 0°. After sampling, the pH value of the enzyme solution was adjusted to 4.2. [b]Not determined.

TABLE IV

CHANGES IN THE ACTIVITY, BINDING AFFINITY, AND NUMBER OF MODIFIED HISTIDINE GROUPS DURING ETHOXYCARBONYLATION OF THE EXPOSED HISTIDINE SIDE-CHAINS OF PORCINE-PANCREATIC ALPHA-AMYLASE[a]

Time of modification (min)	*Number of modified histidine groups*	$K_m \times 10^3$ (%)	k_2 (min^{-1})
0	0	4.44	925
5	0.6	4.28	438
10	1.2	4.75	367
20	2.1	4.30	288
30	2.6	4.55	164
70	3.7	4.79	49

[a]Reaction conditions: actual enzyme concentration, 13.4 nM; 0.01M phosphate buffer (pH 7.0) and 0.1mM $CaCl_2$; substrate concentration: 0.01, 0.015, 0.02, 0.05, and 0.1% of soluble starch; reaction time, 4.3 min at 25°.

TABLE V

CHANGES IN REACTION-KINETIC PARAMETERS DURING ETHOXYCARBONYLATION OF THE EXPOSED HISTIDINE SIDE-CHAINS OF SWEET-POTATO BETA-AMYLASE[a]

Time of modification (min)	*Number of modified histidine groups*	$K_m \times 10^2$ (%)	k_2 (min^{-1})
0	0	2.24	5720
10	2.2	2.68	5240
20	3.9	3.54	4810
30	4.6	2.92	3390
50	5.5	3.19	2820

[a]Reaction conditions: actual enzyme concentration, 3.26 nM; 0.01M acetate buffer (pH 4.8); substrate concentration: 0.01, 0.015, 0.02, 0.05, and 0.1% of soluble starch; reaction time, 4.3 min at 25°.

In determining the degree of ethoxycarbonylation, a molecular weight[26] of 52,000 was used for porcine-pancreatic alpha-amylase. Of the 8–9 histidine residues present in the enzyme[27–28], four exposed groups could be modified. For sweet-potato beta-amylase, a molecular weight[29] of 50,000 was used, and six of the seven histidine residues could be modified[29]. For *A. niger* glucamylase, a molecular weight[30] of 97,000 was used, and four of the six histidine residues[31] could be modified.

The results for the reaction kinetics carried out with modified enzymes were plotted by the Lineweaver–Burk method and the equations of the straight lines were determined by linear regression. The graphically determined, apparent K_m values and calculated rate constants k_2 are given in Tables IV–VI.

TABLE VI

CHANGES IN REACTION-KINETIC PARAMETERS DURING ETHOXYCARBONYLATION OF THE EXPOSED HISTIDINE SIDE-CHAINS OF *A. niger* GLUCAMYLASE[a]

Time of modification (min)	*Number of modified histidine groups*	$K_m \times 10^3$ *(%)*	k_2 *(min^{-1})*
0	0	4.49	353
10	1.2	4.86	360
30	2.6	5.05	366
50	3.4	5.34	349
80	3.8	5.73	352

[a]Reaction conditions: actual enzyme concentration, 56.2 nM; 0.01M acetate buffer (pH 4.2); substrate concentration, 0.01, 0.015, 0.02, 0.05, and 0.1% of soluble starch; reaction time, 4.3 min at 25°.

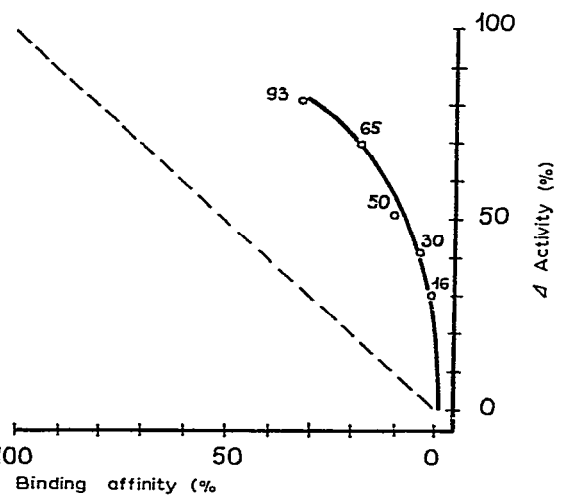

Fig. 1. Changes in binding affinity and catalytic activity for porcine-pancreatic alpha-amylase. The numbers associated with points on the curve indicate the percentage of exposed histidine groups that are modified.

