Influence of Sulfhydryl Groups on the Activity of Sweet Potato β-Amylase*

John A. Thoma,† D. E. Koshland, Jr.,‡ Ryu Shinke,† and John Ruscica‡

ABSTRACT: To investigate the influence of the thiol group on β-amylase activity the chemical and catalytic properties of the native and carboxyamidomethylated enzyme were studied. A yellow substance not necessary for activity could be released from the enzyme. The amino acid composition, chromatographic properties, and thermal stability of the enzyme are described. The *p*H-rate profile shows two groups, one with *pK ca.* 3.7 and a second with *pK* 7.5 which are involved in the enzyme action. On treatment with iodoacetamide, reaction with the surface sulfhydryl groups occurs with concomitant loss of enzymatic activity. When two cysteine residues per monomer unit have reacted, the enzyme activity has decreased by a factor of 50. The modified enzyme, however, has essentially the same *p*H-rate profile as the native enzyme. It is concluded that the surface SH groups are not essential for the enzyme action and that modification of one of these SH groups changes the velocity of the enzyme action without altering the fundamental catalytic mechanism.

Intense chemical investigations of enzymes have been focused primarily on double-displacement enzymes, but because the mechanisms of single-displacement enzymes (Koshland, 1953) may be simpler, they offer an attractive opportunity for enzyme structure and function correlations. β-Amylase has been shown to produce quantitative inversion of configuration upon hydrolysis and hence is probably a single-displacement enzyme (Freeman and Hopkins, 1936; Thoma and Koshland, 1960a; Thoma *et al.*, 1963b). Since β-amylase is a particularly effective catalyst with a turnover number of 250,000 bonds min^{-1} per mole of enzyme (Englard *et al.*, 1951; French, 1960), and since other studies have already suggested that modification of an SH group (Englard *et al.*, 1951; Weil and Caldwell, 1945) can influence enzyme activity, it was of substantial interest to define the precise relationship of this group to the acetal hydrolysis. Our approach to this problem entailed the study of the characteristics of the native and iodoacetamide modified enzyme and the correlation of the kinetics of enzyme inactivation to the loss of SH groups.

Experimental

Chromatography of β-Amylase. To obtain highly pure β-amylase, two-times-crystallized enzyme, prepared by the procedure of Balls *et al.* (1948), was chromatographed according to the following procedure. Enzyme crystals (up to 30 mg, purchased from Worthington Biochemical Corp.) were centrifuged twice and the supernatant was discarded. The crystals were then dissolved in *ca.* 2 ml of deionized water and centrifuged, and the supernatant was added to a dextran gel column (2 × 20 cm, Sephadex G-25, purchased from Pharmacia) which was packed at 0–3°. The main protein fraction (Thoma and Koshland, 1960a) was percolated into an ECTEOLA-cellulose (Peterson and Sober, 1956) column (2 × 20 cm, 0.64 meq/g, purchased from Bio-Rad Co.) at 0–3°. The ECTEOLA-cellulose in the phosphate cycle had previously been equilibrated with 0.00125 M sodium phosphate buffer which had a *p*H of 7.05 at room temperature. The mechanical device to obtain a concave salt gradient was similar to that of Peterson and Sober (1959). The first and second reservoirs contained 150 ml of 0.00125 M phosphate buffer and the third contained 150 ml of the phosphate buffer in 0.75 M sodium chloride.

When the ion-exchange resin had a capacity of 0.41 meq/g, the chromatographic procedure was changed to the following: (a) 2 × 25-cm column; (b) *p*H 6.95; (c) the first and second reservoirs contained 320 ml of the 0.00125 M phosphate buffer, while the third contained 320 ml of the buffer in 0.5 sodium chloride. The same amount of protein was chromatographed on the resin of lower capacity.

Amino Acid Analysis of β-Amylase. For total amino acid analyses on the automatic analyzer of Spackman *et al.* (1958), chromatographed enzyme was dialyzed

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† Indiana University.

‡ Brookhaven National Laboratory.

for 12 hours versus deionized water at room temperature, concentrated by pervaporation at room temperature to a concentration of about 4 mg/ml, and then dialyzed again versus deionized water to remove the remaining salt. Acid hydrolysis and similar procedures were performed according to the method of Hirs *et al.* (1954), and amino acid analyses were run on the automatic analyzer, except that (a) the protein concentration was measured spectrophotometrically ($\epsilon_M = 2.60 \times 10^5$) (Englard *et al.*, 1951; Englard and Singer, 1950) at 280 mμ on a Beckman DU spectrophotometer, and (b) the hydrolysate was not allowed to stand exposed to the air at *p*H 6.5. Tryptophan and tyrosine were determined by the spectrophotometric method of Goodwin and Morton (1946). To determine the concentration of the amino acids at zero time the protein was hydrolyzed for times ranging from 24 to 96 hours and the amount of amino acid released was extrapolated to zero time.

