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A STUDY OF THE ROLE OF TYROSINE GROUPS AT THE ACTIVE CENTRE OF AMYLOLYTIC ENZYMES*

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ABSTRACT

The role of exposed tyrosine side-chains in enzyme-catalysed reactions has been studied for porcine-pancreatic alpha-amylase, sweet-potato beta-amylase, and *Aspergillus niger* glucamylase using *N*-acetylimidazole as the specific protein reagent. The changes in activity, binding affinity ($\Delta k_{-1}/k_{+1}$), and kinetic parameters (K_m, k_2) due to acetylation of the phenolic hydroxyl groups have been determined. Acetylation of each enzyme occurred by an "apparent" first-order reaction with a rate constant of 0.72–1.4 × 10^{-1}min^{-1}. Acetylation increased the apparent K_m (soluble starch as the substrate) for each enzyme (appreciably for alpha-amylase and glucamylase), whereas k_2 remained unchanged. Similarly, for each enzyme, the binding affinity for immobilised cyclohexa-amylose decreased appreciably, whereas the catalytic activity was reduced only to a small degree (and remained unchanged for beta-amylase). It is concluded that the tyrosine groups located in the active centre of each enzyme have a substrate-binding function.

INTRODUCTION

We have described[1] a method for the investigation of the histidine groups of amylolytic enzymes, which allows the enzyme–substrate (ES) complex to be studied by an affinity chromatographic method[2]. It can then be established whether the amino acid side-chain plays a role in the binding of the substrate and the formation of the products, or affects only the stability of the structure of the active centre. The histidine groups of porcine-pancreatic alpha-amylase had a catalytic effect but showed only substrate-binding activity in sweet-potato beta-amylase and *A. niger* glucamylase.

The role of the tyrosine groups has been studied for various types of alpha-amylase, but the results were contradictory. Modification of the enzymes with fluorodinitrobenzene or a diazotised sulphonic acid has been used to assess the role played by tyrosine groups in catalysis in *B. subtilis* and *A. oryzae* alpha-amylase[3–5]. Similar results were obtained in the studies of photo-oxidation[6] and acetylation[7,9].

*The Active Centre of Amylolytic Enzymes, Part III. For Part II, see ref. 1.

Tyrosine side-chains are reported to have no catalytic effect for alpha-amylase of bacterial origin[8,9] and pancreatic alpha-amylase[10–12]. On the other hand, stabilisation of the optimum steric structure has been attributed to tyrosine groups[13,14]. These contradictory statements are mainly due to the application of non-selective reagents. *N*-Acetylimidazole has been used[15] for selective reaction of the tyrosine hydroxyl group. Undesirable side-reactions (mainly, acetylation of SH and NH_2 groups) are thereby avoided[16]. We now report on the application of this reagent to amylases.

EXPERIMENTAL

The characteristics of the crystalline amylases studied and the methods for measuring activity, changes in binding affinity, and reaction-kinetic parameters have been described elsewhere[1].

Modification of tyrosine groups. — Modification of the tyrosine hydroxyl groups in each enzyme was carried out at pH 7.5 in a 0.01M buffer, since the stability of *N*-acetylimidazole (NAI) is highest under these conditions. NAI (analytical grade) was recrystallised from anhydrous benzene, dried *in vacuo* over phosphorus pentaoxide, and kept in a refrigerator. The reaction was carried out with continuous stirring at 25° using a pH-stat and enzyme concentrations of 2.5–3.0 mg/ml. Solid NAI (120 molar excess) was added. Aliquots were immediately diluted with water (10 vol.) and cooled to 0°, and the pH was adjusted to the appropriate optimum value. Excess of NAI was removed by using a rapid dialyser, and the changes in activity and binding affinity were measured[1].

When the *O*-acetyl groups were removed by treatment with 0.5M hydroxylamine at pH 7.5 and 25° for 30 min, 95–98% of the original activity of each enzyme was regenerated. The degree of acetylation was determined[17] by the change in u.v. absorption at 278 nm on deacetylation. The number (N) of acetylated tyrosyl groups is given by $\Delta\varepsilon_{278}.M/1160c$, where $\Delta\varepsilon_{278}$ denotes the difference of extinction measured at 278 nm, M is the molecular weight of the enzyme, c is the protein concentration of the enzyme (mg/ml), and 1160 is the molar extinction coefficient for *O*-acetyltyrosine.

