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A pH-INDUCED CHANGE IN STATE AROUND ACTIVE-SITE TRYPTOPHAN RESIDUES OF *Rhizopus niveus* GLUCOAMYLASE, DETECTED BY STOPPED-FLOW STUDIES OF CHEMICAL MODIFICATION WITH *N*-BROMOSUCCINIMIDE*†

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ABSTRACT

At pH 4.5 (the optimum), stopped-flow studies of the chemical modification with *N*-bromosuccinimide (NBS) of the tryptophan residues of glucoamylase from *Rhizopus niveus* clearly show reaction curves having two phases, fast and slow, in which two separate types of Trp residues are respectively involved. At pH values higher than 4.5 (to 7.0), however, separation of the slow phase becomes less clear. The pH profile of the $\Delta\varepsilon_{280}$ (caused by NBS) gave a pK ~5.8, which corresponds with pK 5.9 for the catalytic residue. At pH 7.5 (no catalytic activity), Trp residues in the slow phase are not modified, but those in the fast are. Trp residues do not have ionizable groups and NBS modification of Trp does not show pH dependency in this pH range. Hence, some change in the environment of Trp residues may be linked to some ionizable residue(s) which has a pK of 5.8 and participates importantly in maltosaccharide hydrolysis.

INTRODUCTION

There is a possibility that conformational change is essential to enzyme catalysis[1,2]; however, it is very difficult to determine a change in the conformation that intimately relates to the catalytic function. Even if a conformational change is essential, it might not be large. Hence, it is important to detect how microscopic changes in conformation or environment around a specified residue occur in enzymes.

One of the five Trp residues modifiable with *N*-bromosuccinimide (NBS) in hen egg-white lysozyme is clearly discriminated by stopped-flow chemical modification; the residue is Trp 62, which is located[3] at Subsite D. The stopped-flow NBS-modification for Trp 62 is very dependent on pH, with an estimated pK of 6.5, that

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is nearly equal to the pK (6.2) for the catalytic residue Glu 35, suggesting that the microenvironment of Trp 62 is intimately related with the catalytic residue. The stopped-flow NBS-modification can thus be useful for detecting a change in microenvironment around a Trp residue[4].

At pH 4.5 (optimum), 4 Trp residues out of a total of 10 per unit in *Rhizopus niveus* glucoamylase are modified with NBS; the stopped-flow reaction-curves clearly consist of two phases, fast and slow[5]. Two residues of Trp are included in each phase: these are named Trp-1 and Trp-2 in the fast phase, and Trp-3 and Trp-4 in the slow phase. It was concluded[5] that Trp-3 is located at "Subsite 1", where the nonreducing terminal D-glucosyl residue of the substrate saccharide is bound. This conclusion is certainly supported by spectrophotometric (kinetic and static) measurements on the binding of substrates or analogs, and inhibition kinetics[6–8].

We have now applied the stopped-flow technique to observation of the NBS-modification of glucoamylase from *Rhizopus niveus* at different pH values. Change in microenvironment around the active-site Trp-residue(s) of the enzyme may be induced by pH, and be related to the catalytic action for maltosaccharide hydrolysis.

EXPERIMENTAL

Materials. — The glucoamylase preparation from *Rhizopus niveus* was a product of Seikagaku Biochemicals Co., Tokyo, and was used without purification. The concentration of the enzyme was determined spectrophotometrically, taking the absorption unit $A^{1\%}_{280\ nm} = 13.6$, and the molecular weight as 56 000. NBS was recrystallized at least twice from water before use. Maltose, purest grade HHH, was obtained from Hayashibara Biochemical Laboratories Co., Okayama, Japan. Other chemicals used were of guaranteed grade, purchased from Kanto Chemicals Co., Tokyo, and used without purification.