Enzyme Assay. The standard assay procedure was as follows: A 1-ml aliquot of the enzyme solution to be assayed was added to 1 ml of reagent containing 1% Lintner soluble starch in 0.1 M sodium acetate–acetic acid buffer at *p*H 4.8. Pipets were always rinsed twice with the enzyme solution to be sampled before the assay aliquot was taken in order to avoid spurious results because of adsorption of protein to glass. After 5 minutes at 25°, the hydrolytic action was terminated by adding 1 ml of Somogyi's copper reagent (Nelson's modification, 1944). The solution was heated for 10 minutes in a vigorously boiling water bath in a sugar tube capped with a marble, then cooled in tap water, and shaken vigorously after addition of 1 ml of molybdate reagent. After 5 minutes, the contents were diluted to 25 ml and the color was read against a starch blank containing buffer in a Beckman DU spectrophotometer at 660 mμ. Aliquots of enzyme which produced 0.1–0.4 mg of maltose gave satisfactory color values.

An alternate assay procedure was used occasionally because Tris, glycine, and citrate buffer interfered with the Somogyi reagent. This procedure was the same as that described above except that (a) 3,5-dinitrosalicylic acid (Kirsop, 1953) was used as color reagent, (b) 2 ml of this reagent was added instead of 1 ml of copper reagent, (c) the time of heating was 5 minutes instead of 10, and (d) color reading was made at 530 mμ against a blank containing the buffer. Nitrogen was bubbled through the salicylic acid for 20 minutes immediately before use (Miller, 1959).

SH *Determination with p-Mercuribenzoate.* Chromatographed β-amylase was dissolved in buffer and diluted to 3 ml. The final solution was 0.5 M in sodium sulfate, 0.05 M in phosphate buffer, and 5.5×10^{-6} M in enzyme at *p*H 7.0 (Boyer, 1954). This solution was then titrated with 25-μl increments of a *p*-mercuribenzoate solution (4.31×10^{-3} M), in identical concentrations of Na_2SO_4 and phosphate buffer. Twenty minutes after addition of the mercurial, the optical density was read at 250 mμ in a Beckman DU spectrophotometer (Boyer, 1954). The blank contained all reagents except the amylase and the optical density readings were corrected for dilution.

To determine the number of sulfhydryl groups in the "unfolded" protein, the experiment described was repeated, except that the enzyme solution and reagents contained 1% sodium dodecylsulfate. In this case the initial protein concentration was determined in the absence of dodecylsulfate since a slight change in the extinction coefficient was observed upon addition of the detergent. The kinetics of reaction of *p*-mercuribenzoate with the SH groups was determined under the conditions mentioned at several enzyme and *p*-mercuribenzoate concentrations.

Iodoacetamide Alkylation of the Native Enzyme. To measure the extent of alkylation as a function of time, desalted chromatographed β-amylase was added to Tris buffer at *p*H 8.8 and twice-recrystallized iodoacetamide was added to initiate the reaction. The final reaction mixture (11.2 ml) of 0.09 M Tris and 0.05 M iodoacetamide contained approximately 50 mg of enzyme. At appropriate intervals aliquots were withdrawn and the reaction was stopped by precipitating the protein with approximately 10 volumes of ice-cold 5% trichloroacetic acid. The solution was allowed to stand at ice temperature for 1 minute and then centrifuged for 3 minutes. The precipitate was washed twice with 2 ml of absolute ethanol and transferred to a test tube where it was allowed to dry overnight under vacuum over NaOH pellets. Protein was hydrolyzed in 0.5 ml of constant-boiling HCl at the temperature of boiling toluene for 22 hours.

For the determination on the amino acid analyzer (Gundlach *et al.*, 1959a,b; Crestfield *et al.*, 1963) of the amount of cysteine and other residues that had been alkylated, the ninhydrin constant of *S*-carboxymethylcysteine was assumed to be equal to the average ninhydrin constant for the other amino acids. Destruction of the *S*-carboxymethylcysteine during acid hydrolysis was compensated for by using a correction factor of 0.91 (Moore and Stein, 1963). The amount of carboxymethylcysteine was related to the total amount of protein by using the known constant of five different stable amino acids and then calculating the average.

Enzyme assays were determined by diluting so that the assay tube produced 0.2–0.4 mg of maltose under the standard assay conditions.

Iodoacetamide Alkylation in the Presence of Sodium Dodecylsulfate. In an attempt to determine the total number of thiol groups in β-amylase the enzyme was alkylated with 0.5 M iodoacetamide under conditions described, except that the solution was made 1% in sodium dodecylsulfate. After the reaction, the solution was exhaustively dialyzed for 60 hours at 3°. Upon drying and extraction of the sodium dodecylsulfate with boiling water the samples were hydrolyzed and analyzed in the fashion described.

Removal of the Yellow Substance from β-Amylase. A minor extinction maximum of the enzyme at 350 mμ suggested the presence of a chromophoric residue attached to the enzyme. This colored material was readily released from the enzyme by gel filtration on a

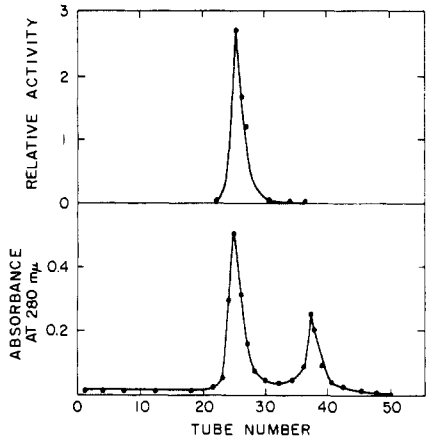

FIGURE 1: Elution diagram of β-amylase from ECTEOLA-cellulose of 0.64 meq/g capacity. For details of procedure, see under Experimental. Starting material was two-times-crystallized β-amylase of low initial specific activity. Samples of 12–13 ml were collected.