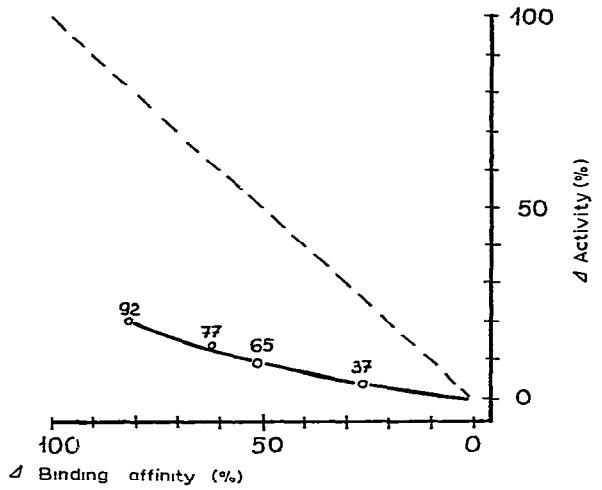

Fig. 2. Changes in binding affinity and catalytic activity for sweet-potato beta-amylase. See legend to Fig. 1.

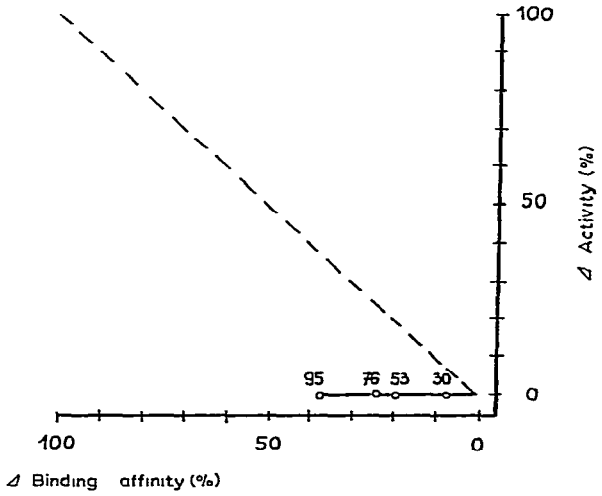

Fig. 3. Changes in binding affinity and catalytic activity for *A. niger* glucamylase. See legend to Fig. 1.

The enzyme concentrations used for the calculation of k_2 were adjusted for the content of inactive protein. For the determination of the role of the histidine residues present in the active centres of the enzymes, binding-affinity changes due to modification ($\Delta k_{-1}/k_{+1}$) were plotted against changes in the activity (Δact) (Figs. 1–3).

DISCUSSION

The activity of porcine-pancreatic alpha-amylase (Table I) is virtually lost on ethoxycarbonylation of the exposed histidine groups and the binding affinity is

slightly reduced. The changes in kinetic parameters also point to the catalytic function of imidazole side-chains located in the active centre of the enzyme. Whereas K_m does not change significantly, the rate constant k_2 is reduced 20-fold (Table IV). This observation supports earlier conclusions drawn on the basis of reaction-kinetic[7-9], photo-oxidative[7,10], and DEP ethoxycarbonylation[19] data.

According to Elődi[19], two of the four exposed histidine groups of porcine-pancreatic alpha-amylase are much more reactive (k 21M^{-1}.min^{-1}) than the others (k 1.5M^{-1}.min^{-1}) towards ethoxycarbonylation, and modification of the more reactive histidine residue virtually inactivates the enzyme. The rate constant (k_i) of inactivation is nearly identical (15M^{-1}.min^{-1}) with the higher reactivity constant.

The kinetics of the deactivation were studied by the method of Ray and Koshland[32]. When the residual activity, binding affinity of the substrate analogue, and percentage of unreacted amino acid are plotted on a logarithmic scale as a function of time, the rate constants of the individual processes and the number of amino acid side-chains participating in the enzymic reaction can be determined. The apparent first-order rate constants were derived from the equation of the straight line by linear regression using the equations $kt = \ln[100/(100 - A)]$, and $2.3 \log (100 - A) = kt + 2.3 \log 100$, where t is the reaction time (min) and A is variously the change in

