Anal. Calcd. for $C_4H_8O_2S_2$: C, 31.56; H, 5.30; S, 42.13. Found: C, 31.58; H, 5.27; S, 42.27.

Oxidized DTE prepared similarly had the same melting point as oxidized DTT, but a mixture of the two melted over the range 110–120°.

Anal. Found: C, 31.84; H, 5.35; S, 42.15.

The ultraviolet spectrum of oxidized DTT is shown in Figure 2. The maximum of the disulfide peak is at 283 mμ (a_M = 273). The spectrum of oxidized DTE is essentially the same.

Redox Potentials of DTT and Cysteine.—Reactions were carried out at room temperature in 3 ml volume in silica cuvets containing 0.2 M buffer (phosphate, *p*H 7.0, or Tris, *p*H 7.9 or 8.1), 0.18 mg/ml crystalline dihydrolipoic dehydrogenase (Massey, 1960) and from 0.4 to 1.2 mM lipoamide. Either DPN^+ or DPNH (0.13–0.2 mM) was present initially. For the DTT experiments, the combined concentration of DTT and oxidized DTT was kept constant at 6.7 mM, and ratios of DTT to oxidized DTT of 4:1, 1:1, and 1:4 were used. The optical density of all components except enzyme was read, the enzyme was added, and the optical density was followed until equilibrium was reached. The amount of dihydrolipoic dehydrogenase used catalyzed a nearly instantaneous equilibration of DPN^+ and lipoamide, so that approach to equilibrium in the over-all reaction was limited by thiol interchange, equilibrium being reached in 1 minute at *p*H 8, and 10 minutes at *p*H 7 with DTT. The measured change in optical density was considered as a first approximation to be caused by DPNH formation or disappearance; corrections were later made for the small optical density changes resulting from changes in the concentrations of lipoamide and oxidized DTT.

In order to observe DPN^+ reduction by cysteine, it was necessary to use 42 mM cysteine and no cystine present initially. The observed reduction of DPN^+ was small, but was completely reversed by addition of 0.83 mM cystine.

ACKNOWLEDGMENT

The author wishes to thank Vicki Lonngren and Alan Bayless for technical assistance, and Dr. Peter Jurtshuk for a generous gift of dihydrolipoic dehydrogenase and lipoamide.

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The Enzymic Condensation of a Thiol Ester–Type Carboxyl-activated Acylamino Acid with an Amino Acid Amide to Form a Peptide*

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Received December 30, 1963

Papain is capable of catalyzing a transfer reaction in which the thiol ester group of benzoylglycine thiol ethyl ester is replaced by glycinamide. This is the first known example of a proteolytic enzyme catalyzing a replacement reaction involving a thiol ester–type activated amino acid carboxyl group. The effect of glycinamide concentration upon this reaction has been studied. These results indicate that a two-step reaction occurs. The first step, the hydrolysis of the thiol ester, is rate determining. The reaction shows a *p*H optimum at *p*H 7.0.

The first indication that proteolytic enzymes can effect the hydrolysis of a thiol ester was reported by Goldenberg *et al.* (1950). These investigators observed, upon incubating acetyl DL-phenylalanine thiol ester with chymotrypsin, the strong odor of ethyl mercaptan. Strecker *et al.* (1955) have shown that ox brain and ox liver contain thiolesterases. In 1956 in this laboratory it was demonstrated that the plant proteolytic enzyme papain possessed powerful thiolesterase activity (Johnston, 1956). Papain was shown to catalyze the hydrolysis of benzoylglycine thiol ethyl ester (BGTEE)[1] and benzoylglycine thiol isopropyl ester as well as benzoylglycine ethyl ester. Previously Bergmann *et al.* (1935) reported that benzoylglycinamide is an active substrate for papain.

Cysteine-activated papain is known to catalyze the transamidation reaction involving the replacement of the amide of benzoylglycinamide by ^{15}N ammonia introduced as isotopic diammonium hydrogen citrate or ammonia (Fruton, 1950). Papain also catalyzes the replacement of amide nitrogen of a number of acylamino acid amides by the —NHOH group of hydroxylamine or by amino acids to form peptides (Johnston *et al.*, 1950a,b; Fruton *et al.*, 1950).