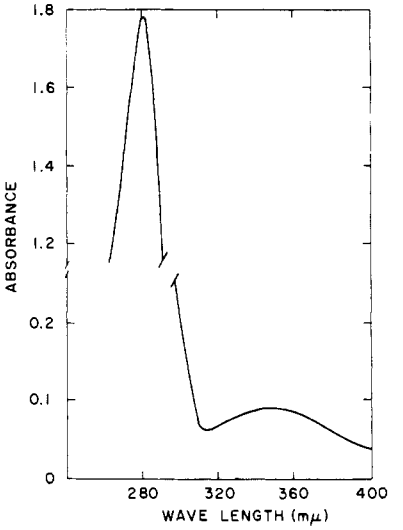

FIGURE 2: Absorption spectrum of chromatographed β-amylase (1.04 mg/ml) at *p*H 7.0, phosphate buffer.

1 × 22–cm column of Sephadex at *p*H 8.8 in the presence of pyrophosphate buffer.

Removal of the chromophoric material can also be achieved by acidification of several ml of enzyme with 5–10 drops of reagent-grade acetic acid. Nearly complete precipitation of the protein occurs on standing overnight at room temperature. The supernatant contains almost all of the chromophoric material.

Thermal Stability of Native and Iodoacetamide Modified Enzyme. To determine the heat stability of native and iodoacetamide modified enzyme, samples containing 0.5 mg/ml were incubated at 65° ± 0.5° in 0.02 M acetate at *p*H 5.5. At various times, aliquots were diluted in 0.02 M acetate buffer, *p*H 5.5, containing 0.01% bovine serum albumin at 25°. The enzyme was then assayed by the standard procedure except that 5×10^{-4} M amylodextrin (Thoma *et al.*, 1959) (average chain length 25) was the substrate and the *p*H was 5.5.

Iodoacetamide Inactivation of β-Amylase as a Function of pH. To determine kinetically the ionization constant of the reactive SH group the rate of inactivation of β-amylase was studied in 0.5 M iodoacetamide at various *p*H values at 25 ± 0.1°. At *p*H 8.0 and 8.5 phosphate buffer, 0.025 M, was employed, and at *p*H 9.0 and 9.3 pyrophosphate buffer, 0.025 M, was employed. At various time intervals aliquots were withdrawn and assayed for enzyme activity using the standard procedure.

Effect of pH on the Apparent V_m of Native and Carboxyamidomethylated β-Amylase. The effect of *p*H on the twice-recrystallized enzyme was studied by measuring initial velocities in buffered solutions at 25 ± 0.1°. The assays were performed under standard assay conditions with the following exceptions: (a) 2% starch in 0.2 M buffer was used at *p*H indicated; (b) the time of incubation was increased at extremely alkaline *p*H to aid analysis but the extent of hydrolysis was never allowed to exceed 5%.

The apparent maximum velocity, V_m', was calculated using equation (1).

$$V_m' = \left(1 + \frac{K_m}{(S)}\right)\left(\frac{k_d C_M}{1 - e^{-k_d t}}\right) \qquad (1)$$

where k_d is the first-order constant for protein denaturation at that *p*H, C_M is the concentration of maltose in moles/liter produced at time t, and K_m is the apparent Michaelis constant (Thoma and Koshland, 1960b). This expression, which corrects for protein denaturation and lack of enzyme saturation by substrate, reduces to the conventional expression $V_m' = C_M/t$ at small times when the enzyme is saturated with substrate.

To study the *p*H effect on the alkylated protein, *S*-carboxyamidomethylated enzyme was obtained by incubating the twice-crystallized enzyme with 0.06 M iodoacetamide for *ca.* 5 hours at 25° in pyrophosphate buffer at *p*H 8.8. Since the enzyme sample was diluted over 100-fold into the 0.1 M buffer solution before assay, and in turn added to starch containing 0.1 M buffer for assay, the maximum change in *p*H owing to pyrophosphate was calculated to be 0.02 *p*H unit. Since K_m' for the modified enzyme has been measured only at *p*H 4.8, the calculation of V_m at various *p*H values assumed that the K_m' of modified enzyme had the same *p*H dependence as the native enzyme. Since the K_m' for the modified enzyme is smaller than that of the native enzyme and since the maximum correction for denaturation of the native enzyme was only 10%, an error in this assumption would have only a very small influence on the calculated values.

TABLE I: Amino Acid Composition as a Function of Time of Hydrolysis.

