Unmasking of Sulfhydryl Groups in Pancreatic α-Amylase*

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Porcine pancreatic α-amylase contains masked sulfhydryl groups which appear to be required for high enzymatic activity. In the presence of ethylenediamine tetraacetic acid (EDTA) these groups are partially exposed and react sluggishly with 5,5′-dithiobis-(2-nitrobenzoic acid). The further addition of sodium dodecylsulfate accelerates the reaction and brings it to completion, indicating the presence of 1.7 sulfhydryl groups per mole of enzyme. In the absence of EDTA, sodium dodecylsulfate fails to expose the sulfhydryl groups. Even in the presence of 7 M urea, EDTA greatly enhances the reaction with 5,5′-dithiobis-(2-nitrobenzoic acid). Parallel studies of enzymatic activity reveal that 5,5′-dithiobis-(2-nitrobenzoic acid) and *N*-ethylmaleimide cause 80% inhibition, and sodium dodecylsulfate causes 100% inhibition, when these agents are preincubated with the enzyme in the presence of EDTA. Preincubation with the sulfhydryl reagents and with the detergent, in the absence of EDTA or with EDTA alone, had no effect on enzymatic activity. The findings suggest that masking of the sulfhydryl groups is mainly due to tightening of the enzyme structure by bound calcium, which can be removed by EDTA. Iodine is the only reagent found which rapidly attacks the sulfhydryl groups in the native enzyme in the absence of EDTA, causing 80% inhibition. Cysteamine reactivates the iodine-oxidized enzyme.

The studies of Caldwell and his collaborators showed that α-amylase from hog pancreas is not inhibited by reagents which react with sulfhydryl groups (Caldwell *et al.*, 1945). It was therefore assumed that the enzyme has no SH groups or that these are not required for activity (cf. Fischer and Stein, 1960). The finding that *Bacillus subtilis* α-amylase contains neither cysteine nor cystine residues (Akabori *et al.*, 1956) further supported this assumption and suggested that the half-cystine groups of pancreatic and other α-amylases might exclusively represent S–S bridges. However, as shown in the present communicaton, hog pancreatic α-amylase contains masked sulfhydryl groups which appear to be required for high enzymatic activity. The factors governing partial or complete exposure of these groups are reported. It has recently been indicated that taka amylase also possesses a masked sulfhydryl group (Isemura *et al.*, 1963).

METHODS

α-Amylase.—Crystalline hog pancreatic α-amylase was prepared and its activity was determined as previously described (Loyter and Schramm, 1962; Levitzki *et al.*, 1964). The suspension of crystals kept at 4° showed no loss of activity during 2–3 months. Enzyme protein was calculated from the absorbance at 280 mμ, applying the extinction coefficient of $E^{1\%}_{1\ cm}$ at 280 mμ = 24.1, calibrated according to dry weight (Hsiu *et al*,. 1964). A unit of amylase was defined as that amount that catalyzed the formation of 1 mg equivalent of maltose hydrate in 3 minutes at 30°. The specific activity was 1300–1500 units/mg of protein. Previously reported values were lower because they were based on protein determination by the method of Lowry *et al.* (1951), calibrated with serum albumin. A molecular weight of 50,000 was used to calculate mole equivalents of enzyme (Fischer and Stein, 1960). Fresh enzyme solution was prepared for each experiment by diluting an aliquot of the suspension of crystals in water to 1% concentration. Incubation at 30° for at least 10 minutes was required to dissolve the crystals. A commercial preparation of *B. subtilis* α-amylase was also tested and treated similarly.

Assay of activity after incubation of enzyme with various inhibitors was usually performed at the same final enzyme concentration as employed for the fully active enzyme (0.2–0.7 μg/ml).

