Bender, M. L., and Glasson, W. A. (1960), *J. Amer. Chem. Soc. 82*, 3336.
Bender, M. L., and Kézdy, F. J. (1965), *Annu. Rev. Biochem. 34*, 49.
Benöhr, H. C., and Krisch, K. (1967), *Hoppe-Seyler's Z. Physiol. Chem. 348*, 1102.
Bergmann, F., Rimon, S., and Segal, R. (1958), *Biochem. J. 68*, 493.
Bruice, T. C. (1961), *J. Polymer Sci. 49*, 100.
Bruice, T. C., Fife, T. H., Bruno, J. J., and Brandon, N. E. (1962), *Biochemistry 1*, 7.
Eigen, M., Hammes, G. G., and Kustin, K. (1960), *J. Amer. Chem. Soc. 82*, 3482.
Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82*, 70.
Epand, R. M., and Wilson, I. B. (1963), *J. Biol. Chem. 238*, 1718.
Epand, R. M., and Wilson, I. B. (1964), *J. Biol. Chem. 239*, 4145.
Franz, W., and Krisch, K. (1968), *Hoppe-Seyler's Z. Physiol. Chem. 349*, 1413.
Gerstein, J., and Jencks, W. P. (1964), *J. Amer. Chem. Soc. 86*, 4655.
Goldberg, M. I., and Fruton, J. S. (1969), *Biochemistry 8*, 86.
Goldberg, M. I., and Fruton, J. S. (1970), *Biochemistry 9*, 3371.
Greenzaid, P., and Jencks, W. P. (1970), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29*, 407.
Heymann, E., and Krisch, K. (1967), *Hoppe-Seyler's Z. Physiol. Chem. 348*, 609.
Holmes, R. S., and Masters, C. J. (1967), *Biochim. Biophys. Acta 132*, 379.
Horgan, D. J., Dunstone, J. R., Stoops, J. K., Webb, E. C., and Zerner, B. (1969a), *Biochemistry 8*, 2006.
Horgan, D. J., Stoops, J. K., Webb, E. C., and Zerner, B. (1969b), *Biochemistry 8*, 2000.
Horgan, D. J., Webb, E. C., and Zerner, B. (1966a), *Biochem. Biophys. Res. Commun. 23*, 18.
Horgan, D. J., Webb, E. C., and Zerner, B. (1966b), *Biochem. Biophys. Res. Commun. 23*, 23.
Inagami, T., Patchornik, A., and York, S. S. (1969), *J. Biochem. (Tokyo) 65*, 809.
Inward, P. W., and Jencks, W. P. (1965), *J. Biol. Chem. 240*, 1986.
Jencks, W. P., and Gilchrist, M. (1964), *J. Amer. Chem. Soc. 86*, 4651.
Jencks, W. P., and Gilchrist, M. (1968), *J. Amer. Chem. Soc. 90*, 2622.
Kaiser, E. T., and Lo, K.-W. (1969), *J. Amer. Chem. Soc. 91*, 4912.
Kézdy, F., and Bender, M. L. (1962), *Biochemistry 1*, 1097.
Koshland, D. E., Jr. (1954), *in* Mechanisms of Enzyme Action, McElroy, W. D., and Glass, B., Ed., Baltimore, Md., Johns Hopkins, p 608.
Krisch, K. (1966), *Biochim. Biophys. Acta 122*, 265.
Lipmann, F., and Tuttle, L. C. (1950), *Biochim. Biophys. Acta 4*, 301.
Malhotra, O. P., and Philip, G. (1966), *Biochem. Z. 346*, 386.
McDonald, C. E., and Balls, A. K. (1956), *J. Biol. Chem. 221*, 993.
O'Neill, J. J., Kohl, H., and Epstein, J. (1961), *Biochem. Pharmacol. 8*, 399.
Pahlich, E., Krisch, K., and Borner, K. (1969), *Hoppe-Seyler's Z. Physiol. Chem. 350*, 173.
Parker, L., and Wang, J. H. (1968), *J. Biol. Chem. 243*, 3729.
Stoops, J. K., Horgan, D. J., Runnegar, M. T. C., de Jersey, J., Webb, E. C., and Zerner, B. (1969), *Biochemistry 8*, 2026.