Amino Acid	Residues per 50,000 g of Enzyme[a]				
	At Time of Hydrolysis:			Average of Extrapolated Value	Nearest Integer
	26 hr	48 hr	96 hr		
Lysine	25.1	24.7	26.2	25.4	25
Histidine	6.4	6.7	6.7	6.6	7
Arginine	15.2	15.2	15.9	15.4	15
Aspartic acid	47.3	48.3	48.1	47.9	48
Threonine	12.6	11.8	10.7	11.7[b]	12
Serine	12.5	10.9	8.2	14.2[b]	14
Glutamic acid	33.4	34.5	33.3	33.8	34
Proline	23.3	23.6	23.1	23.4	23
Glycine	31.4	31.5	30.1	31.0	31
Alanine	30.2	30.8	29.1	30.1	30
Half-cystine	5.1	4.9	4.4	5.4[b]	5
Valine	24.6	25.5	24.9	25.0	25
Methionine	13.3	13.4	12.6	13.7[b]	14
Isoleucine	16.6	17.6	18.0	17.5	18
Leucine	28.0	29.1	28.5	28.5	29
Tyrosine	17.0	17.1	16.5	16.9 (17.0)[c]	17
Phenylalanine	17.0	17.5	17.1	17.2	17
Tryptophan				9.77[c]	10
Total groups per monomer					374

[a] Assumed monomer molecular weight (Thoma *et al.*, 1963a). [b] Extrapolated value. Value for half-cystine agrees with value for cysteic acid obtained by performic acid oxidation (E. Gaetjens and D. E. Koshland, Jr., unpublished). [c] Spectrophotometric determination.

Results

Chromatography. The twice-crystallized enzyme of different Worthington lots showed as much as 2-fold variation in specific activity and even larger variations were encountered in the amount of contaminating second peak of the elution curves. An elution curve of a typical preparation is depicted in Figure 1. Essentially all of the amylase activity was found in the first peak; but when the specific activities were plotted versus elution volume, it was noted that the specific activity decreased somewhat on the back shoulder of the first peak. Whether the impurity on the rear side of the peak was denatured enzyme or another protein is not clear.

Chromophoric Material. The spectral characteristics of β-amylase are recorded in Figure 2, and the peak at about 350 mμ suggested the possibility of a pro thetic group. The colored material, which gives the unusual absorption peak and is normally associated with the enzyme, can be separated from the amylase by chromatography on Sephadex at *p*H 8.8 as shown in Figure 3, or by acid treatment as described in the experimental section. The spectral shifts of this material, recorded as a function of *p*H in Figure 4, account for the enhancement of the yellow color of the enzyme detected visually when the *p*H of an enzyme solution is raised above *p*H 8.3–8.5. Since the activity of the enzyme is not significantly altered upon the release of the substance, it is concluded that this yellow material is not a prosthetic group involved in catalysis. These observations probably also explain the observation of Balls *et al.* (1948) that repeated recrystallization of the enzyme appears to cause bleaching.

The yellow material released from the enzyme can be reattached by incubation at *p*H 8.8 and 0° overnight. If the *p*H of the reaction mixture is then lowered to *p*H 7 the chromophoric material emerges with the protein peak on gel filtration. However, the substance can again be fractionated from the protein by chromatography on Sephadex at *p*H 8.8. At present the yellow substance has not been characterized from its absorption spectra and the paucity of material has prevented further study.

Other instances have been documented in which yellow materials have been released from enzymes, but they apparently play no role in catalysis (Celliors *et al.*, 1963; Kent *et al.*, 1958; Illingworth *et al.*, 1958; King, 1963).

Amino Acid Composition. The amino acid composition of chromatographically purified sweet potato β-amylase as determined on the amino acid analyzer is indicated

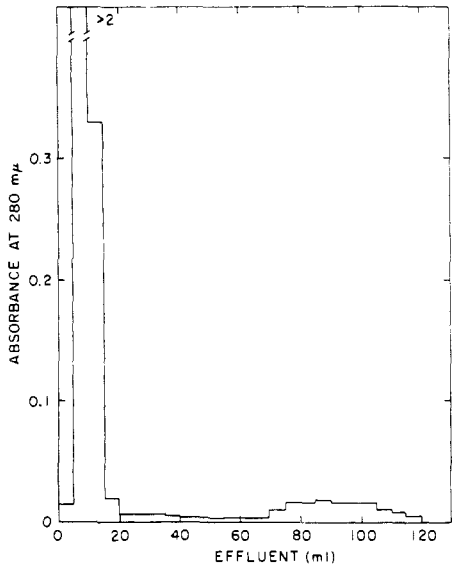

FIGURE 3: Fractionation of yellow substance from β-amylase on dextran gel column. Conditions: *p*H 8.8, 0.05 M pyrophosphate buffer; room temperature; column, Sephadex G-25, 1 × 21.5 cm.

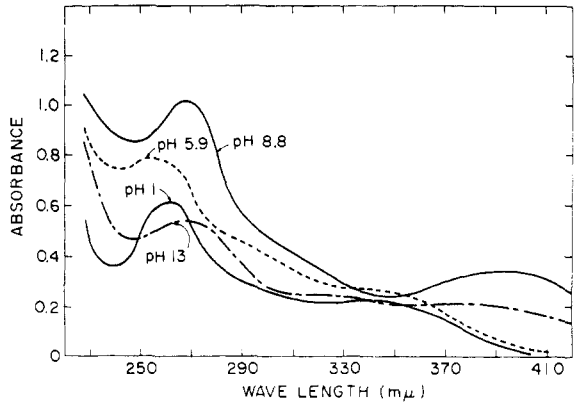

FIGURE 4: Absorption spectra of yellow substance at various *p*H values. Conditions: room temperature; pyrophosphate buffer. Since various amounts of reagent were added the concentration of the samples is not the same.

