Biochimica et Biophysica Acta, 321 (1973) 210–219

BBA 66998

STUDY OF CORRELATION BETWEEN STRUCTURAL MOTILITY AND REACTIVITY OF SH GROUPS IN α-AMYLASE

MARIANNA TELEGDI AND F. B. STRAUB

Enzymology Department, Institute of Biochemistry, Hungarian Academy of Sciences, Budapest (Hungary)

(Received March 12th, 1973)

SUMMARY

1. The two masked SH groups of pancreatic α-amylase (α-1,4-glucan 4-glucanohydrolase, EC 3.2.1.1) react with 5,5′-dithio-bis(2-nitrobenzoate) following first-order kinetics, when EDTA is present. Since the reaction is not accelerated on prolonged preincubation of amylase with EDTA, local fluctuation of the polypeptide chain is supposed to be the rate-limiting step of the reaction.

2. As a result of this reaction of the two SH groups, either one intramolecular, or two mixed disulfides are formed, depending on the relative concentrations of the enzyme and 5,5′-dithio-bis(2-nitrobenzoate).

3. In 7 M urea one SH group becomes unmasked due to the partial unfolding, while the reaction of the other SH group is still limited by fluctuation of the relevant part of the protein molecule.

4. Oxidation of SH groups by 5,5′-dithio-bis(2-nitrobenzoate), or the formation of mixed (enzyme–2-nitro-5-mercaptobenzoate) disulfide does not influence the enzyme activity, but decreases its stability.

INTRODUCTION

Pancreatic α-amylase (α-1,4-glucan 4-glucanohydrolase, EC 3.2.1.1) is known to have a rather rigid and compact structure. Besides the four disulfide bridges, Ca^{2+} bound to the enzyme are responsible for this rigidity[1]. In the presence of Ca^{2+} amylase is resistant towards proteolysis, and its two SH groups are inaccessible for SH reagents, being buried inside the protein molecule[1]. It has been shown that pancreatic amylase contains 1 mole of firmly bound Ca^{2+} per mole enzyme and in addition some loosely bound Ca^{2+} (ref. 2). In the presence of EDTA the SH groups are able to react with certain reagents, resulting in a partial inactivation of the enzyme[3].

The removal of Ca^{2+} by chelating agents, as EDTA, in itself does not cause

Abbreviations: DTNB, 5,5′-dithio-bis(2-nitrobenzoate); NMB, 2-nitro-5-mercaptobenzoate.

irreversible denaturation of the enzyme, although the Ca^{2+}-free amylase is susceptible to irreversible denaturation[4].

There is no indication that incubation with EDTA has any other effect on amylase than the removal of Ca^{2+} bound to the enzyme. Studies of hydrodynamic properties and optical rotation of the Ca^{2+}-depleted and native enzyme failed to indicate any major structural differences[5]. However, differences limited to a small region of the protein molecule cannot be excluded.

In the present work the kinetics and nature of structural changes leading to the unmasking of SH groups of α-amylase were investigated by means of kinetic studies of their modification.

MATERIALS AND METHODS

Hog pancreatic α-amylase was prepared according to Hatfaludi *et al.*[6]. Instead of recrystallization from urea the crystals were washed several times with 10 mM $CaCl_2$ adjusted to pH 6.0. Amylase preparations were treated with DFP to avoid the effect of probable proteolytic contaminations. The specific activity varied between 5500 and 6000 units per mg protein determined according to Smith and Roe[7].

5,5'-Dithio-bis(nitrobenzoate) (DTNB) was from Fluka (Switzerland). 10 mM stock solution (pH 7.5) was used. Concentration of DTNB was determined with an excess of 2-mercaptoethanol. Urea (A grade) was recrystallized from 70% ethanol. Soluble starch was purchased from Merck (GFR), or Reanal (Hungary). A 0.6% solution was prepared freshly in 60 mM NaCl. Maltose was a product of Reanal (Hungary). 2-Mercaptoethanol was from Fluka (Switzerland). Sephadex G-50 and DEAE-Sephadex A-50 were products of Pharmacia (Sweden). Tris was a Merck (GFR) preparation. It was recrystallized from 70% ethanol. All other chemicals were commercial preparations of reagent grade.

