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# THE SULFHYDRYL GROUPS OF PORCINE PANCREATIC α-AMYLASE: UNMASKING, REACTIVITY AND FUNCTION

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## SUMMARY

The two SH groups of porcine pancreatic α-amylase (α-1,4-glucan-4-glucanohydrolase, EC 3.2.1.1) are masked; they react with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) in the presence of EDTA and/or urea. Both SH groups are unmasked with the same rate constant. The unmasking reaction has been shown to be a pseudomonomolecular process. The reactivity of unmasked SH has been studied by preincubating amylase with EDTA. The DTNB reaction of SH in the presence of EDTA is a first-order process with respect to amylase concentration, and both SH groups have the same reactivity. The blocked amylase species have been purified: a part of amylase was found in the S–S form suggesting the close vicinity of the SH groups.

Complete blocking of the SH results in a total loss of enzymatic activity. The equation of the kinetics curve of the loss of enzymatic activity has been determined. The importance of SH for the enzyme activity is discussed. One of the SH groups is not present at the catalytic center. Inhibition by a substrate analog (maltotriose) of the DTNB-blocking reaction suggests that the SH might be part of or close to the binding site(s).

## INTRODUCTION

Porcine pancreatic α-amylase (α-1,4-glucan-4-glucanohydrolase, EC 3.2.1.1) contains calcium [1–3]. Two molecular forms [4, 5] exist as a unique polypeptide chain of 470 residues with identical activities, molecular weight, end groups and amino acid composition, except that amylase I contains five asparagine units more than amylase II [6]. Some insight into the primary structure has recently been obtained. Amylase was cleaved by CNBr, and the nine peptides thus obtained were purified and characterized by end groups and amino acid composition [7]. The ordering of the peptides in the sequence was achieved by the use of an isotopic technique based upon pulse

Abbreviations: DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); TNB, 5-thio-2-nitrobenzoic acid radical.

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labeling [8]. The location of the four disulfide bridges and of the two free SH groups in the chain was also determined.

Previous studies by Schramm [9] have shown that the SH groups are masked in native amylase, and that they react with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) only in the presence of EDTA and/or urea. Schramm also found that the blocking of SH by DTNB inhibits enzyme activity.

The aim of our work was first to get more quantitative information on both SH groups from a structural point of view, and, second to elucidate a possible functional role for each. The accessibility and the reactivity of SH towards DTNB were measured under various conditions. The modified amylase species were next isolated by gel filtration and characterized by their 5-thio-2-nitrobenzoic acid radical (TNB) content and their ultraviolet spectrum. The loss of enzymatic activity due to the blocking by DTNB was measured at various stages of the reaction. The effect of maltotriose on the DTNB reaction was also studied.

The results provide information on the environment, accessibility and the relative spatial vicinity of the SH groups, as well as on possible functional roles. A model which accounts for the loss of activity as well as for the unmasking process is presented. A preliminary report of this work has already been published [10]. Very recently Telegdi and Straub [11] have reported their work on the motility and the reactivity of SH towards DTNB in $\alpha$-amylase. Their conclusion differs on the EDTA effect and on the mechanism of loss of amylase activity.

## MATERIAL AND METHODS

### *Preparation of amylases*

$\alpha$-Amylase was purified from porcine pancreas tissue homogenate according to Fischer and Bernfeld [12]. Amylases I and II were finally separated by chromatography on DEAE-cellulose as previously described [4]. Preparations were routinely checked for purity by polyacrylamide disc electrophoresis at pH 8.6 and by amino acid analysis [5].

### *Assay procedures*

Protein was determined by the method of Lowry et al. [13] calibrated with crystalline serum albumin.

The amylase content of the pure preparations was measured spectrophotometrically ($E^{1\%}_{1\,cm}$ at 280 nm = 25) and by amino acid analysis.

Amylase activity was measured at pH 6.9 and 30 °C by reductometry with dinitrosalicylate as described by Noelting and Bernfeld [14] using purified soluble starch.

### *Titration of SH groups by DTNB*

The reaction of DTNB with SH groups was carried out according to the method of Ellmann [15]. The assay mixture contained 10–20 $\mu$M amylase in 1 mM NaCl 40 mM Tris–HCl buffer, pH 7.9, 0.2 mM DTNB and when present 0.1–10 mM EDTA, 6 M urea and 1 % sodium dodecylsulfate in a final volume of 3 ml.