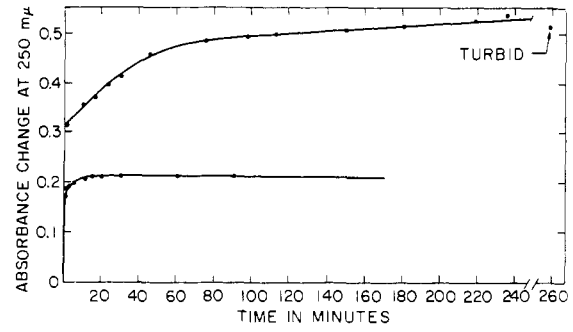

FIGURE 5: Time course of reaction of *p*-mercuribenzoate with β-amylase. Upper curve, 0.75 mg chromatographed β-amylase/ml, phosphate buffer (0.05 M), *p*H 7.0, 0.5 M Na_2SO_4, 1.4×10^{-4} M *p*-mercuribenzoate, room temperature; lower curve, unchromatographed enzyme (0.57 mg/ml) and 3.3×10^{-5} M *p*-mercuribenzoate. Other conditions same as above.

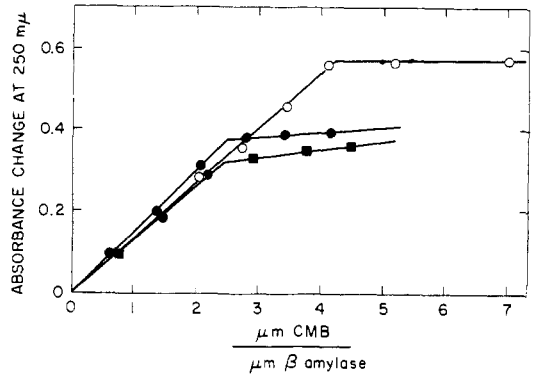

FIGURE 6: Spectrophotometric titration of mercaptan groups on β-amylase by Boyer's method (1954) with *p*-mercuribenzoate. Consult text for details. ○, SDS denatured; ●, native-preparation 1; ■, native-preparation 2.

in Table I. The values of threonine, serine, methionine, and half-cystine have been evaluated by extrapolation to zero time. In the determination of tryptophan by the procedure of Goodwin and Morton (1946) a correction was made for the spurious absorption which entails extrapolation from the region of 360 mμ to the region of tyrosine and tryptophan absorption. Since the enzyme displayed a peak around 350 mμ it was not certain that this procedure was applicable. However, addition of alkali caused a disappearance of the visible peak and the spectrophotometric assay was employed.

The good agreement between chromatographic and spectrophotometric values for tyrosine (Table I) indicate that the latter method is probably reliable for tryptophan also. The amino acid values are reported in terms of a monomer weight of 50,000 since this appears to be the size of the monomer unit (Thoma *et al.*, 1963a).

The numbers of the amino acid residues are roughly comparable to those of the α-amylases most of which have molecular weights of about 50,000 (Fischer and Stein, 1960; Campbell and Manning, 1961). Five amino acids were found to lie outside the usual range, i.e., lysine (high), serine (high), threonine (low), leucine (high), and methionine (high). But these deviations were not much different from those obtained by comparing one α-amylase with another. The only amino acid which appeared significantly outside the range established for the α-amylases was methionine.

Furthermore, the total number of groups classified as ionic, hydrophilic, and hydrophobic were also similar to those characteristics of the α-amylases.

p-Mercuribenzoate Treatment. The time course of reaction with *p*-mercuribenzoate is shown in Figure 5. The lower curve was obtained when the ratio of the mercurial to the monomer was 2.9, while in the upper curve the ratio was approximately 10. When the thiol groups are in excess it appears that reaction requires about 20 minutes to go to completion. However, when the *p*-mercuribenzoate to SH ratio is large, the reaction is completed only after a long time interval and is probably accompanied by denaturation as indicated by the turbidity of the solution. For the spectrophotometric titration of the thiol groups with *p*-mercuribenzoate an end point taken after 20 minutes was chosen. The titrations in the presence and absence of sodium dodecylsulfate are depicted in Figure 6. In the absence of denaturant, replicate experiments indicated there are 2.4–2.5 SH groups per 50,000 g-atoms of protein. In the sodium dodecylsulfate enzyme a total of 4.2 groups were obtained (Table II). The

TABLE II: Number of Sulfhydryl Groups per Monomer of β-Amylase.

Method of Determination	"Reactive"	Total
p-Mercuribenzoate	2.4[a]	4.2[b]
Iodoacetamide alkylation in the presence of urea	2.0[c]	4.5[b]
Chromatographic analysis of acid-hydrolyzed protein		5.4[d]
Amperometric	2.7[e]	

[a] End point of titration taken as 20 minutes (see text). [b] In 1% sodium laurylsulfate (see text). [c] Extrapolated value (see Figure 9). [d] As half-cystine (amino acid analysis measures SH and S-S as S-S; for extrapolated value see Table I). [e] Ito *et al.* (1958).

optical density (8500) at the equivalence point of the curves in Figure 6, divided by the concentration of monomer, corresponds to the average molar extinction coefficient of the mercurated mercaptan groups.