Amylase activity

Amylase activity was determined by the iodine–starch method, measured at 620 nm (ref. 7) at 37 °C, or 15 °C, with 0.2 μg/ml and 1 μg/ml amylase, respectively. Reactions were followed for 10 min, aliquots were taken every 2 min.

Reactions with DTNB

Modification of SH groups with DTNB was carried out according to Ellman's method[9]. The reaction was measured at 412 nm, 25 °C, in 0.1 M Tris–HCl buffer, pH 8.5, in the presence of 3 mM EDTA, when not stated otherwise.

Protein concentration

Protein concentration was determined spectrophotometrically at 280 nm using an extinction coefficient of $E^{1\%}_{1\,cm} = 24$. Spectrophotometric measurements were carried out in a Spectromom 201 spectrophotometer.

Chromatography on a DEAE-Sephadex A-50 column

A 1.8 cm × 45 cm column was equilibrated with 0.1 M Tris–HCl buffer (pH 8.5), containing 1 mM $CaCl_2$ and 20 mM NaCl. The protein sample applied was first washed

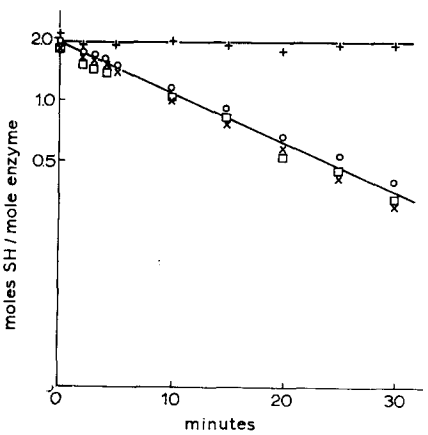
with the initial buffer, then an elution gradient was made by mixing it with the same buffer containing 0.2 M NaCl. 5-ml fractions were collected.

RESULTS

Reaction of amylase with DTNB in the presence of EDTA

The time course of the SH reaction of amylase with different amounts of DTNB was followed at 412 nm (Fig. 1). It is seen that a simple first-order reaction of both SH groups with DTNB takes place in the presence of EDTA. Differences in the reactivity of the two SH groups could not be observed.

As Figs 1 and 2 show, when only 1 mole equivalent of DTNB is added to the enzyme, the reaction is not finished after the release of 1 mole 2-nitro-5-mercaptobenzoate (NMB), as would be expected. The reaction proceeds until a second one is also released.



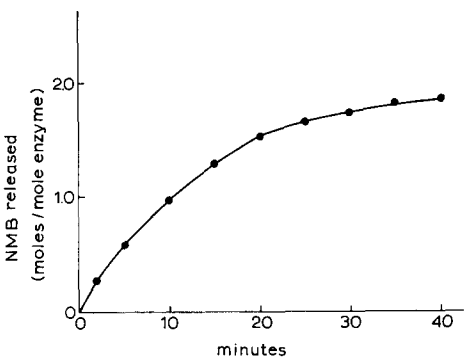


Fig. 1. Reaction of amylase with DTNB. Reaction mixture (3 ml): 20 μM amylase, 3 mM EDTA, in 0.1 M Tris–HCl buffer (pH 8.5). ○—○, 1 mole equivalent of DTNB; ×—×, 30 mole equivalents of DTNB; □—□, 30 mole equivalents of DTNB, 90-min preincubation with EDTA; +—+, 30 mole equivalents of DTNB in the absence of EDTA. Reaction was followed at 412 nm. Molar extinction coefficient for mercaptonitrobenzoate: 13 600.

Fig. 2. Reaction of amylase with 1 mole equivalent of DTNB. Experimental conditions as in Fig. 1.

To explain this unexpected behavior, we assume that first a mixed disulfide is formed with one SH group of the enzyme, and one NMB is released. The enzyme–NMB mixed disulfide is then decomposed by an SS–SH interchange reaction with the formation of a disulfide bridge between the two thiol groups of the enzyme and another NMB is released. We assume that an intramolecular disulfide is formed, since no association of enzyme molecules due to the possible formation of intermolecular disulfides could be detected by gel filtration on a Sephadex G-100 column. Addition of excess DTNB to the enzyme at the end of the reaction did not initiate any increase in the absorbance at 412 nm, no protein SH group was detectable in the reaction mixture. The thiol groups of amylase could be regenerated upon addition of excess 2-mercaptoethanol, confirming that a disulfide bridge was the product of oxidation with DTNB.