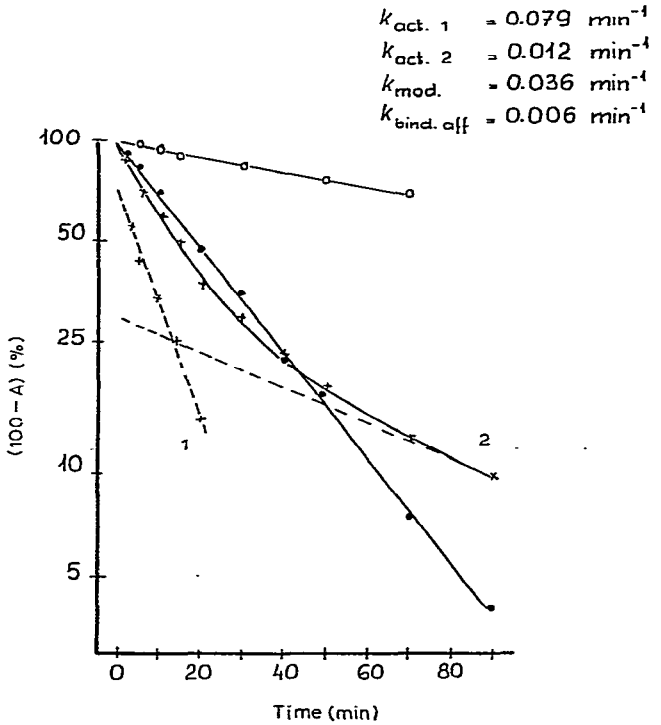

Fig. 4. Kinetics of ethoxycarbonylation of the exposed histidine side-chains of porcine-pancreatic alpha-amylase: —+—, change in remaining activity (%); —●—, change in non-ethoxycarbonylated histidine groups (%); —○—, change in remaining binding-affinity (%).

percentage of activity, binding affinity, and reacted, exposed amino acids at time t. On this basis, the relationship in Fig. 4 was obtained. When the two sections of the activity curve are extrapolated to zero, the rate constants of inactivation characteristic of the modification of the different histidine residues can be determined from the slopes of the straight lines (k_{a1} 7.9 × 10^{-2} min^{-1} and k_{a2} 1.2 × 10^{-2} min^{-1}). From the values at zero time (0.31 and 0.69) obtained by extrapolation, the number of residues with various functions can be calculated[32]. On this basis, it was shown that ethoxycarbonylation of one of the histidine groups results in much quicker inactivation than ethoxycarbonylation of the other three groups. Consequently, one of the four exposed histidine groups has a catalytic function, whereas modification of the other three groups leads to some slight decrease in the activity, probably by changing the tertiary structure of the enzyme.

Considering ethoxycarbonylation as a function of time, it may be stated that the four exposed histidine groups have identical reactivities and that the rate of modification (k 3.6 × 10^{-2} min^{-1}) is somewhat lower than the inactivation rate of the histidine group associated with the activity of the enzyme.

The changes in binding affinity plotted *versus* time also afford a straight line, with a rate constant much lower (k 6.0 × 10^{-3} min^{-1}) than that characteristic of ethoxycarbonylation or rapid inactivation.

The activity of sweet-potato beta-amylase slightly decreased (~25%) during ethoxycarbonylation of the exposed histidine groups, whereas the binding affinity decreased considerably (~80%) (Table II). In accord with these findings were the changes in kinetic parameters, namely a slight decrease in rate constant k_2 and an increase in the K_m value (Table V).

The fact that the increase in the K_m value was lower than expected is probably due to the fact that the molecules of the soluble-starch substrate are more flexible than those of the cyclodextrin substrate analogue.

The above results show that classical reaction-kinetic methods are not sufficient for separate investigation of the rate constants. Due to the very high molecular activity of beta-amylase (where k_2 is the determining factor), the change in k_{-1}/k_{+1} cannot be determined by this method. By using immobilised cyclodextrin, the changes can be much more accurately traced, and the substrate-binding function of the histidine residues can be verified by the substantial change in the binding affinity.

In evaluating the results obtained by the method of Ray and Koshland[32], straight lines were obtained for the ratios of residual activity and binding affinity, as well as for the ratio of unreacted histidine groups (Fig. 5). The rate of ethoxycarbonylation (k 4.9 × 10^{-2} min^{-1}) and the rate of change in binding affinity (k 3.4 × 10^{-2} min^{-1}) were similar, whereas the rate of inactivation (k 5.0 × 10^{-3} min^{-1}) was much lower.