The solutions were placed in a 3-ml thermostated cuvette at 25 °C of a Cary 14 recording spectrophotometer and the reaction was followed by measuring the increase in absorbance at 412 nm against a blank without amylase.

The number of reacting SH groups was calculated from the maximal absorbance, using a molar extinction coefficient value of 13 600 for the liberated TNB anion. This value obtained by Ellmann [15] was checked in the presence or absence of sodium dodecylsulfate, urea or EDTA with known solutions of pure cysteine.

## RESULTS

All experiments reported here were carried out using amylases I and II. Identical results were obtained with both forms.

### (*1*) *Accessibility and reactivity of SH groups towards DTNB*

(*a*) *The EDTA effect.* We have confirmed Schramm's findings [9] that the sulfhydryl groups in native pancreatic $\alpha$-amylase do not react with DTNB in the presence of buffer alone or even with 1 % sodium dodecylsulfate. When EDTA is added to native amylase a sluggish reaction occurs. However, in denaturing media (1 % sodium dodecylsulfate or 6 M urea) containing 10 mM EDTA the reaction is very fast (Fig. 1).

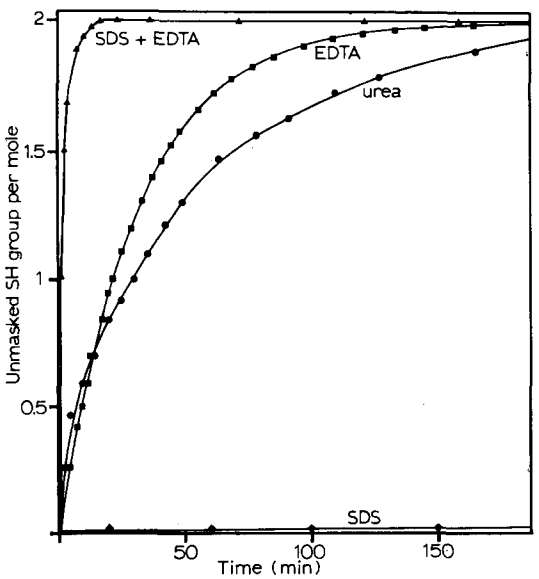



Fig. 1. The blocking of amylase SH groups by DTNB under various conditions. All reactions are carried out at 25 °C in 40 mM Tris–HCl buffer, pH 7.9, and in 0.2 mM DTNB. The amylase concentration is 12 $\mu$M. In addition, the reaction mixture contains: ◆—◆, 1 % sodium dodecylsulfate; ●—●, 6 M urea; ■—■, 10 mM EDTA; ▲—▲, 1 % sodium dodecylsulfate + 10 mM EDTA. The reaction is followed by measuring the absorbance of TNB ions liberated, at 412 nm.

We thus agree with Schramm's conclusions that the SH groups are masked in amylase containing calcium and that in the presence of EDTA alone the unmasking reaction is rate limiting. We have extended this work by performing a more detailed study on the kinetical aspect of this phenomenon. As shown in Fig. 1, a complete titration of the two SH groups by DTNB + EDTA was obtained. We have also found that the

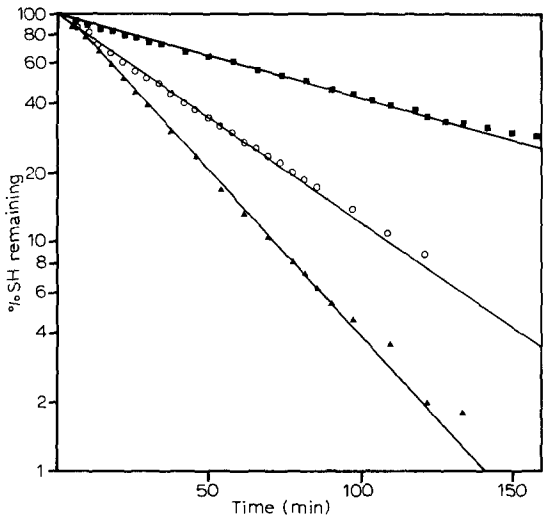



Fig. 2. The effect of EDTA at various concentrations on the DTNB reaction. The reactions are carried out at the same temperature, buffer and DTNB concentration as in Fig. 1. The concentrations in EDTA are: ■—■, 0.1 mM; ▲—▲, 1 mM; ○—○, 10 mM. In all three experiments the initial amylase concentration is 10.7 $\mu$M.