Oxidation of Enzyme by Iodine.—The incubation mixture kept in ice contained 0.04–0.06 μmole of α-amylase; 10 μmoles of phosphate (Na^+, K^+), *p*H 6.5, or 20 μmoles of carbonate, *p*H 9.3; 1.5 μmoles of KI with 0.0–0.4 μmole of iodine, in a final volume of 0.6 ml. The iodine-KI reagent was rapidly delivered into the mixture as the last component in a volume of 10–60 μl. After 30 minutes of incubation at 0°, 5 μmoles of histidine were added to consume excess iodine. When the ratio of iodine to enzyme did not exceed 4 atoms/mole, all the iodine was consumed and histidine could be omitted without effect on subsequent assay of enzyme activity and SH content.

Uptake of ^{131}I.—Carrier-free ^{131}I (40 μc) was added to a solution containing 2 μmoles of iodine and 10 μmoles of KI in a final volume of 0.4 ml. Two hours at 0° were allowed for isotope equilibrium. Iodination of the enzyme with the radioactive reagent was performed as described in the preceding paragraph. Nonprotein iodide and iodine were subsequently removed by dialysis at 4° for 24 hours against 5 mM phosphate, *p*H 6.9, containing 5 mM NaCl, or by passage through a column of Sephadex G-50. Radioactivity was determined with the Packard liquid scintillation counter.

Reactivation System.—The iodine-treated enzyme (0.05 ml) was incubated for 60 minutes at 0° in 40 mM Tris buffer, *p*H 7.8, with 20 mM cysteamine, in a final volume of 0.5 ml. Cystein and mercaptoethanol appeared to be somewhat less efficient than cysteamine.

Quantitative Assay and Reactivity of Sulfhydryl Groups.—The reagent developed by Ellman (1959), 5,5′-dithiobis- (2-nitrobenzoic acid) (DTNB)[1] proved most useful for these studies. The complete assay mixture contained 0.02–0.04 μmole of α-amylase, 40 mM Tris buffer, *p*H

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[1] Abbreviations used in this work: DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); SDS, sodium dodecylsulfate; PMB, *p*-mercuribenzoate.

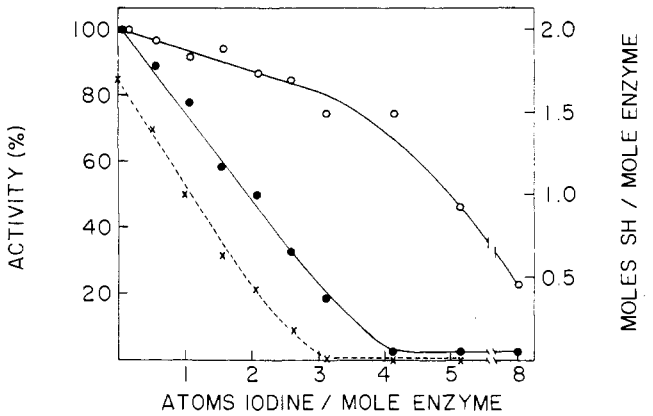

FIG. 1.—Enzyme inactivation, oxidation of sulfhydryl groups, and subsequent reactivation by cysteamine, as a function of the iodine-enzyme ratio in the reaction mixture. X---X, SH content; ●—●, enzyme activity; O—O, reactivation by cysteamine.

7.9, 10 mM EDTA, 1% SDS, and 0.2 mM DTNB, added as the last reagent, in a final volume of 1 ml. The reaction at room temperature was followed to completion at 412 mμ and mole equivalents SH were calculated using cysteine as a standard. The cysteine was standardized by iodimetric titration.

The reaction rate, which reflects the availability of the enzyme sulfhydryl groups, was strikingly affected by EDTA, SDS, and other reagents. Experimental details of such kinetic studies are given under the specific experiments.

MATERIALS

These were obtained from the following sources: *B. subtilis* crystalline α-amylase, Sigma Chemical Co.; cysteamine-(2-mercaptoethylamine) hydrochloride, California Corp. for Biochemical Research, DTNB, Aldrich Chemical Co.; carrier-free ^{131}I, Oak Ridge National Laboratory; SDS, DuPont Chemical Division (Duponal C, USP). The last was recrystallized from ethanol.