Thus, contrary to literature data, it may be stated that histidine groups of beta-amylase have no catalytic effect, but they do have a substrate-binding function. Consequently, the ionisation constant (pK 7.0) derived from the pH-dependence of V_{max} by Thoma and Koshland[10] is probably not related to a histidine residue but

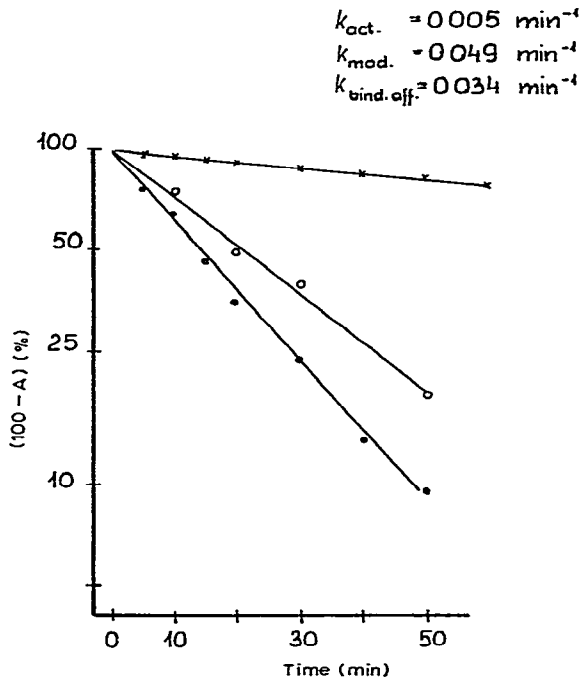

Fig. 5. Kinetics of ethoxycarbonylation of the exposed histidine side-chains of sweet-potato beta-amylase: —+—, change in remaining activity (%); —●—, change in non-ethoxycarbonylated histidine groups (%); —○—, change in remaining binding-affinity (%).

rather to a carboxyl group, and the microenvironment of the subsites is responsible for the higher pK values obtained.

Measurements of inactivation due to photo-oxidation[10] are not conclusive, since the method is not specific for histidine groups. Thus, the statement that beta-amylase and alpha-amylase react by a common mechanism and that the imidazole group has the same catalytic role in both enzymes is questionable[7].

On ethoxycarbonylation of the exposed histidine groups of *A. niger* glucamylase, even less change could be detected than observed for beta-amylase. The catalytic activity was virtually unchanged, the binding affinity was decreased by ~40% (Table III), the rate constant k_2 was almost unchanged, and the K_m value was only slightly altered.

Using the method of Ray and Koshland[32], straight lines were obtained for changes in the ethoxycarbonylation and binding affinity plotted as functions of time (Fig. 6). The rate of modification (k 3.8 × 10^{-2} min^{-1}) was higher than the rate of change in binding affinity (k 6.0 × 10^{-3} min^{-1}).

The results indicate that, for the glucamylase, none of the histidine residues play an important role. The unambiguous decrease in binding affinity verifies the substrate-binding function of the histidine residue, and it may be stated that this amino acid is probably located in the active centre at a distance from the catalytic site.

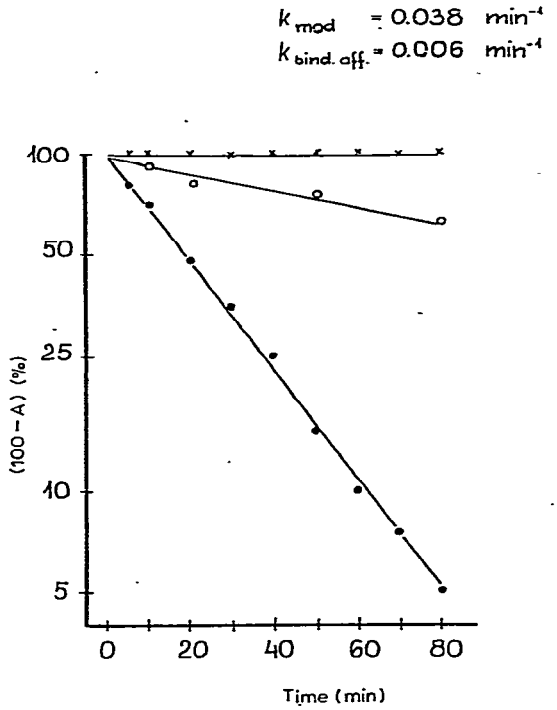

Fig. 6. Kinetics of ethoxycarbonylation of the exposed histidine side-chains of *A. niger* glucamylase: —+—, change in remaining activity (%); —●—, change in non-ethoxycarbonylated histidine groups (%); —○—, change in remaining binding-affinity (%).

The above results are in agreement with those of Hiromi *et al.*[15], who showed, by the reaction-kinetic method, that the catalytic groups belonging to the pK region of imidazole are actually carboxyl side-chains. Barker *et al.*[16] verified that the inactivation observed during photo-oxidation is not due to the oxidation of histidine residues.

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