Peptidoglutaminase. Enzymes for Selective Deamidation of γ-Amide of Peptide-Bound Glutamine*

Mamoru Kikuchi,† Hiroshi Hayashida, Eiichi Nakano, and Kenji Sakaguchi

ABSTRACT: Two different glutaminases have been separated and partially purified from a soil isolate microorganism identified as a strain of *Bacillus circulans*. These enzymes have been named peptidoglutaminases because of their specificity for catalyzing the hydrolysis of the γ-amide group of peptide-bound glutamine. Peptidoglutaminase I was specific for derivatives substituted at the α-amino group of L-glutamine such as carbobenzoxy-L-glutamine, glycyl-L-glutamine, L-leucylglycyl-L-glutamine, and *N*-acetyl-L-glutamine but was inactive toward L-glutamine derivatives substituted at the carboxyl position or at both α-amino and carboxyl positions Peptidoglutaminase II was specific for hydrolysis of the γ amide group of L-glutamine residues substituted at the car boxyl position or both the α-amino and carboxyl groups (*e.g.*, L-glutaminylglycine or L-phenylalanyl-L-glutaminyl-glycine).

Both enzymes catalyzed the deamidation of free L-glutamine poorly and had no activity toward asparagine, asparagine derivatives, or other amino acid amides. No peptidase activity was associated with either peptidoglutaminase.

Glutaminases (L-glutamine amidohydrolase, EC 3.5.1.2) are widely distributed in animals, plants, yeasts, and bacteria (Hartman, 1968; Meister, 1955; Meister *et al.*, 1955; Roberts, 1960). The specificity of previously reported glutaminases was restricted to free glutamine or some derivatives such as methyl-DL-glutamine. Our aim was to find an enzyme which could

* From the Noda Institute for Scientific Research, Noda-Shi, Chiba-Ken, Japan. *Received September 2, 1970.*

† To whom to address correspondence at: College of General Education, University of Tokyo, Tokyo, Japan. *Received September 2, 1970.* This work was supported in part by the Central Research Laboratories of Kikkoman Shoyu Co., Ltd.

catalyze the hydrolysis of γ-amides of protein-bound glutamine. *tert*-Amyloxycarbonyl-L-glutaminyl-L-proline was used as a substrate for screening microorganisms, because of its resistance to hydrolysis by many proteinases and peptidases. After screening many fungi, streptomyces, and bacteria, an organism was isolated which selectively deamidated the γ-amide of peptide-bound glutamine. The organism was subsequently identified as *Bacillus circulans*. During the course of purification of this deamidase activity, two enzymatically active peaks were obtained from DEAE-Sephadex column chromatography. One was specific for glutamine on the carboxy terminal of a peptide, and the other for glutamine on the amino-terminal or endo residue of a peptide. The former was designated as peptidoglutaminase I, and the latter as peptidoglutaminase II. In this paper we describe the purification and some properties of these enzymes.

Materials and Methods

The following compounds were purchased from Cyclo Chemical Corp.: Cbz-L-glutaminylglycine (lot D-2411),[1] Cbz-L-glutamylglycine (lot D-1604), L-glutaminylglycine (lot D-1607), α-L-glutamylglycine (lot P-1958), glycyl-L-glutamine (lot M-2932), L-tyrosyl-L-glutamine (lot M-1458 M), L-prolyl-L-glutamine (lot D-1613), *N*-acetyl-L-glutamine (lot H-3158), and phthaloyl-DL-glutamine (lot R-5099). Glycyl-L-asparagine was obtained from Nutritional Biochemical Corp. and Cbz-L-glutamine, Cbz-L-glutamic acid, Cbz-L-asparagine, L-leucinamide, and glycyl-L-phenylalaninamide acetate from Protein Research Foundation, Osaka, Japan. The following substances were kindly supplied by Dr. S. Sakakibara of Protein Research Foundation, Japan: *t*-AOC-L-glutaminyl-L-proline, L-phenylalanyl-L-glutaminylglycine, L-leucylglycyl-L-glutamine, and L-isoleucylglutaminylasparaginylcysteinylprolylleucylglycine *N*-acetate. L-Pyroglutamyl-L-glutaminyl-L-alanine (Eisenine) was kindly supplied by Dr. T. Ohira of Shinshu University, Japan. *Escherichia coli* glutaminase (grade IV 384 units/mg) and L-glutamic acid γ-monohydroxamate were purchased from Sigma Chemical Co. L-Glutamic acid decarboxylase was from Kyowa Hakko Kogyo Co., Ltd, Japan.