RESULTS

Effects of Iodine on Pancreatic α-Amylase.—Addition of 3 atoms of iodine/mole of enzyme at *p*H 6–7 caused 80% inhibition of the enzyme. Although iodine consumption appeared to be faster at alkaline *p*H, the inhibition decreased above *p*H 7.0. At *p*H 9.3, 3 atoms of iodine/mole of enzyme caused only 20% inhibition.

B. subtilis α-amylase, which contains no cystine or cysteine residues (Akabori *et al.*, 1956), was found completely resistant to iodine at *p*H 6.5, even when tested at a ratio of 8 atoms of iodine/mole of enzyme. It was therefore suspected that the high sensitivity of the pancreatic enzyme was due to the presence of cysteine residues which were oxidized by iodine. Indeed, it was readily demonstrated that the reaction was essentially limited to oxidation of the protein. Incorporation of iodine into the protein was only 0.05 atom/mole of enzyme when ^{131}I was incubated with the enzyme at a ratio of 3 atoms/mole. Thus, at most, 5% of a single tyrosine residue could have been converted to monoiodotyrosyl.

As demonstrated later, special conditions were required for unmasking the sulfhydryl groups to permit their quantitative assay. When such conditions were applied, it could readily be shown that enzyme inactivation by iodine largely coincided with sulfhydryl oxidation (Fig. 1). It should be noted, however, that

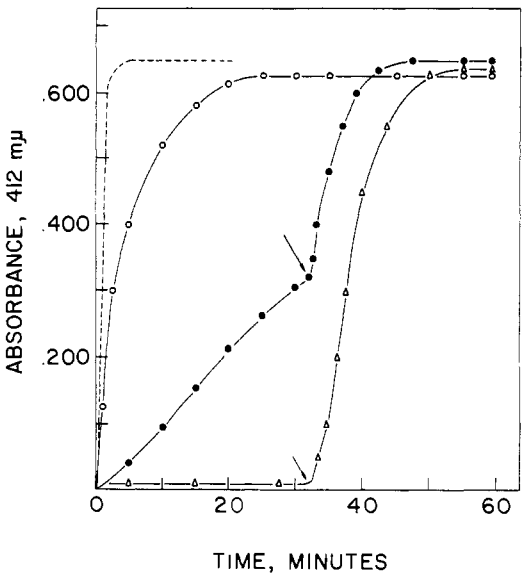

FIG. 2.—The function of EDTA and SDS in the reaction of amylase sulfhydryl groups with DTNB. Incubation mixtures contained 1.4 mg of enzyme. O—O, complete system containing EDTA and SDS, as outlined under Methods; ●—●, SDS omitted, but added subsequently at point marked with arrow; △—△, EDTA omitted, but added subsequently at point marked with arrow; - - - - -, the complete system, containing EDTA and SDS but without DTNB, was preincubated 20 minutes prior to addition of DTNB at zero time.

at the stage of complete sulfhydryl oxidation, inactivation reached 80% and increased further with higher iodine concentrations. Figure 1 also demonstrates reactivation of the iodine-treated enzyme by cysteamine. The activity of the native enzyme, which appeared to contain somewhat less than 2 SH equivalents/mole (Fig. 1), was not affected by cysteamine.