NBS-Modification of Trp residue of glucoamylase. — Equal volumes of the enzyme and NBS solutions were mixed in a Union Giken (recently revised to PHOTAL) SF-70 stopped-flow spectrophotometer. It was used in the fluorescence mode, with an emission cut-off filter (half transmittance wavelength at 310 nm) and with excitation at 292 nm using a D_2-lamp as the light source. Reaction curves of the change in the fluorescence intensity were recorded on a Union Giken (PHOTAL) RA-450 transient-memory apparatus and were usually accumulated 9 times on a Union Giken (PHOTAL) RA-405 data-averaging processor to improve the signal-to-noise (s/n) ratio, as described previously[3,5]. All reactions with NBS were carried out in an appropriate buffer at 25.0°.

U.v. difference absorption-spectrophotometry on glucoamylase. — U.v.-difference absorption-spectra reflecting NBS modification of the enzyme and of *N*-acetyltryptophan ethyl ester (ATE) were measured at the specified pH values on a Shimadzu UV-200 spectrophotometer equipped with tandem quartz cells, as

described elsewhere[9]. The NBS reactions were performed at 25.0° and at pH 4.5, 5.5, 6.0, and 7.5.

The time course of the enzyme-catalyzed reaction for hydrolysis of amylose (d.p. ~17) or maltose as a substrate was monitored by reducing-value determinations by the Nelson–Somogyi method[10]. All reactions and measurements were carried out at 25.0° and at the pH values described in the text.

RESULTS AND DISCUSSION

NBS-Modification of glucoamylase using the fluorescence stopped-flow method. — Chemical modification of Trp residues of glucoamylase with NBS was observed by the stopped-flow method, using change in fluorescence as a probe, because only Trp residues can be detected when the fluorescence (~340 nm, excited at 290 nm) is employed as a probe for the NBS-modification[11]. Typical examples of the reaction curves are illustrated in Fig. 1. Reaction curves thus obtained are clearly very dependent on pH. At pH 4.5, the fast phase and the slow phase are distinctly recognized in the reaction curves, as may be seen in Fig. 1a and 1b (note the time scales), respectively. However, the reaction curves obtained at pH 5.5 and pH 6.0 are quite different from those at pH 4.5. Particularly, the slow phase at pH 6.0 is difficult to detect (Fig. 1b). We had observed[5] the reactions at pH 4.5 under the various concentrations of NBS ($[NBS]_0 > [GA]_0$) and have obtained the apparent first-order rate-constants (κ_{app}) from the reaction curves for evaluation of the second-order rate-constant[5]. The rate-constants k ($M^{-1}.s^{-1}$) for Trp residues, named Trp-1 and Trp-2 in the fast phase, are 3.5×10^5 and 8.8×10^4, while those for Trp-3 and Trp-4 in the slow phase are 2.8×10^3 and 7.4×10^2, respectively.

At pH 7.5, where the enzyme has no catalytic activity, the molar activity, k_0 for maltose hydrolysis is less[11a] than 1/50 of that at pH 4.5. The NBS modification for glucoamylase as observed with the fluorescence stopped-flow instrument is shown in Fig. 2. Based on the Guggenheim plot[13,14], first-order rate-constants, k_{app} were determined from the reaction curves under the various concentrations of NBS, and the second-order rate-constants were calculated to be 2.2 ($\pm$0.3) $\times 10^5$ and 3.7 ($\pm$0.7) $\times 10^4 M^{-1}.s^{-1}$. This indicated that Trp residues involved in the fast phase were undoubtedly modified at pH 7.5. The slow phase was not observed; hence, their rate constants cannot be evaluated here.

We had concluded[5] that one Trp residue, which is modified in the slow phase (rate constant $2.8 \times 10^3 M^{-1}.s^{-1}$, and named Trp-3), is located in the active site at "Subsite 1", where the nonreducing-terminal D-glucosyl residue of substrate is bound[5]. Substrate will, therefore, protect Trp-3 from NBS modification. In fact, at

*On the basis of observations made on other proteins, we assume that the reaction of NBS with the Trp residues is second-order. However, we have not experimentally verified this assumption for *R. niveus* glucoamylase.