The pure alkaline proteinase from *Aspergillus sojae* was kindly supplied by Mr. K. Hayashi of the Central Research Laboratories of Kikkoman Shoyu Co., Ltd.

Transglutaminase was prepared according to the procedure of Clarke *et al.* (1959) from the soluble fraction of guinea pig liver homogenate. This enzyme preparation showed a specific activity of 250 units/mg of protein when assayed by means of the N-Z Amine substrate of Mycek and Waelsch (1960). [2-^{14}C]Ethanolamine-HCl (CFA. 329, batch 5, 27.3 mCi/mmole) was obtained from The Radiochemical Centre, Amersham.

Peptide Synthesis. Cbz-L-glutaminyl-L-proline was prepared from Cbz-L-glutamine and L-prolinebenzyl ester hydrochloride by the coupling procedure of Woodward *et al.* (1961); L-prolinebenzyl ester hydrochloride was prepared according to the procedure of Miller and Waelsch (1952) to obtain a cyrstalline specimen of mp 143°, lit. mp 148–148.5° (Greenstein and Winitz, 1961). Cbz-L-glutamine (7.52 g) was dissolved in acetonitrile (500 ml) containing triethylamine (4.18 ml), and 8.15 g of the Woodward reagent (Protein Research Foundation, Osaka, Japan) was added with stirring until the reagent had dissolved. L-Prolinebenzyl ester hydrochloride (7.23 g) and triethylamine (4.18 ml) were added and the reaction mixture was stirred overnight at room temperature. After the solvent was removed *in vacuo*, ethyl acetate (200 ml) was added and washed two times with water, five times with 0.1 N HCl, five times with 5% $NaHCO_3$, and three times with water. The resulting solution was dried over anhydrous sulfate, concentrated *in vacuo*, and evaporated to dryness after addition of methanol. The residue was dissolved in methanol and saponified with 1 N NaOH for 2 hr at room temperature. The solution was made weakly alkaline to thymol blue test paper with dilute acid, and evaporated. The product was dissolved in water and extracted twice with ethyl acetate. After acidification to thymol blue with 1 N HCl, the aqueous layer was reextracted five times with ethyl acetate. The extract was washed two times with water, dried over anhydrous sodium sulfate, and concentrated *in vacuo*. The oily precipitate was obtained by the addition of ether (100 ml) and petroleum ether (bp 30–60°). The desired product was crystallized from benzene (150 ml) by the addition of petroleum ether so as to obtain crystals (yield 5.67 g), mp 62°. *Anal.* Calcd for $C_{18}H_{23}N_3O_6$, C, 57.3; H, 6.2; N, 11.1. Found: C, 57.2; H, 6.1; N, 10.6.

Other materials used which are not specifically described were the best available commercial products.

Enzyme Assay. Peptidoglutaminase activity was determined by ammonia liberation from glutaminyl peptide. The reaction mixture usually contained: 10 μmoles of substrate, 40 μmoles of phosphate buffer (pH 7.5), and suitably diluted enzyme solution, in a final volume of 1.0 ml. The reaction mixture was incubated for 10 min at 30°. Aliquots of the reaction mixtures were added to equal volume of 10% trichloroacetic acid and the pH was adjusted to 6.0–6.5 by the addition of 1 N NaOH. Ammonia was determined on these samples by nesslerization after a modified Conway microdiffusion (Cedrangolo *et al.*, 1965).