RESULTS

The changes in activity on acetylation of the phenolic hydroxyl groups, the changes in binding, and the degrees of acetylation are presented in Tables I–III. Under the chosen experimental conditions, only the exposed tyrosine groups are blocked.

For porcine-pancreatic alpha-amylase, a mol. wt. of 52,000 ±2500 was calculated[18] and 6 of the 16–18 tyrosine groups present in the enzyme[19,20] could be modified. These values are in good agreement with those obtained by Krysteva and Elődi[21]. On application of the perturbation method, ~30% of the tyrosine groups proved to be exposed[21]. For sweet-potato beta-amylase, on the basis[22] of a mol. wt.

of 50,000, 6 of the 17 tyrosine groups[23] could be modified. For *A. niger* glucamylase, a mol. wt. of 97,000 was calculated[24] and 12 of the 33 tyrosine groups[25] could be modified.

The reaction-kinetic data for the modified enzymes were plotted by the double-reciprocal method of Lineweaver–Burk and the equation of the straight lines was derived by linear regression (Packard 65). The apparent Michaelis constants (K_m), V_{max}, and rate constants (k_2) are presented in Tables IV–VI.

The changes in binding ($\Delta k_{-1}/k_{+1}$) due to modification of the tyrosine groups are plotted against changes in the activity (Δ_{act}) in Figs. 1–3.

TABLE I

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

Time of modification (min)	*Number of modified tyrosine groups*	*Remaining activity (%)*	*Remaining binding-affinity (%)*
0	0	100	100
2	1.8	90.7	52.1
5	2.5	80.9	31.6
10	4.4	72.8	23.2
15	5.2	64.2	16.2
20	5.6	61.1	[b]
25	5.8	60.0	[b]
30	5.9	58.0	10.6

[a]Reaction conditions: enzyme concentration, 44 μM; 0.01M phosphate buffer (pH 7.5) and 0.1mM $CaCl_2$; 120 mol of NAI per mol of enzyme; 25°; pH-stat method. After sampling, the pH value of the enzyme solution was adjusted to 7.0. [b]Not determined.

TABLE II

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

Time of modification (min)	*Number of modified tyrosine groups*	*Remaining activity (%)*	*Remaining binding-affinity (%)*
0	0	100	100
2	0.8	100	93.2
5	1.8	100	74.9
10	3.0	100	52.5
15	4.1	100	40.2
20	4.6	100	[b]
25	5.0	100	[b]
30	5.3	100	13.5

[a]Reaction conditions: enzyme concentration, 50 μM; 0.01M acetate buffer (pH 7.5); 120 mol of NAI per mol of enzyme; 25°; pH-stat method. After sampling, the pH value of the enzyme solution was adjusted to 4.8. [b]Not determined.

TABLE III

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

Time of modification (min)	*Number of modified tyrosine groups*	*Remaining activity (%)*	*Remaining binding-affinity (%)*
0	0	100	100
2	2.5	100	[b]
5	5.0	97.3	66.7
10	7.5	93.6	51.4
15	9.1	93.1	[b]
20	10.7	91.8	22.0
25	11.0	90.0	[b]
30	11.3	89.7	13.1

[a]Reaction conditions: enzyme concentration, 29 μM; 0.01M acetate buffer (pH 7.5); 120 mol of NAI per mol of enzyme; 25°; pH-stat method. After sampling, the pH value of the enzyme solution was adjusted to 4.2. [b]Not determined.

TABLE IV

CHANGES IN REACTION-KINETIC PARAMETERS DURING ACETYLATION OF THE EXPOSED TYROSINE SIDE-CHAINS OF PORCINE-PANCREATIC ALPHA-AMYLASE[a]

Time of modification (min)	*Number of modified tyrosine groups*	$K_m \times 10^3$ *(%)*	k_2 *(min^{-1})*
0	0	4.5	930
2	1.8	5.5	912
5	2.5	6.7	860
10	4.4	9.3	889
15	5.2	11.6	721
30	5.9	13.5	674

[a]Reaction conditions: actual enzyme concentration, 34.8 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 ACETYLATION OF THE EXPOSED TYROSINE SIDE-CHAINS OF SWEET-POTATO BETA-AMYLASE[a]

Time of modification (min)	*Number of modified tyrosine groups*	$K_m \times 10^2$ *(%)*	k_2 *(min^{-1})*
0	0	2.22	6250
2	0.8	2.58	6100
5	1.8	3.34	6390
10	3.0	3.99	6330
15	4.1	4.21	6470
30	5.3	5.23	6330

[a]Reaction conditions: actual enzyme concentration, 4.08 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°.