semi-logarithmic plot of this curve is linear (Fig. 2); both SH groups are then unmasked with the same rate constant ($K = 0.02$ min$^{-1}$ using EDTA 10 mM and amylase 10.7 $\mu$M), but this rate constant is dependent upon the EDTA concentration (Fig. 2). Surprisingly 1 mM EDTA is more efficient than higher concentrations. This point is unclear and suggests a complex mechanism in the removal of $Ca^{2+}$. However, 10 mM EDTA will be used in all other experiments (standard conditions). Also we have found that the rate of the unmasking reaction increases with amylase concentration in the range of concentrations studied (10–20 $\mu$M); when referred to standard conditions amylase has been used at 10.7 $\mu$M concentration. The unmasking reaction is, therefore, a pseudomonomolecular process. In the next experiment, we have been able to measure the reactivity of the SH groups unmasked after preincubation with EDTA.

(*b*) *The EDTA effect with preincubation.* Amylase, at various concentrations, was preincubated for 30 min at 25 °C in 10 mM EDTA, before adding 0.2 mM DTNB. The kinetics of liberation of TNB ions was then followed (Fig. 3). The kinetics are different depending upon the amylase concentrations. At a concentration of 9 $\mu$M the kinetic plot is almost linear, but at slightly higher concentrations three processes are apparent: a fast process at the beginning of the reaction, an intermediate process and a slow one at the very end. Because of its small contribution this last process was neglected. Only the first two parts of the kinetics were analyzed. The contribution of the early reaction was calculated by subtracting the values due to the intermediate process. As shown in Fig. 3, the semi-log kinetic curve of the first reaction is linear and independent of the amount of amylase in this range of concentration. It is a first-order process with respect to amylase concentration ($K_0 = 0.11$–$0.15$ min$^{-1}$ at 0.2 mM DTNB, 10 mM EDTA). This result indicates that the two unmasked SH groups have

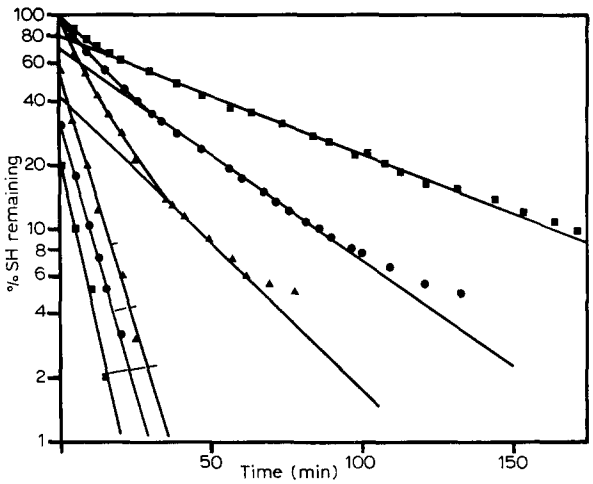
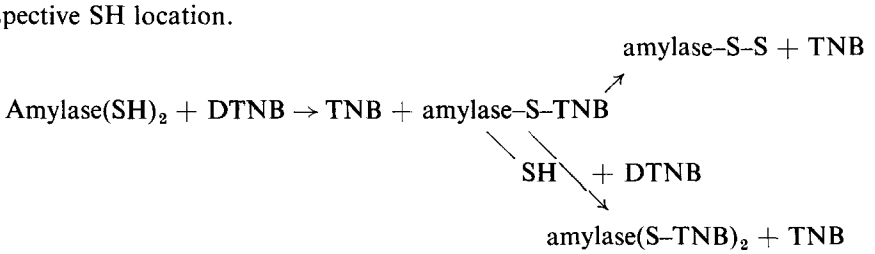



Fig. 3. Effect of preincubating amylase at various concentrations with EDTA. Amylase (■—■, 9.1 $\mu$M; ●—●, 12.6 $\mu$M; ▲—▲, 20.1 $\mu$M) is preincubated 30 min at 25 °C in 40 mM Tris–HCl buffer, pH 7.9, and in 10 mM EDTA. At this time 0.2 mM DTNB is added and the TNB ion liberation is routinely measured.

the same reactivity towards DTNB. The second process presumably corresponds to the unmasking reaction measured without preincubation (Fig. 2). As previously found the rate of this reaction increases with the initial amylase concentration ($K$ = 0.013, 0.022, 0.031 $min^{-1}$). The intercept of the extrapolated curve allows the calculation of the amount of amylase unmasked during the preincubation period.