The reported values for ammonia production have been corrected for ammonia produced nonenzymatically and for ammonia in the enzyme preparations in the absence of substrate. Nonenzymic ammonia formation from all substrates was very small (less than 0.03 μmole of ammonia/10 μmoles of substrate under these experimental conditions). One unit of enzyme is defined as that which catalyzes the liberation of 1 μmole of ammonia from glutaminyl peptide per 1 min at 30°. Specific activity is defined as units per milligram of protein.

Hydroxamate Formation. Hydroxamate formation of glutaminyl peptides was measured by the colorimetric procedure of Lipmann and Tuttle (1954) with γ-glutamohydroxamate as the standard. To 1.0-ml aliquots of incubation mixtures were added 0.9-ml portions of the ferric chloride–trichloroacetic acid reagent. The absorbances were measured in 1.0-cm cuvets at 525 nm.

Thin-Layer Chromatography. Peptides were chromatographed on silica gel film (Kieselgel G Nach Stahl, Merck) with the following solvent systems: 1-butanol–acetic acid–water (4:1:1, v/v), 1-butanol–acetic acid–5% (w/v) ammonia water (5.5:3:1.5, v/v), ethyl acetate–benzene–acetic acid (50:50:2, v/v), and chloroform–methanol–acetic acid (95:5:3, v/v). Amino acids and peptides were detected using ninhydrin reagent. Cbz-peptides were detected with ninhydrin reagent after treatment with 48% HBr.

Paper Electrophoresis. Amino acids and peptides were applied to Toyo Roshi No-50 filter paper rinsed in a pyridine–acetic acid–water (25:1:225, v/v) buffer and subjected to electrophoresis in the same buffer for 70 min at 2000 V.

[1] The abbreviations used are: Cbz, carbobenzoxy; *t*-AOC, *tert*-amyloxycarbonyl; GTF, glutamotransferase.

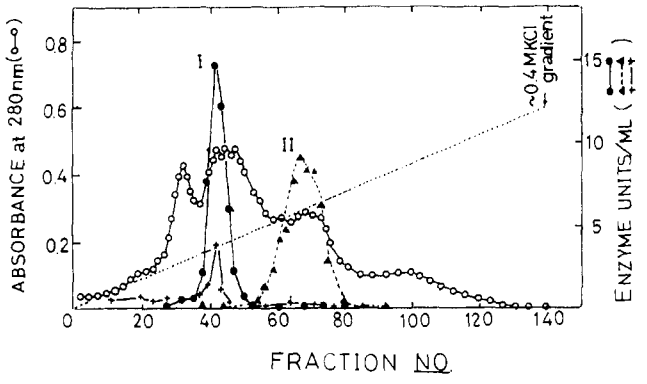

FIGURE 1: DEAE-Sephadex A-50 chromatography of peptidoglutaminase. The elution of protein was followed at 280 nm. The enzyme activity was measured with Cbz-L-glutamine (●—●), Cbz-L-glutaminyl-L-proline (▲---▲), and L-glutamine (+—+). Fractions of 6 ml were collected.

Cbz-peptides were detected by oxidation with I_2 or spraying with Morin reagent (Schellenberg, 1962).

Enzymatic Hydrolysis of Casein. Hammarsten milk casein (Merck and Co., Inc.) was hydrolyzed by the purified alkaline proteinase of *Aspergillus sojae* (Hayashi *et al.*, 1967a,b) in 0.1 M phosphate buffer (pH 7.0) containing 50 μmoles of $CaCl_2$ and toluol for 3 days at 37°. The incubation was stopped by heating in boiling water for 5 min.

Free L-glutamic acid content of samples was measured manometrically by a modification of Gale's method (Gale, 1945). Warburg manometers were used containing 0.5-ml samples together with 1.0 ml of 0.2 M acetate buffer (pH 5.0) in the main cup, and in the side-bulb 0.5-ml enzyme preparation of L-glutamic acid decarboxylase made up in the same buffer. The CO_2 liberated from L-glutamic acid by L-glutamic acid decarboxylase at 37° for 60 min was measured manometrically.