The discovery of the thiolesterase action of this proteolytic enzyme suggested the possibility that the enzyme might be capable of catalyzing the transfer of the acyl group of a thiol ester to an amino compound to form a peptidelike structure. Previous to the work reported here, the catalysis by proteolytic enzymes of a transfer reaction of this general type has not been reported. In this report the papain-catalyzed synthesis of BGGA from BGTEE and glycinamide is described.

* This work has been supported in part by a grant (HE-2970) from the National Institutes of Health.

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[1] The following abbreviations will be used: BGTEE = benzoylglycine thiol ethyl ester; BGGA = benzoylglycylglycinamide; BG = benzoylglycine.

Experimental

The BGTEE was prepared by a method which has been previously described (Johnston, 1956). The papain was prepared according to the method of Grassmann (1935) from crude dried papaya latex obtained from Wallerstein Laboratories. The BGGA was prepared by treating benzoylglycylglycine methyl ester with methanol saturated with ammonia in a pressure flask for 24 hours. The carboxyl ^{14}C-labeled glycinamide was prepared as described by Johnston *et al.* (1950b) from ^{14}C-labeled glycine which had been prepared according to Bloch (1949). The specific activity under the standard conditions of determination of radioactivity was 1,200,000 cpm. In most experiments the radioactive glycinamide was diluted 1:3 with nonradioactive glycinamide.

Analysis of BGTEE.—The method described earlier (Johnston, 1956) was employed.

Enzyme Incubation.—The enzymic reactions were carried out at 37°. The final concentrations of the components of the incubation solution were HCN, 0.05 M; sodium citrate or sodium phosphate buffer, 0.025 M; radioactive glycinamide, 0.025 M; papain, 0.5 mg/ml; BGTEE, 0.23 M; and methanol, 30%. Total volume was 10 ml. The incubation time was usually 25 minutes. The reaction was initiated by the addition of a 0.075 M solution of BGTEE in methanol. Duplicate 0.1-ml samples of the incubation mixture were withdrawn immediately after mixing and at the termination of the experiment in order to determine the extent of hydrolysis of the thiol ester.

Radioactive BGGA Assay.—The reaction was stopped by adding the incubation mixture to 5 ml of 2 M neutralized hydroxylamine. The hydroxylamine was added to effect the conversion of any unhydrolyzed BGTEE into the hydroxamic acid. This prevented the thiol ester from reacting nonenzymatically with the radioactive glycinamide. Preliminary experiments in which no hydroxylamine was added demonstrated that unreacted BGTEE can nonenzymatically acylate the radioactive glycinamide under the conditions employed for the isolation of BGGA. This resulted in a very high radioactivity in the isolated BGGA even in the experiment when no enzyme was added. The conversion of the thiol ester to the hydroxamic acid before isolating the BGGA greatly diminished the radioactivity in the BGGA due to this nonenzymic condensation. After 20 minutes 100 ml of 95% ethanol containing 100 mg of BGGA was added and the sample was placed in a refrigerator overnight. The mixture was filtered in the cold through Celite which had previously been washed with ethanol. The sample was then evaporated to dryness under vacuum at 40–50°. The resulting solid was recrystallized from water, from nonlabeled glycinamide, and again from water. The infrared spectra of the isolated BGGA was identical to that of a pure sample of BGGA. Approximately 8 mg of the isolated BGGA was burned in a Fischer microcombustion furnace in an atmosphere of oxygen and the resulting CO_2 was trapped as $BaCO_3$. The $BaCO_3$ was plated on planchets ($^3/_4$-inch diameter) and counted in a Packard Model 200 gas-flow counter and the results were corrected to infinite thickness (Reid, 1948).

Results

The replacement reaction in which the acylamino group of the acylamino acid thiol ester is transferred to the amino group of an amino acid to form a peptide was studied by measuring the extent of incorporation of carboxyl-labeled ^{14}C-glycinamide into BGGA. The ^{14}C-glycinamide was incubated with papain, buffer, and BGTEE, and after the addition of carrier BGGA and the isolation of the BGGA the extent of the exchange was measured by the isotope-dilution technique. Since BGGA is a substrate for papain and since the carrier was not added until the termination of the enzyme incubation, the experiments must be carried out under conditions under which a minimum amount of the enzymatically synthesized BGGA is hydrolyzed. For this reason the enzymic reactions were allowed to proceed only to about 30–40% completion. Under the conditions described in the experimental section this required an incubation time of about 30 minutes. The cleavage of the BGGA is less than 1% under these conditions since the rate of hydrolysis of the thiol ester is about twenty times faster than the rate of hydrolysis of the amide.