TABLE VI

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

Time of modification (min)	*Number of modified tyrosine groups*	$K_m \times 10^3$ (%)	k_2 (min^{-1})
0	0	4.82	356
5	5.0	6.48	356
10	7.5	8.07	338
20	10.7	8.82	334
30	11.3	10.37	325

[a]Reaction conditions: actual enzyme concentration, 56.5 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°.

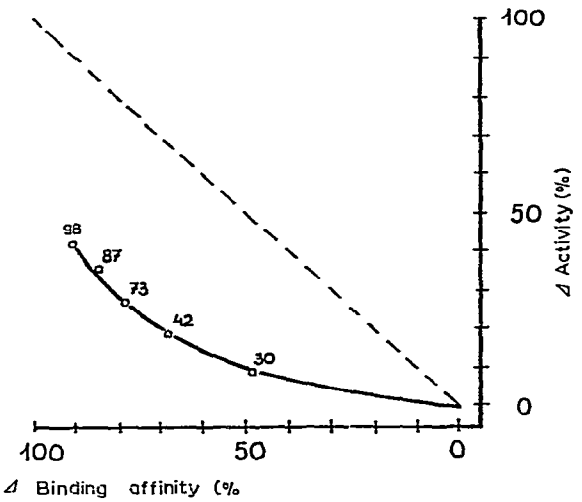

Fig. 1. Binding affinity and catalytic activity of porcine-pancreatic alpha-amylase. The numbers associated with points on the curve indicate the percentage of exposed tyrosine groups that are modified.

DISCUSSION

The foregoing data indicate that, for each amylase, the tyrosine side-chains have a substrate-binding function.

Acetylation of the exposed tyrosine groups in porcine-pancreatic alpha-amylase (Table I) decreased the catalytic activity to a much less extent (42%) than binding affinity (90%), which is characteristic of the formation of an ES complex. The changes in the reaction-kinetic parameters also prove that phenolic hydroxyl groups play an important role in substrate binding. On acetylation of the enzyme, K_m increased 3-fold, but k_2 remained practically constant (Table IV).

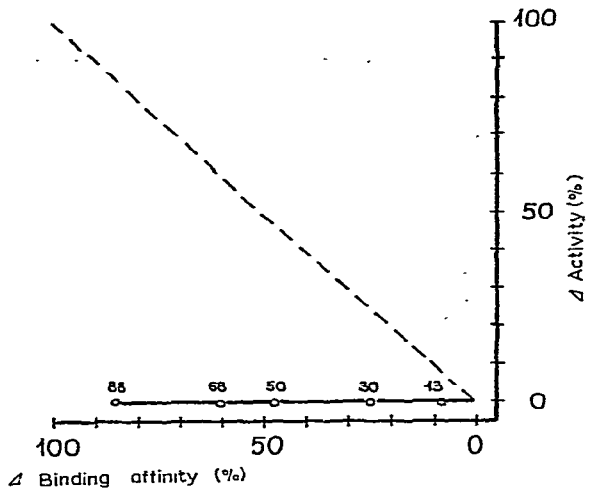

Fig. 2. Binding affinity and catalytic activity of sweet-potato beta-amylase (see Fig. 1).

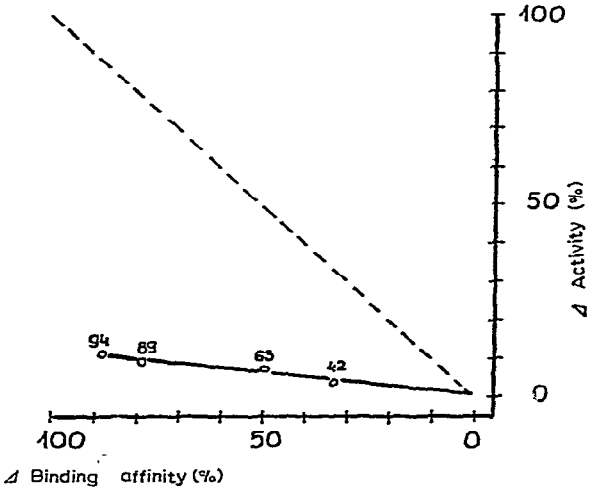

Fig. 3. Binding affinity and catalytic activity of *A. niger* glucamylase (see Fig. 1).