[^{14}C]Ethanolamine Incorporation into Cbz-peptide. The incorporation of [^{14}C]ethanolamine into Cbz-peptide was assayed after Folk and Cole (1965) using Dowex 50X-8 (H^+) resin for the elimination of excess ethanolamine. Radioactivity was measured in 0.5 ml of 6% NH_4OH and 10 ml of naphthalene–dioxane counting fluid (Bray, 1960) with the use of a Nuclear-Chicago liquid scintillation system. Control experiments showed that more than 99% of the unreacted radioactive amine was removed from a typical incubation mixture by the ion-exchange resin treatment. The counts obtained 0-min incubations were subtracted from those obtained at the end of each incubation period.

Assay of Protein. The concentration of protein was determined by the Biuret Method of Layne (1957a) and by a spectrophotometric procedure (Layne, 1957b) using a Hitachi Perkin-Elmer spectrophotometer type 139.

Results

The Organism. A strain of bacteria was isolated from soil which specifically hydrolyzes the γ-amide of glutamine residue of *t*-AOC-L-glutaminyl-L-proline. The bacteriological properties of the organism were studied on the basis of the Manual of Microbiological Methods (Pelczar, 1957) and then classified according to Bergey's Manual of Determinative Bacteriology, (Breed *et al.*, 1957). As a result, it was identified as a *Bacillus circulans* species. This organism was rod-shaped cell, capable of producing endospores in definitely swollen sporangia, motile by means of peritrichous flagella, gram negative, a non-gas producer from carbohydrates, a starch hydrolyzer, a non-indole and non-acetylmethylcarbinol producer, and incapable of growing at 65°.

Cells were grown in a medium containing: polypepton, 1.0%; yeast extract, 0.3%; lactose, 0.5%; $MgSO_4 \cdot 7H_2O$, 0.025%; $FeSO_4 \cdot 7H_2O$, 0.001%; KH_2PO_4, 0.025%; $Na_2HPO_4 \cdot 12H_2O$, 0.17%, pH 7.2. For large-scale growth, an inoculumn of 750 ml in 3-l. erlenmeyer flask was prepared and grown for 14 hr on a rotary shaker (140 rpm) at 30°. This was transferred into 15 l. of sterile culture medium in a jar fermentor of 30-l. capacity (Marubishi Laboratory Equipment Co., Ltd, Japan). Cultivation was continued at 30° with airation (8 l./min of air) and stirring at 350 rpm. When the optical density at 660 nm exceeded 0.9 (maximum growth), the cells were harvested, washed once with 0.01 M phosphate buffer (pH 7.2), frozen, and stored.

Preparation of the Enzymes. A total of 110 g wet cells was suspended in 400 ml of 0.05 M phosphate buffer (pH 7.2) and sonicated for 10 min at 4° using a 10-kc sonic oscillator (Toyo Riko Co., Japan). All further operations were carried out at 0–4°. The sonified material was centrifuged in a Spinco Model-L preparative ultracentrifuge for 30 min at 105,000*g*. The resulting supernatant fluid was collected and dialyzed overnight against 0.005 M phosphate buffer (pH 7.2). This dialysate (crude extract) contained 6680 mg of protein. To this crude extract 10% streptomycin sulfate (w/v) was added slowly with stirring until precipitation was complete, allowed to stir for 30 min, then centrifuged. Ammonium sulfate solution (3.5 M, pH 7.2) was added with stirring to the supernatant fluid. The fraction precipitating between 38 and 54% of saturation was allowed to stand at 0° for 30 min then collected by centrifugation at 30,000*g* for 20 min. This precipitate was dissolved in 30 ml of 0.01 M phosphate buffer (pH 7.6) containing 0.1 M KCl and 2 mM EDTA. The sample was applied to a column (4 × 90 cm) of Sephadex G-200 (Pharmacia) previously equilibrated with 0.01 M phosphate buffer (pH 7.6) containing 0.1 M KCl and 2 mM EDTA, and the column was developed with the same buffer. A flow rate of 25 ml/hr was maintained and fractions of 10 ml were collected. The enzyme activity was retarded on the Sephadex G-200 column and appearred behind the major protein fractions. Fractions no. 52–70 were combined and applied to a column (2 × 40 cm) of DEAE-Sephadex A-50 (Pharmacia) previously equilibrated with 0.01 M phosphate buffer (pH 8.0) containing 0.1 M KCl and 2 mM EDTA. Chromatography was carried out at a flow rate of about 30 ml/hr using a linear gradient of increasing KCl concentration generated by a mixing 500 ml of starting buffer with 500 ml of this buffer containing 0.5 M KCl. Each fraction was assayed for enzymatic hydrolysis of Cbz-L-glutamine, Cbz-L-glutaminyl-L-proline, and L-glutamine. As shown in Figure 1, two kinds of enzyme have been separated on this DEAE-Sephadex column chromatography. Peptidoglutaminase I which was specific for Cbz-L-glutamine was eluted from 0.17 to 0.21 M KCl concentration and peptidoglutaminase II which was specific for Cbz-L-glutaminyl-L-proline from 0.22 to 0.27 M KCl.