*(2) Preparation and molecular properties of modified amylase*

The liberation of TNB ions does not necessarily imply that the unmasked SH groups have been blocked with TNB. In particular, an exchange reaction between two neighbouring SH groups (one of which is TNB blocked) can occur to form one disulfide bridge [16]. If the two groups belong, respectively, to two protein molecules, intermolecular bridges can be formed, and dimers or even polymers can accumulate. If the SH groups are in close vicinity in the same protein molecule, intramolecular bridges can be formed. This latter case is of special interest from the point of view of the respective SH location.




Amylase was incubated with DTNB and EDTA under standard conditions until the reaction was complete, as indicated by the stoichiometric liberation of two TNB ions. The modified amylase species were then purified by gel filtration in order to check the amount of TNB covalently bound to amylase. By measuring the absor-

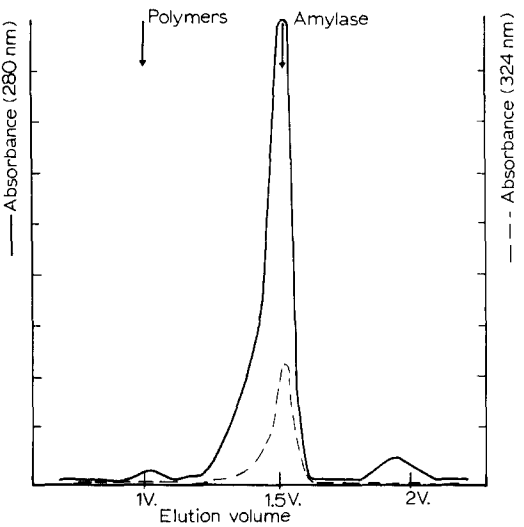



Fig. 4. Gel filtration of modified amylase. Amylase is incubated in 40 mM Tris–HCl buffer, pH 8, 10 mM EDTA, 0.2 mM DTNB at 25 °C until the stoichiometric liberation of two TNB ions. The reaction mixture (6 mg amylase in 8 ml) is filtered through a Sephadex G-100 column (1.5 cm × 90 cm) in 40 mM Tris–HCl buffer, pH 8.0, containing 1% sodium dodecylsulfate. The flow rate is 11 ml/h. The absorbance of the eluted fraction is measured at 280 nm (———) and at 324 nm (— — —). The arrows indicate the position at which the polymers and the unreacted amylase elute.

bance at 324 nm, it was found that the average TNB content of the modified amylase (the extinction coefficient $E$ (S–TNB) = 9050 $M^{-1} \cdot cm^{-1}$, ref. 16), was one-half of that expected if the two blocked SH were present as S–TNB. However, the purified modified amylase does not react further with DTNB. This indicates that the two SH groups are actually blocked. The gel filtration pattern is given in Fig. 4. Only one major peak (absorbance at 280 nm) is present at 1.5 $V_0$. As a control amylase was chromatographed in the same conditions, its elution peak appears at the same position (arrow) as one of this modified species. It is then concluded that in our conditions the only species formed is of the monomeric type and that no polymer is present (a polymeric form is obtained when the DTNB reaction is carried out with EDTA in 1% sodium dodecylsulfate). It is then proposed that half of the amylase molecules are present as di-TNB species and the other half contains a new fifth intramolecular disulfide bridge. Actually, these two species are partially separated by filtration as shown by the asymmetrical 324-nm absorbance pattern (Fig. 4). The di-TNB amylase is more retained on Sephadex than the S–S species. Incubation of the TNB amylase-containing fraction (1.5–1.6 $V_0$), with $\beta$-mercaptoethanol in excess liberates 1.6–1.8 moles of TNB per amylase molecule. Whereas no TNB liberation is obtained in the case of S–S amylase (1.2–1.4 $V_0$). Moreover, amylase species in 1.2–1.4 $V_0$ and in 1.5–1.6 $V_0$ fractions have been characterized by their ultraviolet spectrum (Fig. 5). The 1.5–1.6 $V_0$ fraction gives a typical di-TNB spectrum with a new maximum in the 325–330-nm region, between 260 and 310 nm the absorbance pattern is identical to the one obtained with native amylase. In contrast, the spectrum of the 1.2–1.4 $V_0$ fraction (S–S species) differs from native amylase in having a 5-nm shift of the maximum towards the low wavelength. This shift is certainly indicative of a change in the protein conformation.