These results supplement the data obtained by Krysteva and Elődi[21] using differential spectroscopic and perturbation methods, and those of Wakim *et al.*[12] for spectrophotometric titration. These authors found most of the tyrosine groups of porcine-pancreatic alpha-amylase to be located in the interior of the molecule and that no ionisation of the phenolic hydroxyl group was observed below pH 8. The possibility that tyrosine groups were involved in the enzyme reactions was therefore ruled out, but it was assumed that they were involved either in substrate binding or in the development of the optimum steric structure. Evaluation of the obtained results by the method of Ray and Koshland[26] gave the relationship in Fig. 4. On plotting

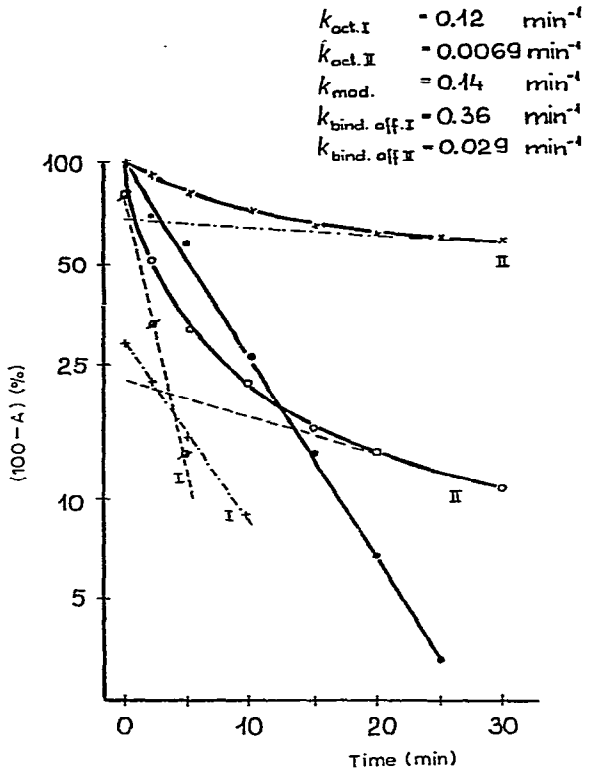

Fig. 4. Effect of acetylation of the exposed tyrosine side-chains of porcine-pancreatic alpha-amylase: —+—, change in remaining activity; —●—, change in non-acetylated tyrosine groups; —○—, change in remaining binding-affinity.

the changes in acetylation against time, a straight line was obtained which proves that all exposed tyrosine groups have the same reactivity and that the rate of modification (with a rate constant of apparently first-order: k 0.14 min^{-1}) is nearly identical with the rate of rapid changes in binding affinity and activity.

The plots of activity and binding affinity against time are not linear (Fig. 4). Each curve can be divided into two sections with different slopes, which can be extrapolated to zero. For the changes in binding affinity, the rate constants characteristic of acetylation of the various tyrosyl residues (k_1 0.36 and k_2 2.9 × 10^{-2}min^{-1}), as well as the number of residues with various functions[26], can be calculated from the values extrapolated to zero time (0.19 and 0.81). Thus, the rate of decrease in binding affinity caused by acetylation of one of the tyrosyl groups is much higher (12-fold) than that induced by acetylation of the other five groups. By graphic analysis of the changes in activity, the rate constants (k_1 0.12 and k_2 6.9 × 10^{-3}min^{-1}) can also be calculated, although the accuracy is not high due to the low change in activity.

Comparison of the results with those in Fig. 1 shows that one of the tyrosine groups is located in the active centre, presumably near the catalytic site, and partici-

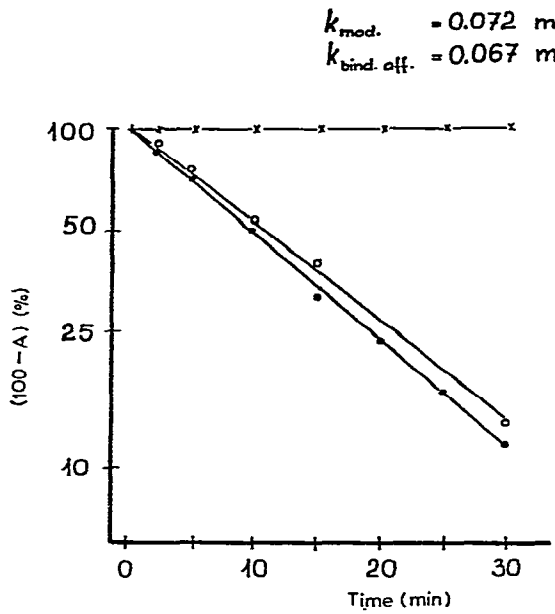

Fig. 5. Effect of acetylation of the exposed tyrosine side-chains of sweet-potato beta-amylase: —+—, change in remaining activity; —●—, change in non-acetylated tyrosine groups; —○—, change in remaining binding-affinity.