The consecutive processes of the formation of mixed and intramolecular disulfide take place as a single first-order reaction. The rate constant is $(6 \pm 2) \cdot 10^{-2}$ min^{-1}.

From these observations a structural change facilitated by the removal of Ca^{2+} (as the first-order rate limiting process of SH oxidation) can be supposed. As is shown in Fig. 1, the reaction is not accelerated if the enzyme is preincubated with EDTA, although its presence is necessary to obtain any reaction. Therefore, exposure of SH groups as a result of gradual unfolding after the removal of Ca^{2+} is excluded. The structural change can be interpreted on the basis of fluctuation of the polypeptide chain, as an equilibrium between at least two conformations, *i.e.* between molecules containing masked and unmasked SH groups after the removal of Ca^{2+} by EDTA.

The two SH groups of amylase, as it was interpreted above, can be oxidized by one equivalent of DTNB producing an intramolecular disulfide bond.

If DTNB is added in excess (30 moles of DTNB per mole of amylase) in the presence of EDTA, the reaction shows the same time course, as shown in Fig. 1. The reaction is again a first-order one and the rate constant is independent of the reagent concentration. 2 moles of NMB are finally released, but in this case (excess of DTNB) the result is an enzyme containing two mixed disulfides (enzyme–NMB_2).

This interpretation is supported by the following further experiments:

(1) We found that in 0.1 M NaOH the absorption maximum of DTNB and the enzyme–NMB mixed disulfide is shifted from 325 nm to 412 nm, to the absorption maximum of NMB. We studied the effect of NaOH on the 412-nm absorption of amylase, oxidized with 1 and 30 mole equivalents of Ellman reagent, respectively. 0.1 M NaOH was added to the gel-filtered enzyme samples after the release of 2 equivalents of NMB. (a) The absorption of the sample treated previously with 1 equivalent of DTNB did not change at 412 nm on addition of NaOH, showing that no DTNB or mixed disulfides were present. The same result was obtained if the gel-filtration was omitted. (b) When amylase was treated with 30 equivalents of DTNB, addition of NaOH to the gel-filtered enzyme increased the absorption at 412 nm, indicating the presence of amylase–NMB mixed disulfide bridges.

(2) The presence of reducible amylase–NMB mixed disulfides was also studied by using 2-mercaptoethanol as a reducing agent. The enzyme was treated with 1 and 30 equivalents of DTNB, respectively. After release of 2 equivalents of NMB the mixtures were passed through a Sephadex G-50 column, in the presence of Ca^{2+}, to remove NMB and excess DTNB from the solution. Then 0.1 M 2-mercaptoethanol was added to the enzyme samples. (a) The amylase sample treated previously with 1 mole equivalent of DTNB, did not show any increase in the 412-nm absorption. It shows that the mixed disulfides, formed during reaction with DTNB, were decomposed. (b) On addition of the reducing agent to the enzyme pretreated with 30 equivalents of DTNB, an increase was observed in the absorption at 412 nm, indicating the presence of reducible amylase–NMB mixed disulfide bonds.

(3) A mixture of amylase solutions, treated with 1 and 30 equivalents of DTNB was chromatographed on a DEAE-Sephadex column (Fig. 3).

As it is seen, two types of oxidized enzyme molecules, those containing the additional intramolecular disulfide and those containing two mixed disulfides could be separated.