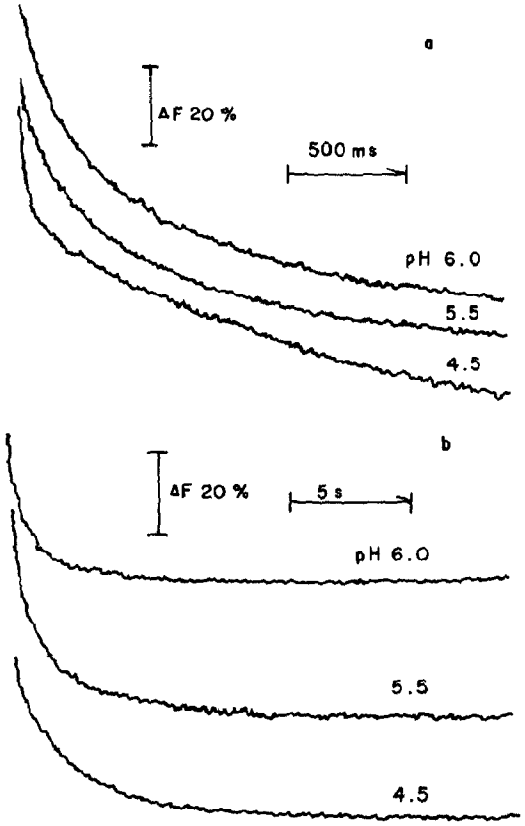

Fig. 1. Stopped-flow reaction-curves for the NBS modification of glucoamylase. (a) Fast phase, (b) slow phase. [NBS]/[GA], 40:1; glucoamylase, 6.8μM; excitation, 292 nm; 25.0°. ΔF means the change in fluorescence intensity, represented by % of the intensity of the intact enzyme.

pH 4.5, the Trp residue (Trp-3) of the active site is protected from NBS modification by the presence of maltose, amylose, or D-glucono-1,5-lactone[5]. At pH 7.5, the NBS modification was also carried out in the presence of the substrate maltose (19.4 or 19.8mM) to test the protection, in the same experimental procedure as already described (data not shown here). We had previously shown[12] that, at pH 7.5, maltose is bound to the active site of the enzyme in the same manner as at pH 4.5. The stopped-flow reaction-curves were analyzed, and found to consist of two second-order processes with rate-constants, 7.6 ($\pm$3.1) $\times$ 10^5 and 1.2 ($\pm$0.1) $\times$ $10^5 M^{-1}.s^{-1}$, that are nearly identical to the values obtained in the absence of maltose already described; thus, maltose does not protect the Trp residues from NBS modification. At pH 7.5, Trp residues in the slow phase are not modified, but those involved in the fast phase, located outside the active site, are certainly modified with NBS.

The difference absorption caused by the NBS-modification of ATE, a model

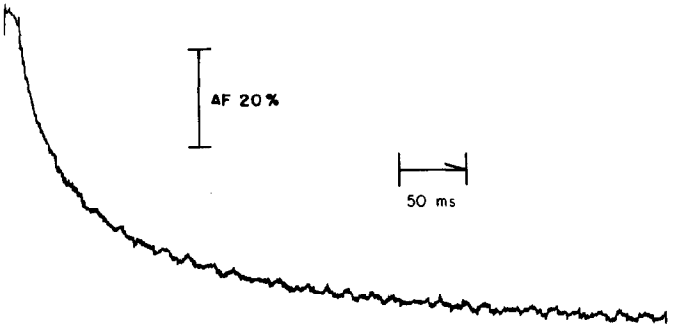

Fig. 2. Stopped-flow reaction-curve for the NBS modification of glucoamylase at pH 7.5 [NBS]/[GA], 36.6:1; glucoamylase, 10.6μM; excitation, 292 nm; 25.0°.

compound for the Trp residue, does not depend[4,11] on pH over the range of pH examined, namely, 3.5–8.5 (data not shown here). Moreover, Trp residues in enzymes do not ionize at these pH values.

These findings suggest that a change in state of Trp residues at the active site of the enzyme is induced by pH; under acidic conditions, the Trp residues react with NBS, but at alkaline pH a conformational change occurs to prevent reaction with NBS. The change may decrease the accessibility of two Trp side-chains.