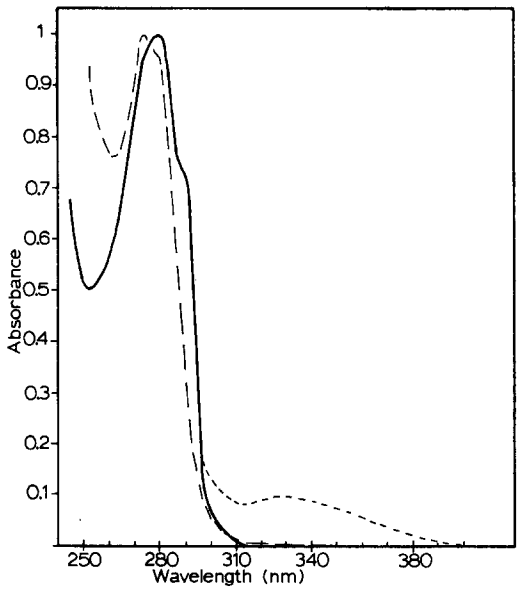



Fig. 5. Ultraviolet spectra of native (———) di-TNB (·····) and S–S (— — —) amylase. The spectra are recorded with a Cary Model 14 spectrophotometer. The solution medium is 40 mM Tris–HCl buffer, pH 8.0, containing 1% sodium dodecylsulfate. The protein concentration is 0.4 mg/ml. An identical spectrum was obtained in the case of native amylase with or without sodium dodecylsulfate.

*(3) Effect of DTNB on enzymatic activity*

The selective blocking of the two SH groups by DTNB, whatever the amylase species obtained, results in a complete loss of activity. Amylase was incubated with DTNB in standard conditions and the remaining enzymatic activity was assayed at various times on aliquot fractions, while the TNB ion liberation was simultaneously measured. The semi-log plot of both of these processes is given in Fig. 6. As previously found (Fig. 2) the TNB liberation is linear ($K = 0.02$ min$^{-1}$). The loss of amylase activity ($A/A_0$) is at first lower than the TNB liberation, but then the activity decreases in a parallel fashion to the liberation of TNB.

Analysis of the activity plot indicates that the overall process is characterized by two different rate constants $k_1$ (0.043 min$^{-1}$) and $k_2$ (0.021 min$^{-1}$) according to the velocity equation:

$$v = v_1 e^{-k_1 t} + v_2 e^{-k_2 t}$$

The rate constant $k_1$ of the first process is given by the slope of the straight line obtained by subtracting the experimental values from the extrapolated ones corresponding to the second process.

As a control native amylase was incubated under the same conditions except that the DTNB was omitted. The slight loss of activity observed (Fig. 6) was taken into account for correcting the experimental values. The final calculations for the corrected values are: $k_1 = 0.040$ min$^{-1}$, and $k_2 = 0.020$ min$^{-1}$. The equation of the loss of activity obtained empirically is thus:

$$A/A_0 = 1.5e^{-0.02t} - 0.5e^{-0.04t} \qquad (1)$$

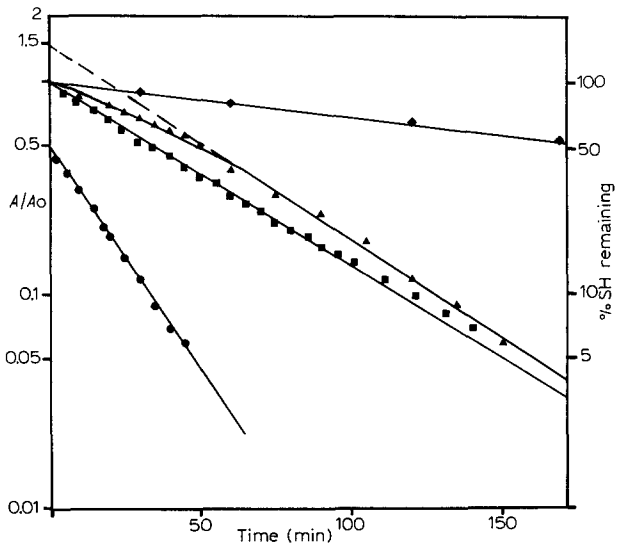



Fig. 6. Loss of amylase activity as the result of the blocking of SH. ▲—▲, loss of amylolytic activity (DTNB-treated amylase). The full line represents the calculated values, the triangles are experimental; ●—●, loss of amylolytic activity (obtained by subtraction); ◆—◆, loss of amylolytic activity (amylase incubated without DTNB and in the presence of EDTA); ■—■, percent of SH remaining (from TNB liberation). The reaction mixture (10.7 $\mu$M amylase, 40 mM Tris–HCl buffer, pH 8.0, 0.2 mM DTNB, 10 mM EDTA) was incubated at 25 °C. Aliquots of the reaction mixture are taken up at the time indicated, immediately cooled by cold phosphate buffer and assayed for amylolytic activity.

The slow decrease in enzyme activity observed at the beginning of the reaction indicates that the intermediate mono-TNB amylase is more than 50% active. By matching the empirical equation with the simple two-step model:

$$\text{amylase} \xrightarrow[k_1 = 0.04]{} \text{mono-TNB amylases} \xrightarrow[k_2 = 0.02]{} \text{di-TNB amylase}$$

it follows that the average mono-TNB amylase species is 75% active. The fact that

$$k_1 = 2k_2$$

and the similarity between the unmasking constant ($K$) and the loss of activity constant ($k_2$) will be examined in the discussion.

(*4*) *Enzymatic properties of modified amylase*

In order to obtain more information on a possible function of SH in enzymatic activity, we have determined the kinetic parameters $V$ and $K_m$ in native and partially blocked amylase for comparison. The DTNB reaction was stopped at various times by adding a large volume of cold 0.02 M phosphate buffer, pH 6.9. The amount of blocked SH was measured and the amylolytic activity was assayed as described above, using soluble starch as substrate. Since the molecular weight of the starch used is not

TABLE I

KINETICS PARAMETERS OF MODIFIED AMYLASE

Partially blocked amylase was obtained using the incubation conditions as in Fig. 6. In the assays the starch concentration was within 0.5–0.03 %. The hydrolysis rates were taken from the initial slopes.

| Number of SH blocked | $V$ | $K_m$ (mg/ml) |
|---|---|---|
| 0 | 1640 | 0.54 |
| 0.41 | 1530 | 0.83 |
| 0.5 | 1530 | 0.98 |
| 0.9 | 1000 | 1.00 |
| 1.2 | 816 | 1.02 |

defined, we have expressed $K_m$ in mg/ml (Table I). When 0.5 SH are blocked the Michaelis constant $K_m$ appears increased by a factor of 2, while no significant change is seen in the apparent maximum velocity $V$. At a higher amount of blocked SH the $V$ decreases. Due to the multiplicity of the modified amylase species present no clear relationship appeared.

*(5) Effect of maltotriose on the amylase–DTNB reaction*

The existence of two substrate binding sites has been demonstrated by Loyter and Schramm [17] using maltotriose as substrate. We have then attempted to check the hypothesis that the SH groups might be a part of or close to the binding site(s) or involved in some way in the binding of the substrate. If such were the case the maltotriose should interfere with the amylase–DTNB reaction. Maltotriose at a 20-fold molar excess was preincubated for 3 h with amylase at 0 °C in 40 mM Tris–HCl buffer, pH 7.9 (without EDTA) then DTNB and EDTA were added and the reaction