It is concluded that the two SH groups of amylase can be oxidized by DTNB

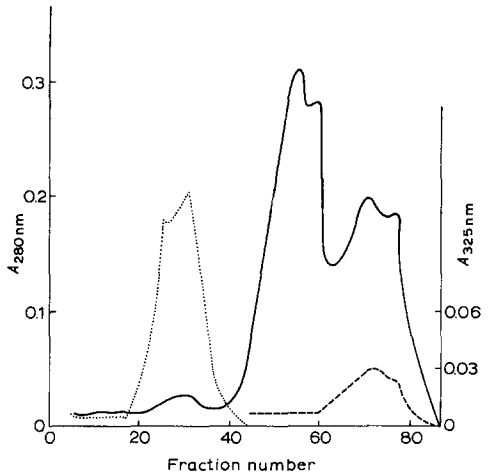


Fig. 3. Separation of amylase molecules oxidized with 1 and 30 mole equivalents of DTNB. 0.12 mM amylase solutions were treated with 1 and 30 equivalents of DTNB, respectively, until 2 moles of NMB per mole protein were released. After gel-filtration, the two solutions were mixed in equal amounts and 11 mg protein in 5 ml was chromatographed on DEAE-Sephadex as described in Materials and Methods. ———, $A_{280\,nm}$; — — —, $A_{325\,nm}$; . . . . . , $A_{280\,nm}$ of native control.

in two ways, producing either two mixed, or one intramolecular disulfide bond, depending on the relative amount of the reagent added.

It was found that the reaction of amylase with DTNB could be stopped at any stage by the addition of Ca^{2+}. This phenomenon proved to be a method to study the enzyme species present at different stages of the SH reaction.

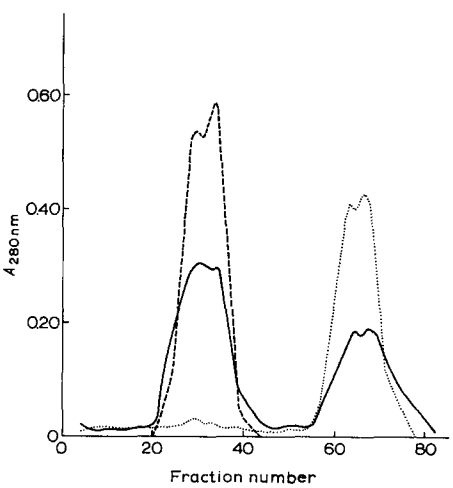


Fig. 4. Chromatography of DTNB-treated amylase on DEAE-Sephadex. 50 mole equivalents of DTNB were added to 80 μM amylase solutions, in the presence of 3 mM EDTA. After release of 1 and 2 moles NMB per mole enzyme, respectively, the reaction was stopped adding 10 mM $CaCl_2$ to the mixture. The solution was passed through a Sephadex G-50 column equilibrated with 0.1 M Tris–HCl buffer (pH 8.5), containing 1 mM $CaCl_2$ and 20 mM NaCl. The protein sample (10 mg in 5 ml) was then chromatographed as described in Materials and Methods., — — —, native control; ———, DTNB-treated, after release of 1 mole NMB; . . . . . , DNTB-treated, after release of 2 moles NMB.

Two amylase samples were treated with excess of DTNB. The reactions were stopped at the half time and after the completion, respectively, adding 10 mM $CaCl_2$ to the reaction mixture. The excess of DTNB used, leads to the formation of mixed disulfides with both SH groups. The solutions were applied to a DEAE-Sephadex column and NaCl gradient elution was performed (Fig. 4).

It is seen that amylase, chromatographed after release of 2 NMB per enzyme molecule, was eluted in one peak. The data reveal that two peaks of protein were obtained, when the reaction was stopped at half time. About half of the amount of the eluted protein was collected in the fractions of unreacted enzyme. The fractions pooled from the second peak contained the oxidized amylase molecules in which two mixed disulfides were formed.

Similar separation was made when only 1 equivalent of DTNB was added, leading to intramolecular disulfide formation, and the product was chromatographed as described above. At half time two peaks were observed representing a native and a disulfide amylase. After completion of the reaction only the latter one could be observed.

It is concluded that the reaction of 1 SH group per enzyme molecule is followed by the rapid reaction of the other SH group of the same molecule. Thus, at any stage of SH reaction only two types of molecules (those containing no SH groups and intact ones, containing 2 SH groups) are present. Enzyme molecules containing one mixed disulfide and one SH group could not be detected. This kind of molecule could not be found even if the enzyme was only partially oxidized with one equivalent of DTNB and gel filtered after the addition of Ca^{2+}. No change of absorption at 412 nm occurred on addition of 2-mercaptoethanol or NaOH to this partially oxidized enzyme preparation.