One of the problems encountered in the early phases of this investigation was the nonenzymic reaction of the BGTEE with the radioactive glycinamide, which reaction occurred during the isolation of the BGGA. This resulted in nonenzymic blanks whose radioactivity was too high for accurate interpretation of the results. A similar type of condensation of an amino acid thiol ester has been previously reported (Wieland and Schäfer, 1951). In order to avoid this difficulty the unreacted thiol ester which remained after the enzyme incubation period was immediately decomposed by the addition of 2 M hydroxylamine. This prevented any further reaction of the BGTEE with glycinamide. A typical experiment performed under these conditions is shown in Table I. The

Table I

The Enzymic Condensation of BGTEE with Glycinamide[a]

Substrate	Conditions	Specific Activity of BGGA (cpm)
BGTEE	Complete system	8853
BGTEE	No enzyme	41
BG	Complete system	18

[a] Conditions are described in the experimental section. Specific activity of the glycinamide was 400,000 cpm; pH = 7.5.

observed radioactivity corresponds to the formation of 13.8 mg or 58.7 μM of BGGA. This represents a 23% conversion of the original thiol ester to BGGA. The time of incubation corresponds to 43% hydrolysis of the substrate. Consequently about 54% of the substrate which reacted with the enzyme reacted with the glycinamide to form the product. These observations clearly demonstrated that an enzyme-dependent condensation of the BGTEE with glycinamide to form BGGA had occurred. In order to establish that the formation of BGGA is dependent upon the presence of BGTEE and is not due to the reversal of the hydrolytic reaction the experiment was repeated with BG as the substrate. The results given in Table I show clearly that BGTEE participated in the reaction.

The effect of glycinamide concentration on the rate of incorporation of radioactive glycine into BGGA is summarized in Figure 1. The rate of incorporation of the radioactive glycinamide into BGGA was dependent upon the concentration. The variation of concentration of glycinamide throughout the range of concentrations studied had no appreciable effect upon the rate of the hydrolysis of the thiol esters. At the

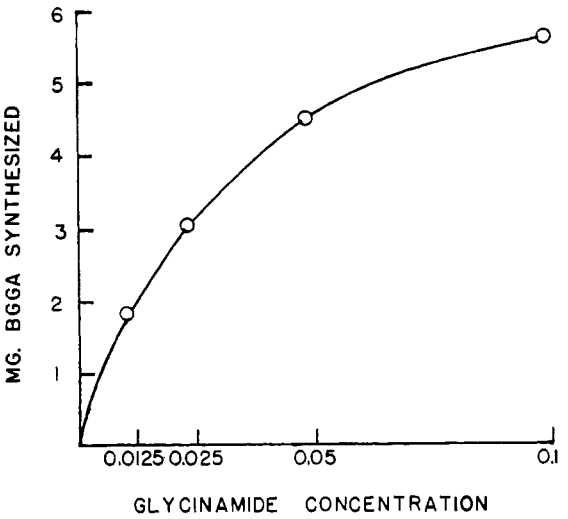

FIG. 1.—BGGA synthesis at varying glycinamide concentrations. The mg BGGA synthesized refers to the synthesis in 10 ml incubation solution. Conditions are described in the experimental section. Specific activity of the glycinamide was 40,000 cpm; *p*H = 7.5.