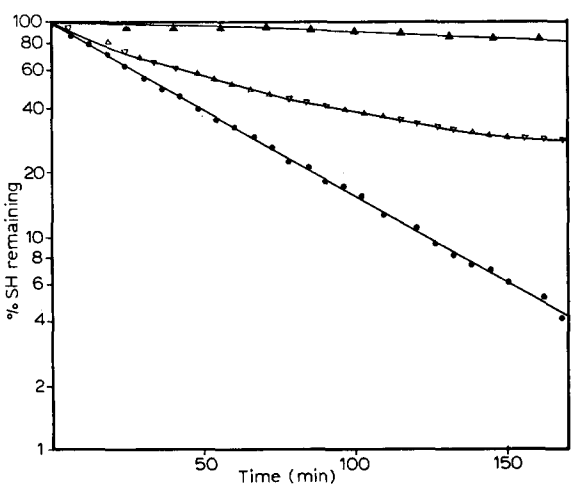



Fig. 7. Effect of maltotriose on the amylase DTNB reaction. Except for maltotriose and amylase the reaction conditions are as in Fig. 6. The amylase concentration is 10 $\mu$M. ●—●, no maltotriose added; △—△, 20-fold molar excess of maltotriose (after a 3-h incubation without EDTA); ▲—▲, 10 000-fold molar excess of maltotriose (no preincubation).

was allowed to proceed at the standard conditions. The liberation of TNB ions is strongly inhibited (Fig. 7), and at this low maltotriose concentration the reaction never reaches completion (1.45 SH blocked). Without preincubation with a 20-fold molar excess of maltotriose no inhibition is apparent. Maltotriose at a 10 000-fold molar excess completely inhibits the blocking of SH even without preincubation.

## DISCUSSION AND CONCLUSION

### *(1) The masking of SH by calcium and their spatial location*

The masking of SH groups by $Ca^{2+}$ appears to be a rather general feature of SH-containing α-amylases such as Taka-amylase [18] and the saccharifying amylase from *Bacillus subtilis* [19]. In the case of porcine amylase, calcium is known to be of structural importance in the maintenance of the tertiary structure, since its removal strongly diminishes the resistance [3] of amylase towards denaturing agents and proteolytic enzymes but very little is known on the role of calcium in the masking process [20]. Our results indicate that the calcium implied in SH masking is easily removed and is thus of the weakly-bound type as compared to the tightly-bound calcium necessary for enzyme activity [1, 2]. Also in the range of amylase concentration tested it appears that the rate constant of unmasking is dependent upon the initial amylase concentration (Fig. 3). This concentration effect is consistent with the model presented in the next part of the discussion. Since SH groups can be unmasked in the presence of urea alone, one must conclude that calcium helps to stabilize an amylase form (native form) of which the SH are not accessible to DTNB.

It should be noted that in all the conditions employed the accessibility and the reactivity of both SH towards DTNB in native amylase was found to be identical: this certainly implies a similar environment of these two groups. Moreover, the formation of a fifth intramolecular disulfide bridge in the amylase-blocked molecules should be emphasized. This result indicates that both SH are in a close spatial vicinity. A similar conclusion was recently obtained by Steer and Levitski [20] using a mercurial reagent for SH.

From the ultraviolet spectra, the overall conformation of the di-TNB amylase does not appear to differ from the native one; however, the formation of the fifth intramolecular disulfide bridge introduces visible changes in the spatial structure. This difference between the two amylase species is unclear.

### *(2) The dimeric model*

The monomeric model (amylase → mono-TNB amylase → di-TNB amylase) assumes that the loss of activity during the first process corresponds to the blocking of one SH by DTNB and that the second process corresponds to the blocking of the second SH. This model has not been retained for the following reasons: (1) It would imply a two-step unmasking of amylase SH which is not apparent from the kinetics of TNB liberation, moreover, both SH are found in a close spatial vicinity. (2) The concentration effect of amylase in the unmasking (DTNB) reaction is not explained by the monomeric model.

We then propose a dimeric model which accounts for the kinetics of TNB liberation and for the loss of activity as well as the molecular forms obtained after complete blocking in the standard conditions.

The concentration effect of amylase in the DTNB reaction can be explained if the enzyme is more easily unmasked in a polymeric state. We propose a dimer whose subunits are loosely bound, each subunit being unmasked independently in the subsequent reaction (Fig. 8).