The variable rates of reaction of the SH groups may be a reflection of the rapidity with which the residues become accessible to the reagent (Cecil and McPhee, 1959). The time course of reaction of the thiol groups (Figure 5) with *p*-mercuribenzoate suggests there are three different classes of SH groups in the enzyme. The triphasic nature of the curve is much more apparent if the data are analyzed according to the method of Ray and Koshland (1961). Using this approach it was found that the reactive groups (class I) are lost within 0.5 minute while the residues of intermediate reactivity

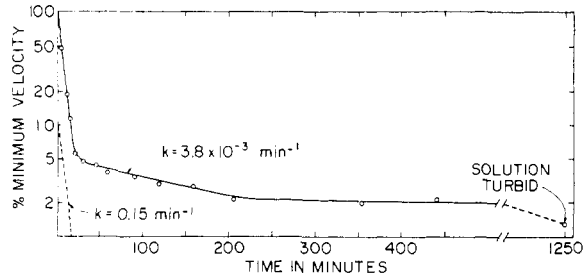

FIGURE 7: Kinetics of inactivation of β-amylase with iodoacetamide. Conditions: 25°, 0.05 M iodoacetamide, 0.09 M Tris buffer, *p*H 8.8. Lower curve, % change × 2 for first phase on inactivation.

(class II) are lost with a rate constant of 4.6×10^{-1} min^{-1} and those residues of low reactivity (class III) are lost with a rate constant of 8.0×10^{-3} min^{-1}. Moreover, the reaction with *p*-mercuribenzoate in conjunction with the alkylation data suggests that there are approximately two SH groups in class I, two SH groups in class II, and one SH group in class III. The highly reactive SH groups may be exposed on the surface and the less reactive SH side chains may become available as the conformation of the protein is altered. Thus the number of groups titrated vary with time and the ratio of mercurial to enzyme. Unfolding may be detected by the mercurial but not by iodoacetamide. Because of the speed of reaction the mercurial may react with a reversibly denatured form not detected by the more slowly reacting iodoacetamide. These possibilities may account for the small but significant discrepancy observed between the number of reactive groups determined by *p*-mercuribenzoate and iodoacetamide. However, it is interesting to note that the number of residues determined by spectrophotometric titration at 0.5 minute in the presence of a large excess of *p*-mercuribenzoate are identical, within experimental error, to the number titrated using the 20-minute end point.

Iodoacetamide Treatment. The kinetics of reaction of the enzyme with the sulfhydryl reagent, iodoacetamide, were studied and the results are presented in Figure 7. Inactivation apparently occurs in three phases: (a) a rapid inactivation leading to approximately 95% loss in activity, (b) a slower inactivation causing an additional 3% loss in activity, and (c) a very slow inactivation probably concomitant with unfolding of the protein. After about 300 minutes at the end of the second phase of the inactivation the activity had apparently reached a plateau of about 2% of the activity of the native enzyme.

The amino acid analyses during the iodoacetamide inactivation are indicated in Figure 8. Those amino acids not listed were unchanged within an error of approximately 2%. During the first two phases of inactivation only sulfhydryl residues reacted in significant amounts with the alkylating reagent. After protracted intervals of time in the third phase of the

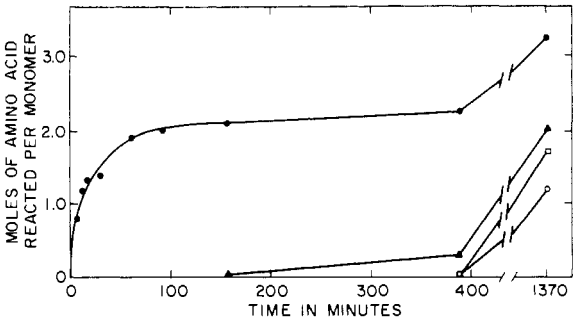

FIGURE 8: Carboxyamidomethylation of β-amylase as a function of time. ●, Carboxymethylcysteine; ▲, dicarboxymethyllysine; □, carboxymethylhomocysteine; ○, homoserine. Conditions as in Figure 6. To test for carboxymethylhistidine (which appears under the proline peak) optical density at 440 mμ was compared to that at 570 mμ. An authentic sample of proline gave a ratio of 4.32 while the ratio for the proline peak at 1370 minutes was 4.30. Hence we conclude histidine was not alkylated under assay conditions.