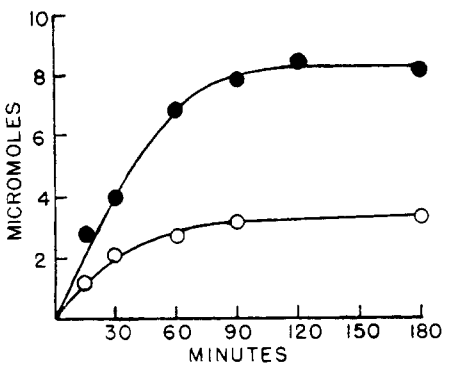

FIG. 2.—Comparison of the enzymic hydrolysis of BGTEE and the formation of BGGA at various stages of the reaction. ● = the enzymic hydrolysis of BGTEE expressed in μmoles/ml; ○ = the enzymic formation of BGGA expressed in μmoles/ml. Concentrations of the components of the enzyme incubation solution were as described in the experimental section. Specific activity of the glycinamide was 400,000 cpm. One-ml aliquots were removed at the indicated time intervals and mixed with 100 mg of carrier BGGA.

higher glycinamide concentrations the observed increase in the formation of BGGA must be the result of a greater proportion of enzyme substrate complexes reacting with the glycinamide.

The rate of synthesis of BGGA and the rate of hydrolysis of BGTEE as a function of the time of incubation is shown in Figure 2. From Figure 2 it can be seen that the synthesis of BGGA and the hydrolysis of BGTEE reached a maximum in 90 minutes. The similarity in the shapes of the two curves would suggest that the formation of BGGA is dependent upon the extent of the interaction of the BGTEE with the enzyme.

The enzymic and nonenyzmic incorporation of glycinamide into BGGA at *p*H 5.5–7.5 is given in Figure 3. At the higher *p*H range a relatively large nonenzymic formation of BGGA occurred. This reflects the instability of the thiol ester substrate at this *p*H. The enzymic incorporation is determined by subtracting the nonenzymic incorporation from the total incorporation observed in the incubation

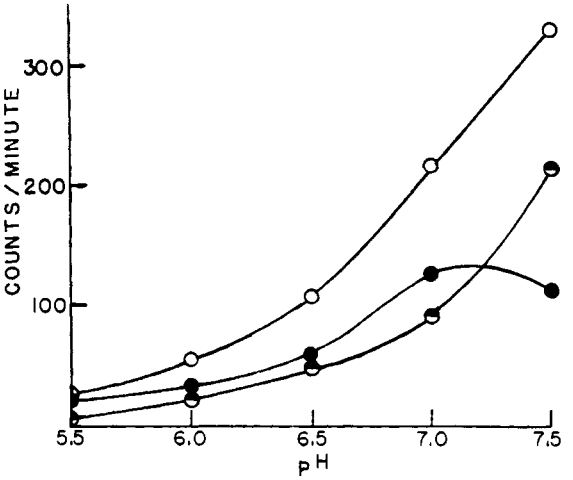

FIG. 3.—The effect of *p*H on the incorporation of glycinamide into BGGA. ○ = total incorporation; ● = enzymic incorporation; ◓ = nonenzymic incorporation. The conditions of incubation are described in the experimental section. Specific activity of the glycinamide was 40,000 cpm.

solution containing the enzyme. Contrary to the behavior of the nonenzymic formation of BGGA the enzymic incorporation of glycinamide into BGGA reaches a maximum at *p*H 7.0. This suggests that interaction of the glycinamide with the thiol ester–enzyme complex is probably altered by ionization in the vicinity of the active site of the enzyme. This effect can be illustrated somewhat more dramatically when the per cent of conversion of the enzyme substrate complexes to BGGA is plotted versus *p*H (Fig. 4). At *p*H 7.0 nearly five times as many enzyme-substrate complexes react with glycinamide as at *p*H 5.0.

DISCUSSION

The formation of a peptide bond from a thiol ester-activated intermediate with papain as the catalyzing entity has been clearly demonstrated. The reaction appears to proceed by the following pathway:

(1) PAPAIN–S′H + –CO–NH–CH_2–CO–S–CH_2–CH_3

→ –CO–NH–CH_2–CO–S′–PAPAIN + CH_3–CH_2–SH

(2a) –CO–NH–CH_2–CO–S′–PAPAIN + NH_2–CH_2–CO–NH_2 →

 –CO–NH–CH_2–CO–NH–CH_2–CO–NH_2 + PAPAIN–S′H

(2b) –CO–NH–CH_2–CO–S′–PAPAIN + H_2O →

 –CO–NH–CH_2–CO_2H + PAPAIN–S′H

The intermediate thiol ester which involves the sulfhydryl group of papain was first proposed by Smith *et al.* (1955).