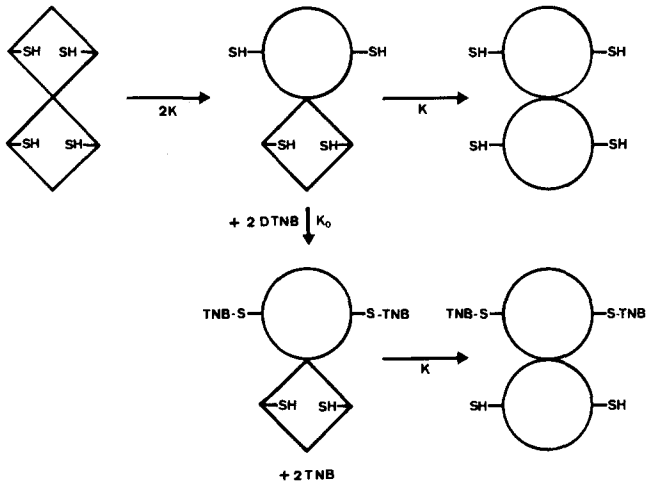



Fig. 8. The dimeric model. The squares represent the native amylase with masked SH and the circles represent the EDTA-treated amylase with unmasked SH.

The subsequent blocking reaction by DTNB is very fast ($K_0 = 0.11$–$0.15\ \text{min}^{-1}$) as shown by the preincubation experiment. Therefore, in this situation the TNB liberation kinetics is a first-order process ($K = 0.020\ \text{min}^{-1}$) in the standard conditions, and $2K = k_1$ and $K = k_2$.

This dimeric hypothesis is also consistent with the percentage of disulfide bridges obtained: an exchange might occur in the three TNB species. The reaction is very rapid and the dimers dissociate as 50% of each species (Fig. 9).

How is it now possible to explain the 75% activity of the intermediate species $E_1$? $E_1$ is mainly present as the di-TNB form since the unmasking rate constant ($K$) is much lower than the blocking reaction rate constant ($K_0$). A simple dissociation of this species should give 50% activity. A possible explanation is that the dimeric species in the activity assay medium (probably under the influence of the substrate) is partially reactivated.

The dimeric model hypothesis needs more experimental support to be accepted

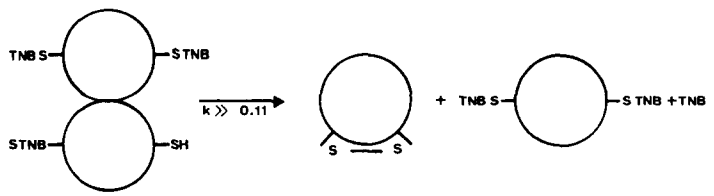



Fig. 9. The formation of disulfide bridges. The three TNB species is very rapidly formed ($K_0$) as soon as the second amylase molecule is unmasked.

since no direct evidence is available at the moment. This model might be completed by introducing possible secondary reactions with monomeric and higher polymeric forms.

*(3) A possible functional role of SH groups*

The complete blocking of both SH groups results in a total loss of activity. According to Telegdi and Straub [11] 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. Although this cannot be excluded completely, our results support a different conclusion. EDTA-treated amylase is known to be poorly stable [3] and this is shown in the control experiment (Fig. 6): the enzyme is 50% active after a 3-h incubation in EDTA, but in a parallel assay the DTNB amylase was found to be completely inactive. From the di-TNB amylase ultraviolet spectrum, no important changes in the overall structure are apparent. One may then postulate that the loss of activity is due to a local modification such as the blocking of an SH group necessary for enzyme activity and/or to steric hindrance of the active site.

The 25% loss of activity found to be produced by the blocking of one or any of the two SH groups indicates that at least one of them is not present at the catalytic center.

The masking of SH by maltotriose appears to be very specific, since a rather strong inhibition of TNB liberation is obtained with only a 20-fold molar excess. Maltotriose is, of course, a very poor substrate and this may explain why preincubation is necessary at such a low concentration. We then propose that the SH groups may be part of or at least close to the binding site(s). More direct evidence is of course necessary to precisely define the functional role of amylase SH. The SH groups in Taka-amylase and in *B. subtilis* saccharifying amylase have been found to be essential for enzyme activity [18, 19]. However, our results differ in this respect from those obtained by Telegdi and Straub [11]. Also the mercurial complex of $\alpha$-amylase prepared by Steer and Levitski [20] was found to be active in the presence of calcium.

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