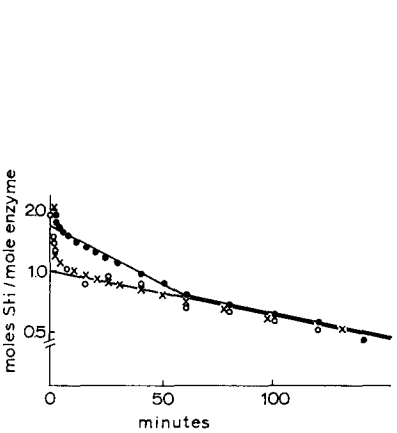
Effect of urea on the reactivity of SH groups of amylase

The reaction of amylase with DTNB was measured in 7 M urea with slight excess of the Ellman reagent, in the absence of EDTA (Fig. 5).

When amylase is not preincubated in urea, initially there is a very rapid reaction, showing that a small fraction of the molecules is unfolded when the reaction starts, because of the presence of urea. This fraction contains unmasked thiol groups and their reaction is a second order one. This is followed by a biphasic process that can be separated into two first-order reactions.

After preincubation of the enzyme in 7 M urea for 1 h, one of the SH groups becomes exposed, as a result of partial unfolding of the molecules, while the reaction of the other SH group proceeds at the same rate as in case of no preincubation in urea. The first part of the biphasic reaction disappears showing that it might have been the rate of unfolding of the molecules. The picture does not change after 2 h of preincubation of amylase in urea (Fig. 5).

The rate of reaction of the second SH group is independent of the concentration of the reagent showing that it is a real first-order reaction, the rate constant is $5 \cdot 10^{-3}$. It should be mentioned that after a 1-h incubation of the enzyme in 7 M urea, the buried position of both SH groups can be restored on dilution of the enzyme with 1 mM $CaCl_2$. Moreover, the enzyme activity is completely restored by such treatment. It follows that the enzyme structure is not irreversibly destroyed in 7 M urea. These



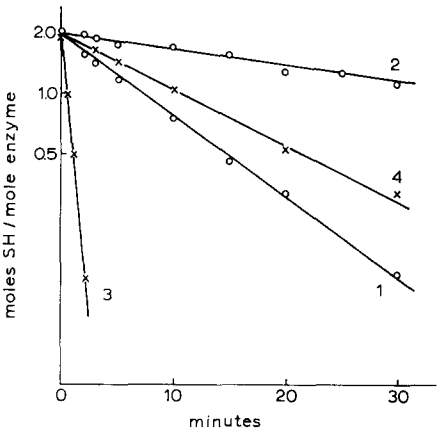


Fig. 5. Reaction of amylase with DTNB in 7 M urea. 15 mole equivalents of DTNB were added to 20 μM amylase solution in 7 M urea. The reaction was measured at 25 °C and preincubation in urea was made at the same temperature. ○—○, 60-min preincubation in urea; ×—×, 2-h preincubation in urea; ●—●, no preincubation.

Fig. 6. Effect of maltose on the reactivity of SH groups of amylase. Amylase: 20 μM, DTNB: 0.3 mM. ○—○, in 0.1 M Tris (pH 8.5) + 3 mM EDTA; 1, no maltose added; 2, 60 mM maltose added. ×—×, in 2 M urea + 0.3 mM EDTA; 3, no maltose added; 4, 60 mM maltose added.

results suggest the interpretation that the reaction of the second SH group is still controlled by the fluctuation of the relevant part of the enzyme molecule.

Effect of maltose on the fluctuation of amylase around the SH groups

The influence of maltose on the kinetics of SH reaction with Ellman reagent was studied in 0.1 M Tris buffer (pH 8.5) containing 10 mM EDTA and in 2 M urea containing 0.3 mM EDTA, respectively (Fig. 6).

The reactivity of SH groups is decreased on the addition of maltose. The equilibrium of fluctuation is shifted to the enzyme form containing the buried SH groups. In other words, maltose stabilizes the more compact structure of the protein.

TABLE I

ACTIVITY OF AMYLASE MODIFIED BY DTNB

Modification was performed in the presence of 3 mM EDTA. Concentration of amylase was 20 μM. Enzymic activity was measured at 15 °C.