Unmasking of Enzyme Sulfhydryl Groups.—The sulfhydryl groups in the native pancreatic α-amylase did not react with DTNB in the presence of buffer alone. When EDTA was added to the system, a sluggish but progressive reaction occurred (Fig. 2). Oxalate and pyrophosphate, which cannot remove the tightly bound calcium from the enzyme (Fischer and Stein, 1960), did not replace EDTA. Although SDS also could not be substituted for EDTA, it greatly enhanced the reaction in the presence of the chelator. Preincubation of the enzyme with EDTA had little effect on the rate of reaction when DTNB was subsequently added (not shown in Fig. 2). However, preincubation with EDTA plus SDS led to an almost instantaneous and quantitative reaction upon addition of DTNB. As shown in Figure 3, a sluggish reaction of the enzyme sulfhydryl groups occurred in the presence of 7 M urea. The reaction was greatly stimulated by EDTA. These observations indicate that the rate-limiting factor is the unfolding of the protein, so that the sulfhydryl groups become completely exposed. Indeed, when enzyme was first denatured by incubation for 5 minutes at 25° with HCl, *p*H 2, and then brought to *p*H 7.9, it reacted instantaneously with DTNB even in the absence of EDTA. The color yield was the same as that obtained with enzyme incubated in the presence of EDTA and SDS.

Enzyme-Inhibition Studies.—In the absence of EDTA, enzyme activity was not affected by incubation for 120 minutes at 25° with iodoacetate, iodoacetamide, *N*-ethylmaleimide, DTNB, or *p*-mercuribenzoate (other conditions as described in Table I). Since EDTA caused partial unmasking of the sulfhydryl groups (Fig-

TABLE I

EFFECT OF SH-REACTIVE AGENTS AND SDS ON ENZYMIC ACTIVITY[a]

Additions	Inhibition (%)
DTNB	0
EDTA	0
EDTA, DTNB	76[b]
N-Ethylmaleimide	0
EDTA, *N*-ethylmaleimide	80
EDTA, iodoacetate	0
EDTA, iodoacetamide	0
EDTA, PMB	0–10
SDS	0
EDTA, SDS	100

[a] Incubation mixtures contained 2 mg of enzyme and 40 mM Tris buffer, *p*H 7.9. The concentration of other additions specified in the table were as follows: iodoacetate, iodoacetamide, and *N*-ethylmaleimide, 1 mM; PMB, 0.2 mM; DTNB, 0.2 mM; EDTA, 10 mM; SDS, 1%. The final volume was 1 ml. After incubation for 100 minutes at 22°, aliquots were diluted in 0.02 M phosphate buffer, *p*H 6.9, containing 1 mM $CaCl_2$ and 7 mM NaCl. Activity was then determined by the standard procedure. [b] Spectrophotometric assay at the end of incubation showed that 82% of the SH groups had reacted with DTNB.

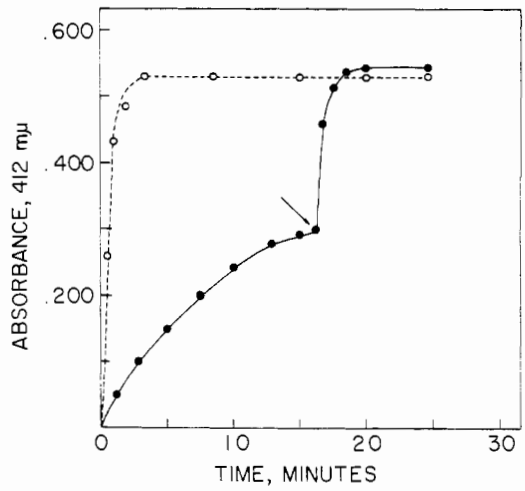

FIG. 3.—Effect of urea on the reaction of amylase sulfhydryl groups with DTNB, in the presence and absence of EDTA. Incubation mixtures contained 1.25 mg of enzyme, 7 M urea, 40 mM Tris buffer, *p*H 7.9, 0.2 mM DTNB, and 10 mM EDTA, as specified by the symbols. O---O, EDTA added at zero time; ●—●, EDTA added at point indicated by arrow.

ure 2), the above sulfhydryl reactive agents were retested in the presence of the chelator (Table I). It should be noted that incubation of the enzyme with EDTA alone had no effect on enzymatic activity when subsequently measured in the presence of calcium. However, incubation of the enzyme with *N*-ethylmaleimide or DTNB, in the presence of EDTA, resulted in extensive inhibition of activity. Iodoacetate and the other SH-reactive agents still had no effect. The reaction mixture containing iodoacetate was passed through a Sephadex G-50 column to remove the alkylating agent, and the sulfhydryl groups of enzyme in the effluent were determined. The amount of SH was the same as that in a sample of untreated enzyme, proving that iodoacetate had indeed failed to react with the sulfhydryl groups. Table I further demonstrates that the enzyme was unaffected by 1% SDS but was completely inactivated by this detergent if EDTA was present.