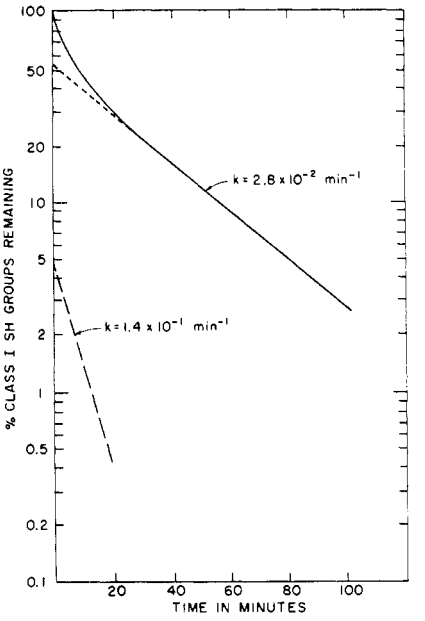

FIGURE 9: Kinetics of reaction of iodoacetamide with β-amylase. Data replotted from first portion of Figure 9. Lower curve, (% change)/10 for fastest SH group. The second phase of the curve extrapolates to 53% while the theoretical value should be 50%.

inactivation, both lysine and methionine residues were found to have been carboxyamidomethylated.

To evaluate the number of groups responsible for activity loss the analytical method of Ray and Koshland (1961) was applied. The per cent of the remaining SH groups was calculated from the curve of Figure 8 and corrected for the number of SH groups of intermediate reactivity which were lost. Since it is apparent that two residues are destroyed during the initial phase of the reaction, the per cent was calculated by the following formula:

$$\% \text{ remaining} = 100\left(1 - \frac{\text{no. of residues reacting}}{2}\right) \quad (2)$$

The resulting data, which are plotted in Figure 9, produce a biphasic curve. Since only two groups are reacting, the extrapolated portion of the second phase of the curve should intersect at an ordinate of 50%. The observed ordinate of 53% is consistent with the reactivity of two residues. The pseudo-first-order rate constants for alkylation of the two sulfhydryl groups under the conditions of the experiment were 1.4×10^{-1} min^{-1} and 2.8×10^{-2} min^{-1}, respectively. Since the half-life of the enzyme inactivation was 1.5×10^{-1} min^{-1}, the modification of the most reactive SH group in class I of the enzyme is probably responsible for the bulk of the loss of activity. It is interesting to note that under conditions in which iodoacetamide caused this inactivation treatment with comparable amounts of iodoacetic acid completely failed to cause any change in activity. This rather unusual difference in these two reagents which have rather similar reactivities suggests the presence of a negative charge in the vicinity of the sulfhydryl groups.

Thermal Stability of Native and Modified β-Amylase. The effect of thiol alkylation on the thermal stability of the enzyme is shown in Figure 10. Since the heat stability of the enzyme is sensitive to protein and salt concentration (Nakayama and Kono, 1957, 1958; Nakayama, 1961), care was taken to ensure that these conditions were identical for the native and modified preparation. On the basis of Figure 10 it appears that alkylation has introduced thermal instability into the molecule and that the modified protein is inactivated by biphasic kinetics as is the native preparation.

pK of Reactive SH *Group.* The *pK* of the reactive SH group can be determined by a *p*H study assuming that the ionized form, RS^-, reacts many times faster with iodoacetamide than the protonated form, RSH. When the log of remaining activity was plotted versus time, graphs similar to those in Figure 7 with variable slopes were obtained. The pseudo-first-order rate constants of inactivation calculated from the initial inactivation rate in these plots are displayed in Table III. From two experiments at different hydrogen ion concentrations the apparent ionization constant of the reactive sulfhydryl group can be calculated according to equation (3)

$$K_{app} = \frac{k_1H_1 - k_2H_2}{k_2 - k_1} \quad (3)$$

in which k_1 and k_2 are the pseudo-first-order rate constants of inactivation observed at hydrogen ion concentrations of H_1 and H_2, respectively. The results of these calculations for each pair of *p*H values are also

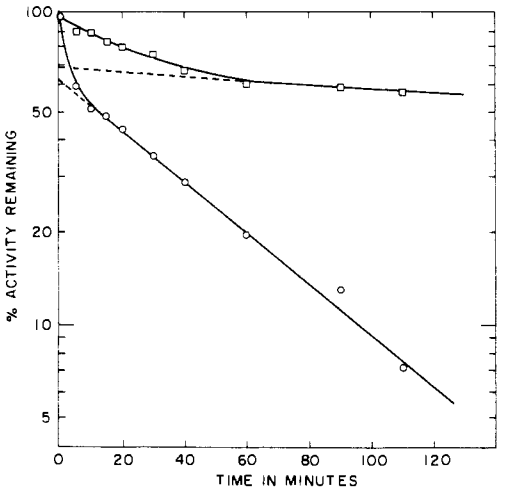

FIGURE 10. Thermal stability of native and carboxyamidomethylated β-amylase. Temperature 65°; *p*H 5.5, 0.02 M acetate buffer; □, native enzyme; O, modified enzyme.