Temperature of SH reaction (%)	*DTNB added (moles/mole enzyme)*	*NMB released (moles/mole enzyme)*	*Activity of modified amylase (%)*	
				24-h incubation at 5 °C*
5	1	1.8	98	50
5	30	1.8	95	45
25	1	1.8	43	42
25	30	2.0	47	40
25	—	—	100	94

* Modified amylase was incubated in the presence of 5 mM $CaCl_2$.

Enzymic activity of oxidized amylase

Enzymic activity was followed during the reaction of amylase in the presence of EDTA with 1 and 30 mole equivalents of Ellman reagent, respectively (Table I).

If the reaction of SH groups is performed at 5 °C, the activity is practically unchanged during the reaction. After prolonged incubation of the modified enzyme, the activity decreases, but even after 24 h of incubation the enzyme still has 40–50% activity. When the SH groups react with DTNB at 25 °C, the amylase activity gradually decreases to about 50%, and there is no more decrease even after several hours of incubation.

These data show that none of the two SH groups is essential for the enzyme function, but their modification results in the decrease of the stability of the protein structure. It seems that the partially active enzyme possesses a rather stable conformation.

DISCUSSION

Some results of this work have led to the conclusion that the reactivity of SH groups of amylase is controlled by the fluctuation of enzyme structure.

The idea of motility of protein structure has been raised by several authors[10–12] and it has been supported by several experimental results[13–17].

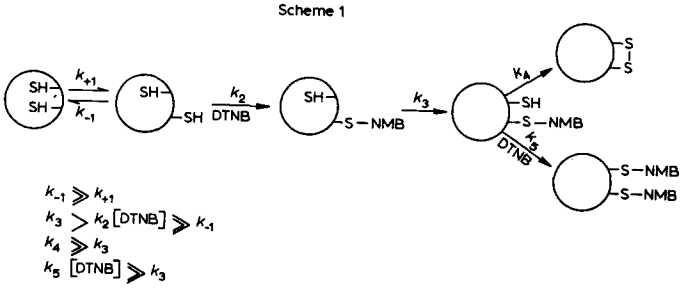
In the present work the increase of motility of amylase (at least in the region near to the two masked SH groups) due to removal of Ca^{2+} was observed. Although this motility of the amylase molecule could be detected only in the absence of added Ca^{2+}, the possibility of fluctuation in the presence of excess Ca^{2+} cannot be excluded. Addition of EDTA actually does not remove the firmly-bound Ca^{2+}, only the loosely-bound Ca^{2+} are removed in this way[18]. Evidently, the fluctuation is greatly influenced by the Ca^{2+} loosely bound to amylase.

According to the kinetics of formation of mixed and intramolecular disulfides, it is assumed that the rate of both processes is limited by the rate of fluctuation of the enzyme structure. Formation of an intramolecular disulfide shows that the SH groups are (or become) sterically close to each other during reaction.

Robyt *et al.*[19] have found that amylase forms more mixed disulfides with DTNB than could be expected. They postulated that disulfide groups were cleaved by the NMB released from DTNB during SH reaction. Under the conditions used in our experiments disulfide bridges of amylase were not attacked by NMB.

The following scheme is proposed for the reaction of SH groups of amylase with DTNB in the presence of EDTA (Scheme 1).

Scheme 1



The rate of the overall reaction is characterized by the first-order rate constant of the rate-limiting fluctuation (k_{+1}). As Scheme 1 shows, the reactivity of the two SH groups is different. The reaction of only one SH group is controlled by the fluctuation. Its reaction is followed by the rapid unmasking and reaction of the other SH group. No accumulation of amylase containing one mixed disulfide occurs ($k_3 > k_2$ [DTNB]). The end product of the reaction is dependent on the concentration of DTNB.

The kinetics of the reaction of amylase with DTNB in urea suggests that fluctuation exists even after prolonged incubation of the protein in concentrated urea solution, although one of the SH groups becomes exposed at the surface of the molecule. This is in accordance with the spectrophotometric observation of Elödi and Krysteva[20], who have shown by perturbation of chromophores in 8 M urea that amylase was not completely unfolded even after incubation for 24 h.

Elödi[21] has shown that reaction of diethylpyrocarbonate with two histidyl residues inactivates the amylase and as a result one SH group becomes exposed and reacts with DTNB in the absence of EDTA. It shows that one of the two buried thiol groups is probably close to the surface and the active center of the enzyme molecule. The different reactivity of SH groups in urea may be due to the difference in their location in the interior of the molecule, and one of them may not be exposed to the attack of urea.