pates in the formation of the enzyme–substrate complex. Acetylation of the other groups changes the tertiary structure of the enzyme and leads to a further increase in inactivation and a decrease in the binding affinity.

The catalytic activity of sweet-potato beta-amylase did not change on acetylation of the exposed tyrosine groups, whereas there was a considerable decrease (86%) in the binding affinity (Table II). In studying the changes in reaction-kinetic parameters (Table V), there was a two-fold increase in the K_m value, probably because of the flexibility of the soluble starch used as the substrate analogue. The rate constant k_2 remained practically unchanged.

Using the method of Ray and Koshland[26], the changes in binding affinity and acetylation plotted *versus* time were characterised by an apparently first-order rate constant, and the two rate constants were nearly identical: $k_{bind.aff.}$ 6.7 × 10^{-2} min^{-1} and $k_{mod.}$ 7.2 × 10^{-2}min^{-1} (Fig. 5).

Thus, the tyrosine group seems to be located in the active centre, probably far from the catalytic site. This assumption is supported by the fact that a macromolecular substrate (starch) can form an enzyme–substrate complex with the other substrate-binding sites, by overlapping the active centre, even if the subsite involving the tyrosine group is blocked. For smaller substrates (cyclodextrin), the formation of an analogous enzyme–substrate complex is appreciably hindered by acetylation of the tyrosine group. Acetylation of the exposed tyrosine groups of *A. niger* glucamylase also

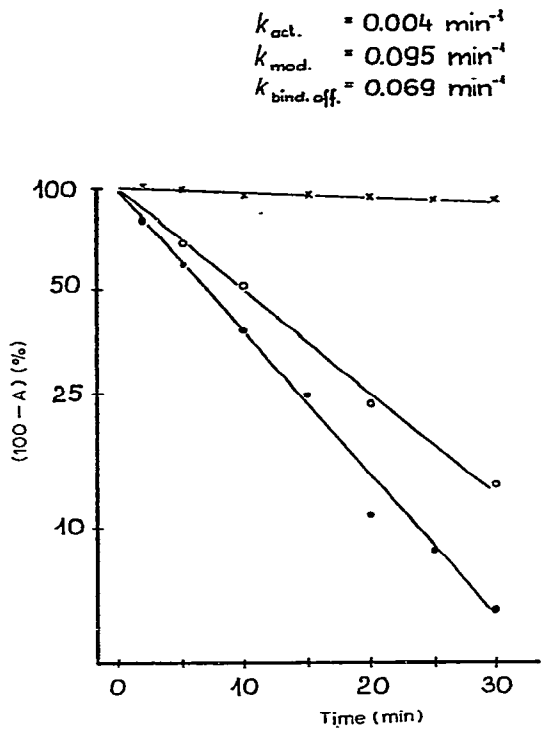

Fig. 6. Effect of acetylation of the exposed tyrosine side-chains of *A. niger* glucoamylase: —+—, change in remaining activity; —●—, change in non-acetylated tyrosine groups; —○—, change in remaining binding-affinity.

resulted in a large decrease in binding affinity (87%) and a relatively low decrease (10%) in catalytic activity (Table III).

A study of the changes in kinetic parameters (Table VI) showed that K_m reached twice its original value, whereas k_2 remained unchanged. The changes in residual activity and binding affinity on acetylation, as a function of time, showed each process to change according to "apparent" first-order rate constants where the rate of inactivation is lowest (k_{act} 4 × 10^{-3}min^{-1}), whereas the rate of acetylation and the change in binding affinity were considerably higher (Fig. 6). The rate constant of acetylation was $k_{mod.}$ 0.1 min^{-1} and that of binding affinity was $k_{bind.aff.}$ 6.9 × 10^{-2}min^{-1}.

These results show that, as for sweet-potato beta-amylase, the tyrosine group in glucamylase has a substrate-binding function and is probably located in the active centre far from the catalytic site.

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