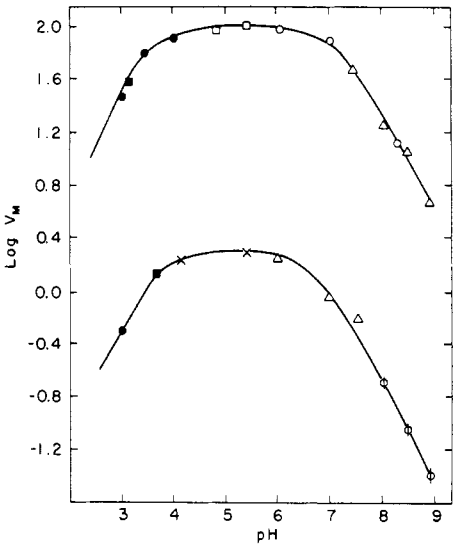

FIGURE 11: *p*H-rate profiles of native and carboxyamidomethylated β-amylase at 25°. Upper curve, native enzyme; lower curve, alkylated enzyme. Buffers as follows: O, Tris; △, phosphate; ×, acetate; ■, citrate; ●, phthalate; □, pyrophosphate-acetate; ϕ, pyrophosphate.

TABLE III: Comparison of *p*H-dependent Pseudo-First-Order Rate Constants for Inactivation of β-Amylase by Iodoacetamide and Calculation of Apparent Dissociation Constant of Reactive SH Group.

*p*H	Inactivation Constant (min^{-1})	Apparent Dissociation Constant ($\times 10^{10}$)	*p*H Values Used for Calculation
9.3	0.099	1.1	9.0 and 8.5
9.0	0.056	2.9	8.5 and 8.0
8.5	0.019	1.6	9.0 and 8.0
8.0	0.006	1.3	9.3 and 9.0
		1.2	9.3 and 8.5
		1.5	9.3 and 8.0
		Average 1.6	

given in Table III and the mean value of the ionization constant was found to be 1.6×10^{-10}. The good agreement between the apparent dissociation constants calculated for various pairs of *p*H values is taken as excellent evidence that only the ionized form of the thiol is alkylated. Comparing these data with the ionization constants of SH groups in other proteins (Boyer, 1959), it appears that the thiol group which most influences activity has a normal ionization constant.

*p*H *Dependence of Native and Modified β-Amylase.* The *p*H-rate profiles for native and iodoacetamide inactivated β-amylase are shown in Figure 11. From the inflection points, the dissociation constants of the groups involved in catalysis are found to be 3.7 and 7.0 for the native and 3.8 and 6.5 for the alkylated preparation, respectively. The alkylated enzyme possesses only 2% of the original activity.

Discussion

β-Amylase appears to be a protein whose amino acid composition is not distinctively different from that of the α-amylases and whose monomer molecular weight is about the same as that of the α-amylases.

This study of inactivation of β-amylase with iodoacetamide leads to the conclusion that alkylation of a sulfhydryl group is the primary event responsible for the loss of enzyme activity. The time course of inactivation (Figure 7) indicates that the loss of catalytic power occurs by two first-order processes characterized by rate constants of 0.15 min^{-1} and 3.8×10^{-3} min^{-1}. Since the first-order rate constant for the most reactive sulfhydryl group is 0.14 min^{-1}, it seems probable that the first phase of enzyme inactivation leading to about 95% loss of activity occurs concomitantly with the loss of this residue.

The remaining activity (*ca.* 2%) after long exposure of the enzyme to inactivating reagent is believed to represent the activity of a modified protein. It is possible that the residual activity is due to a contaminating α-amylase or glucosidase which is present and unaffected by iodoacetamide. This possibility seems remote, because an aliquot of the protein obtained after the second phase of inactivation had been completed was found to yield maltose as the sole product of hydrolysis of starch. This action pattern is characteristic of β-amylase but not of α-amylase or glucosidases.

Another possibility, that the residual activity is due

to native enzyme which has somehow failed to react, is thought to be unlikely because (a) the enzyme achieved essentially constant activity for several hours after prolonged incubation with iodoacetamide, (b) the *p*H-rate profile of the modified enzyme is significantly different from that of the native enzyme, (c) the Michaelis constant of the modified preparation and the inhibition constant of cyclohexaamylose were found to be significantly different from those of the native enzyme (Thoma and Koshland, 1960c), (d) the heat stability of the modified preparation differs from that of the native enzyme, and (e) the enzyme appears homogeneous by ultracentrifuge analysis, electrophoresis, and ECT-EOLA-cellulose chromatography.

Thus it appears fairly certain that alkylation of an SH group causes the catalytic efficiency of the enzyme to drop by a factor of approximately 50. Because the modified protein does have residual activity it is doubtful that the sulfhydryl groups which are alkylated are essential to catalytic activity of the enzyme. Although the *p*H-rate profile of the modified protein is different from the *p*H-rate profile of the native enzyme, consistent with modification of the active site, perhaps the most significant feature of the profiles is their gross similarity. From Figure 11 it appears that a single group of *pK* approximately 4 and a second group of *pK* approximately 7 must exist in the unprotonated and protonated forms, respectively, for maximum activity of the enzyme. Since the *pK* of the SH group whose modification results in 95% loss of activity was determined to be about 10, it is certain that ionization of this SH group cannot be responsible for loss of enzyme activity above *p*H 7. This conclusion is a satisfying one since it is difficult to conceive of an essential catalytic role of a sulfhydryl group which would not be strikingly altered either by ionization or by alkylation. Since there is partial protection of the SH group against alkylation by cyclohexaamylose (Thoma and Koshland, 1960c) it is probable that either the SH group is near the active site or that alkylation produces a conformation change which alters the active site. Thus the SH group affects enzyme activity but is not essential for it.

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