We found that maltose decreases the reactivity of thiol groups. It is known that maltose as a product of amylase reaction is a competitive inhibitor with respect to the substrate, indicating that it can be bound to the substrate binding site. However, we have shown that the SH groups do not participate in substrate binding, since their modification does not alter the activity of amylase. Therefore, the effect of maltose on SH reactivity can be explained by a secondary effect taking place when the substrate binding site is occupied. This secondary effect results in the stabilization of the more compact structure of amylase. As seen in Fig. 6, the reactivity of SH groups is markedly decreased by maltose even in the presence of urea and EDTA, though in this medium the architecture of the amylase molecule is otherwise far from the native form. Moreover, according to our results, not documented here, Cl^-, the activator of amylase, also decreases the reactivity of SH groups in a similar manner to the effect of maltose. Therefore, the stabilization of the compact structure by the substances mentioned above is probably a more general indirect effect.

ACKNOWLEDGMENT

Thanks are due to Miss J. Vurszta and Mrs E. Pekáry for their skillful technical assistance.

REFERENCES

1 Fischer, E. H., Summerwell, W. N., Junge, J. and Stein, E. A. (1958) *Proc. 4th Int. Congr. Biochem. Vienna*, Symp. 8, pp. 124–137, Pergamon Press, London
2 Hsiu, J., Fischer, E. H. and Stein, E. A. (1964) *Biochemistry* 3, 61–66
3 Schramm, M. (1964) *Biochemistry* 3, 1231–1234
4 Stein, E. A., Hsiu, J. and Fischer, E. H. (1964) *Biochemistry* 3, 56–61
5 Fischer, E. H. and Stein, E. A. (1960) in *The Enzymes* (Boyer, P. D., Lardy, H. and Myrbäck, K., eds), Vol. 4, p. 313–343, Academic Press, New York

6 Hatfaludi, F., Strashilov, T. and Straub, F. B. (1966) *Acta Biochim. Biophys. Acad. Sci. Hung.* 1, 39–44
7 Smith, B. W. and Roe, J. H. (1949) *J. Biol. Chem.* 179, 53–59
8 Závodszky, P. and Elödi, P. (1970) *Acta Biochim. Biophys. Acad. Sci. Hung.* 5, 225–229
9 Ellman, G. L. (1959) *Arch. Biochem. Biophys.* 82, 70–77
10 Schellman, J. A., Lumry, R. and Samuels, E. T. (1954) *J. Am. Chem. Soc.* 76, 2808–2813
11 Linderstrøm-Lang, K. U. and Schellman, J. A. (1959) in *The Enzymes* (Boyer, P. D., Lardy, H. and Myrbäck, K., eds), Vol. 1, p. 443–510, Academic Press, New York
12 Straub, F. B. and Szabolcsi, G. (1964) in *Mol. Biol.* pp. 182–187, Nauka Moscow
13 Hvidt, A. and Linderstrøm-Lang, K. U. (1954) *Biochim. Biophys. Acta* 14, 574–575
14 Barnard, E. A. (1964) *J. Mol. Biol.* 10, 235–262
15 Barnard, E. A. (1964) *J. Mol. Biol.* 10, 263–281
16 Závodszky, P., Abaturov, L. B. and Varshavsky, Y. M. (1966) *Acta Biochim. Biophys. Acad. Sci. Hung.* 1, 389–402
17 Vas, M. and Boross, L. (1970) *Acta Biochim. Biophys. Acad. Sci. Hung.* 5, 203–213
18 Vallee, B. L., Stein, E. A., Summerwell, W. N. and Fischer, E. H. (1959) *J. Biol. Chem.* 234, 2901–2905
19 Robyt, J. F., Ackerman, R. J. and Chittenden, C. G. (1971) *Arch. Biochem. Biophys.* 147, 262–269
20 Elödi, P. and Krysteva, M. (1970) *Acta Biochim. Biophys. Acad. Sci. Hung.* 5, 449–462
21 Elödi, P. (1972) *Acta Biochim. Biophys. Acad. Sci. Hung.* 7, 241–245