Correlation of the NBS modification with the glucoamylase-catalyzed hydrolysis. — The NBS modification of Trp residues of glucoamylase results in a u.v.-difference absorption-spectrum that is characterized by a trough at 280 nm and two isosbestic points, at 265 nm and 300 nm (data not shown here, see ref. 5). From the absorbance change (which was complete within ~5 min) per mole of enzyme at 280 nm, $-\Delta\varepsilon_{280}$ was calculated for a fixed concentration of glucoamylase (abbreviated to GA) and various quantities of NBS. In Fig. 3, $-\Delta\varepsilon_{280}$ thus obtained is plotted against the concentration of NBS, which is represented with the molar ratio of NBS to GA, namely [NBS]/[GA]. The $-\Delta\varepsilon_{280}$ values become larger with increasing

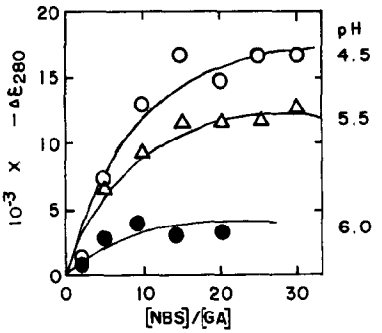

Fig. 3. Effect of pH on the change in absorption difference at 280 nm caused by the NBS modification of glucoamylase. Glucoamylase, 3.2μM; 25.0°. The molar absorption difference $\Delta\varepsilon_{280}$ was evaluated from the difference-absorption spectra.

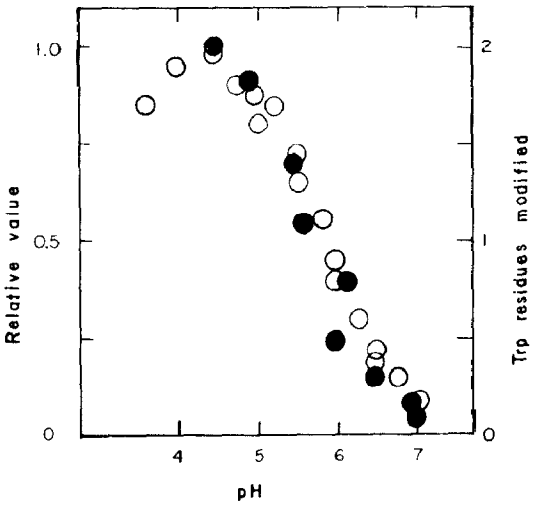

Fig. 4. Effect of pH on change in difference absorption and on enzyme activity. Closed circles, $-\Delta\varepsilon$ evaluated from a plot of maxima $-\Delta\varepsilon$ *vs.* [NBS]/[GA] (see Fig. 3); open circles, glucoamylase activity on maltose at 25.0°. $-\Delta\varepsilon$ and activity are expressed as relative values.

[NBS], and reach a maximum value. Hence, the number of tryptophan residues modified at pH 4.5 increases with increasing concentration of NBS to a maximum of 4, where the number is calculated by using the conversion factor 4 500/Trp, estimated with ATE, a model compound for the Trp residue[11]. At pH 5.5 and 6.0, $-\Delta\varepsilon$ values were also evaluated as at pH 4.5, are plotted against the NBS concentration, and are presented in Fig. 3 (triangles and closed circles). This shows that the number of Trp residues modified at pH 5.5 and 6.0 is much different from that at pH 4.5 (open circles).