DISCUSSION

The present study clearly demonstrates that pancreatic α-amylase possesses sulfhydryl groups and that their oxidation or substitution results in greatly diminished catalytic activity. Analyses with two batches of enzyme gave a figure of 1.7 sulfhydryl groups/mole of enzyme. The reasons for this fractional number are still not clear.

The conditions which affect the reactivity of the sulfhydryl groups are of special interest as they reflect fine structural changes in the protein. In the native enzyme the sulfhydryl groups are readily reached by iodine but not by other typical SH-reactive agents. When EDTA acts, presumably by removing the tightly bound calcium from the enzyme (Fisher and Stein, 1960), the sulfhydryl groups become somewhat further exposed, so that they react with DTNB and *N*-ethylmaleimide. The sluggishness of the reaction with DTNB, and the fact that other SH-reactive agents are still ineffective, indicate, however, that the secondary and tertiary structures surrounding the sulfhydryl groups remain largely intact. Complete disruption of this structure occurs when SDS is also added. The increased rate of reaction with DTNB in the presence of both EDTA and SDS is essentially a measure of the rate of unfolding of the protein by SDS in the vicinity of the sulfhydryl groups. In support of this deduction are the observations that preincubation of the enzyme with EDTA and SDS results in an almost instantaneous quantitative reaction with DTNB, and that such preincubation causes complete inactivation of the enzyme.

The requirement for EDTA to unmask the sulfhydryl groups also gives some indications concerning the role of calcium in the structure of pancreatic α-amylase. An –S–Ca– group which would be transformed to an –SH by EDTA appears unlikely since urea exposed the sulfhydryl groups even in the absence of EDTA. Furthermore, tightly bound calcium is also found in *B. subtilis* α-amylase, which contains no sulfhydryl groups (Hsiu *et al.*, 1964).

Calcium is probably bound to other sites and contributes to the masking of the SH groups by tightening the structure of the molecule (Hsiu *et al.*, 1964). In this role calcium appears to be extremely effective. As shown in the present study, the metal prevented even partial exposure of the sulfhydryl groups by 1% SDS, and greatly delayed the action of urea. It should be noted that analyses in the ultracentrifuge of enzyme treated with EDTA and SDS have so far failed to show any evidence for dissociation of the enzyme into subunits (H. Loyter and M. Schramm, unpublished).

Since complete oxidation of the sulfhydryl groups coincided with 80% inactivation of the enzyme, it seems evident that they are not absolutely required in the enzymatic reaction. Recent findings by Robyt and French (1963, 1964) indicate that α-amylases from different sources might vary greatly in their substrate and product specificity. The possibility that the sulfhydryl groups of hog pancreatic α-amylase function in determining such specificity is currently being explored.

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REFERENCES

Akabori, S., Okada, Y., Fujiwara, S., and Sugae, K. (1956), *J. Biochem. (Tokyo) 43*, 741.

Caldwell, M. L., Weill, C. E., and Weill, R. S. (1945), *J. Am. Chem. Soc. 67*, 1079.

Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82*, 70.

Fischer, E. H., and Stein, E. A. (1960), *Enzymes 4*, 313.

Hsiu, J., Fischer, E. H., and Stein, E. A. (1964), *Biochemistry 3*, 61.

Isemura, T., Takagi, T., Maeda, Y., and Yutani, K. (1963), *J. Biochem. (Tokyo) 53*, 155.

Levitzki, A., Heller, Y., and Schramm, M. (1964), *Biochim. Biophys. Acta 81*, 101.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Loyter, A., and Schramm, M. (1962), *Biochim. Biophys. Acta 65*, 200.