The variation of glycinamide concentration is without effect on the rate of BGTEE disappearance while the rate of incorporation of radioactive glycinamide into BGAA is dependent upon the concentration of glycinamide. These results indicate that a two-step

reaction occurs. These reactions are represented by reactions 1 and 2a. As the glycinamide concentration is increased a greater proportion of the enzyme-substrate complex reacts with the glycinamide to form BGGA (reaction 2a). At the higher glycinamide concentrations there is a corresponding decrease in the amount of complex which reacts with water to give hippuric acid (reaction 2b). Results of the experiments in which the effect of glycinamide concentration upon the rates of BGTEE disappearance and the incorporation of radioactivity indicates that the formation of the enzyme-substrate complex from BGTEE and papain (reaction 1) is the rate-controlling step.

The enzymic formation of BGGA is more markedly *p*H-dependent than either the disappearance of BGTEE or the total incorporation of glycinamide. Although at *p*H 7.5 the nonenzymic condensation of BGTEE with glycinamide proceeds at a faster rate than at *p*H 7.0, the enzymic formation of BGTEE exhibits a distinct maximum in the region of *p*H 7.0. The reactivity of the thiol ester–type enzyme-substrate complex would be expected to be greater at *p*H 7.5 than at 7.0 as is indicated by the nonenzymic condensation at *p*H 7.5. The occurrence of a maximum in the enzymic formation of BGGA may be due to an ionization in the region of *p*H 7.5 and near the active center of the enzyme, which ionization may alter the charge distribution at the active site in such a way that the glycinamide cannot react so readily at *p*H above 7.0 with the enzyme-substrate complex.

The results reported here represent an extension of the various types of exchange reactions catalyzed by proteolytic enzymes to include the more recently discovered thiol ester substrates. In this case the energy-rich thiol ester bond is utilized for the formation of the peptide bond. The enzyme system reported here can be considered as a model system for the biological formation of peptides by utilization of a thiol ester–type carboxyl-activated amino acid energy-rich bond.

Matthaei and Nirenberg (1961) have reported that β-mercaptoethanol will stabilize the enzyme system capable of incorporating amino acids into proteins and Hülsmann and Lipmann (1960) have found that the ribosomal system which effects the incorporation of aminoacyl-s-RNA into protein requires a sulfhydryl component. These observations suggest the participation of a sulfhydryl group in the mechanism of condensation of amino acids to form proteins and raise the possibility that an amino acid thiol ester may be an intermediate in the biological condensation of amino acids to form proteins. In recent years considerable experimental evidence has accumulated which has clarified the biochemical mechanism of protein synthesis. However, most of these studies have been concerned with the activation and the orientation of the amino acids prior to their condensation. The precise mechanism by which the activated and oriented amino acids condense to form the peptide chain remains obscure. If the requirement for mercaptans in the protein-synthesizing systems is because they participate in the transient formation of amino acid thiol esters either with the mercaptan or with an active sulfhydryl group on the enzyme, then it would appear that the experiments on model systems such as the one reported here may be important in further studies on the mechanism of the condensation of activated amino acids to form proteins.

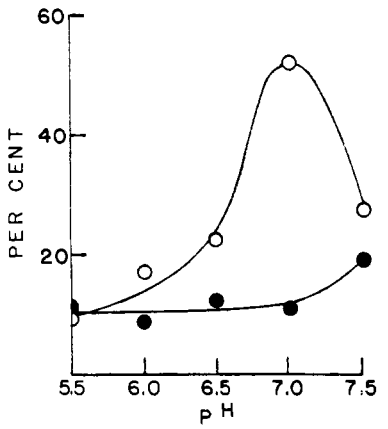

FIG. 4.—Comparison of the per cent hydrolysis and the per cent exchange at *p*H values between 5.5 and 7.5. The per cent exchange is the μmoles BGGA synthesized per μmoles BGTEE hydrolyzed × 100. ○ = the per cent exchange; ● = the per cent hydrolysis. Conditions are described in the experimental section. Specific activity of the glycinamide was 40,000 cpm.

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