Difference absorption-spectra caused by the NBS modification were observed at many different pH values, and the maximum values of $-\Delta\varepsilon$ were evaluated from plots (not presented here) like those in Fig. 3. Fig. 4 shows the maximum value of $-\Delta\varepsilon$ plotted against pH (closed circles). The measurements were carried out only at pH values higher than 4.5, because the enzyme is likely to be denatured at low pH values. The $-\Delta\varepsilon_{280}$ values are very dependent on pH. $-\Delta\varepsilon$ Apparently de-

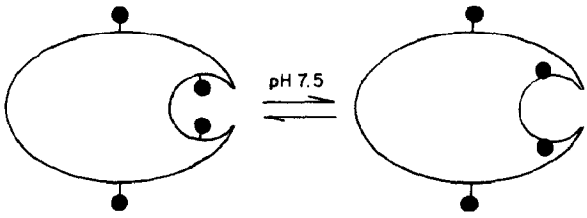

Fig. 5. Schematic illustration of the pH-induced change in state of Trp residues. Closed circles represent the four Trp residues modifiable with NBS, of which two are located at the active site and the other two are situated on the surface of the enzyme molecule. At acidic pH, all four Trp residues are available for reaction with NBS.

creases with increasing pH, and becomes almost zero at pH 7. As already described for the NBS-modification reactions studied by stopped-flow, two Trp residues in the fast phase were clearly found to be modified at pH 7.5. Thus, this appears to be a discrepancy, however, this is due to the NBS modification of tyrosine residues. Difference absorption $+\Delta\varepsilon$ caused by the NBS modification of a tyrosine residue is[11] 5 000. Hence, it is reasonable that $-\Delta\varepsilon$ caused by the NBS modification for Trp residues ($-\Delta\varepsilon$ for Trp ~4 500–5 000) in the fast phase is cancelled out by $+\Delta\varepsilon$ of the Tyr residue modification. The NBS modification for Tyr ($k < 2 \times 10^3 M^{-1}.s^{-1}$ at pH 7, 25°) was found to be much slower than that for Trp ($k \sim 7 \times 10^5 M^{-1}.s^{-1}$ at pH 7) and was not observed with the fluorescence stopped-flow as a probe, because Tyr residues in this enzyme do not produce fluorescence at 340 nm when excited at 292 nm. Hence, using fluorescence stopped-flow as a probe, observation of the NBS modification of the Trp residues is not disturbed by modifications to Tyr residues. We can therefore conclude that the pH dependency of $-\Delta\varepsilon$ (shown in Fig. 4, closed circles) is attributable to the Trp residues in the slow phase, namely, Trp-3 and Trp-4. The number of Tyr residues (suggested to be ~2) modified at pH 7.5 was estimated spectrophotometrically on a multidimensional correlation spectrophotometer developed by Union Giken and Prof. A. Wada, Tokyo University (unpublished data). The Trp residues in the fast phase, Trp-1 and Trp-2, were concluded to be modified over the entire range of pH examined (4.5–7.5).

The pH dependency of $\Delta\varepsilon$ is compared in Fig. 4 with that of the catalytic activity for maltosaccharide hydrolysis. The $-\Delta\varepsilon$–pH profile (Fig. 4, closed circles) is apparently consistent with the catalytic activity–pH profile (open circles) and gives a pK value estimated to be 5.8, which is almost identical to the pK of the catalytic residue (pK = 5.9; another pK is 2.9) evaluated[15] by Hiromi *et al.*, from the steady-state kinetics of glucoamylase-catalyzed reaction for maltose hydrolysis. On the other hand, with ATE, a model compound for Trp residues, when difference-absorption changes produced by the NBS modification are evaluated by the procedures already described, it is found that $-\Delta\varepsilon$ values are not dependent on pH, but are nearly constant[11] through the wide range of pH values examined, 3.5 to 8. Moreover, there is very little pH-induced change in the state of Trp residues of glucoamylase as determined by u.v.-difference absorption (or fluorecence) measurements on the enzyme in the absence of NBS modification at the pH values examined, 2.5 to 8.5 (data not shown). These experimental results, also, were confirmed on the multidimentional correlation spectrophotometer (unpublished data).

The difference absorption-spectrophotometry of the NBS modification suggests that some change in the state of Trp residues (slow phase) is induced by pH (schematically illustrated in Fig. 5), which involves a residue ionized at pK 5.8. The change, which may be due to a small conformational movement in state or in environment of the Trp residue, seems to be intimately related to the catalytic function of the enzyme.

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