Robyt, J., and French, D. (1963), *Arch. Biochem. Biophys. 100*, 451.

Robyt, J., and French, D. (1964), *Arch. Biochem. Biophys. 104*, 338.

Myeloperoxidase of the Leucocyte of Normal Human Blood. II. Isolation, Spectrophotometry, and Amino Acid Analysis*

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The first reported preparation of myeloperoxidase of A_{430}/A_{280} of 0.83 from the leucocytes of normal human blood donors was accomplished by column chromatography on XE-64 of extracts obtained from the crude enzyme precipitated from tryptic digests of frozen-thawed cells in 50% cold ethanol. The preparation was chemically characterized by Fe (0.093%) and N (14.3%), and by the following amino acid composition expressed as residues per molecule: lysine (11), histidine (4), arginine (35), aspartic acid (51), threonine (23), serine (21), glutamic acid (37), proline (31), glycine (26), alanine (26), valine (17), methionine (12), isoleucine (16), leucine (43), tyrosine (9), tryptophan (13), phenylalanine (17), and cystine as cysteic acid (13). The absorption spectra of the oxidized and reduced enzyme are the same as those described in the literature, but the pyridine hemochromogen spectrum is similar to that of formyl-diacetyl hemes and the hemochromogen of cytochrome oxidase. Solutions of alkaline dithionite convert the spectrum to a protohemochromogen type.

Procedures reported for the isolation of myeloperoxidase vary with the source of starting material. Summaries have been reported (Paul, 1963; Maehly, 1955). In the original preparation of the peroxidase from tubercular empyema (Agner, 1941) a mixture of ether, ammonium sulfate and water were used to precipitate the enzyme, which was dissolved in water and precipitated with alcohol after the sulfate was removed by precipitation with barium ions. The enzyme was then redissolved in water, and any insoluble material was recycled. More recently, Agner (1958) prepared crystalline myeloperoxidase from the cells of infected dog uteri which were digested at room temperature in ammoniacal ammonium sulfate and fractionally precipitated with ammonium sulfate. After purification, this product was passed through kieselguhr; material crystallizable from ammoniacal ammonium sulfate was obtained. In the case of rat chloroma, a tumor rich in myeloperoxidase, Schultz *et al.* (1957) prepared the crystalline enzyme (Schultz, 1958) by combining the preparation of crude enzyme described by Agner (1941) with the use of CG-50 (XE-64) Amberlite resin.

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Application of the above procedures to the isolation of the peroxidase from leucocytes of normal human blood, however, was found to be unsatisfactory. Treatment of normal white cells with trypsin, precipitation of the digest with alcohol, and extraction of the enzyme from the precipitate with phosphate buffer as described by Maehly (1955) proved to be a good starting point. In our hands this product was readily purified on the ion-exchange column (XE-64) previously described (Schultz *et al.*, 1957). In the present report a process is described which has been in use for 3 years, during which each week 300 ml of buffy coat of the blood of 300 donors was continuously processed. The enzyme content based on activity and cell counts of twenty-five such batches, as has been reported, varied over a wide range; but the average value was $2.13 \pm 0.14 \times 10^{-6}$ enzyme units per cell (Schultz and Kaminker, 1962).

The purpose of this report is to describe in detail the preparation of highly purified enzyme, having a ratio of A_{430}/A_{280} of 0.83 (equal to Agner's crystalline product), and to present hitherto unreported spectrophotometric properties of the enzyme and its chemical composition. Subsequent reports will deal with the nature of the heme peptides obtained from tryptic digests and preliminary data on primary structure.

METHODS AND PROCEDURES

Nitrogen was determined by micro-Kjeldahl, Fe by the procedure described by Drabkin (1941), and the amino acids by the method of Spackman *et al.* (1958) using the Phoenix automatic amino acid analyzer. Tryptophan was assayed both spectrophotometrically