Further purification of these enzymes was accomplished using hydroxylapatite (Bio-Rad Laboratory) column chromatography. The enzyme solution of peptidoglutaminase I or II was applied separately to a columns (1.5 × 23 cm) which were previously equilibrated with 0.001 M phosphate buffer (pH 7.0) and elution was carried out using a phosphate buffer gradient (0.01–0.2 M phosphate buffer, pH 7.0). The elution profiles are depicted in Figures 2 and 3. The eluate was col-

TABLE I: Purification of Peptidoglutaminases I and II.

Fraction[a]	Total Act.[b] (units)	Total Protein (mg)	Sp Act.[c] (units/mg)	Recov (%)
Peptidoglutaminase I				
1. Crude extract	935	6680	0.14	100
2. Streptomycin	986	3400	0.29	105
3. Ammonium sulfate	954	954	1.0	102
4. Sephadex G-200	540	200	2.7	57
5. DEAE-Sephadex	309	19	16.3	33
6. Hydroxylapatite	129	2	64.5	13
Peptidoglutaminase II				
1. Crude extract	1203	6680	0.18	100
2. Streptomycin	1159	3400	0.34	96
3. Ammonium sulfate	858	954	0.9	71
4. Sephadex G-200	784	200	3.9	65
5. DEAE-Sephadex	638	19.6	33.6	53
6. Hydroxylapatite	256	3.5	73.2	21

[a] 110 g wet cells were treated as indicated in the text. The recovery at each fraction is calculated on the basis of the amount of enzyme present in the crude extract. [b] In the determination of activity, Cbz-L-glutamine was used as a substrate for peptidoglutaminase I and Cbz-L-glutaminyl-L-proline as a substrate for peptidoglutaminase II. The assays were carried out at pH 7.5 as described in the text. [c] The specific activity is the micromoles of ammonia produced per minute per milligram of protein.

lected in 5-ml fractions and those in which the activity of peptidoglutaminase I was greater than 52 units/mg of protein were pooled. In the case of peptidoglutaminase II, fractions in which the activity was more than 55 units/mg of protein were pooled. At this stage of purification, peptidoglutaminase I was purified approximately 460-fold over the crude extract and had been obtained in 13% yield, and peptidoglutaminase II was purified approximately 400-fold and had been obtained in 21% yield. Most of the experiments to be described in this report were conducted on these enzyme fractions. The yields and activities during purification are summarized in Table I.

Both purified enzymes had a broad pH–rate profile with the optimal pH at about 7.5. A number of divalent ions were tested for an effect of the peptidoglutaminases. Using dialyzed preparation to remove any EDTA, it was found that 10 mM Zn^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Ca^{2+}, Cd^{2+}, had very little effect on enzyme activity. Cu^{2+} and Hg^{2+} ions were slightly inhibitory (about 70% of maximal activity).

Identification of the Product. The enzymatically deamidated products of the Cbz-peptides, Cbz-L-glutamine, and Cbz-L-glutaminylglycine were identified by a catalytic decarbobenzoxylation, thin-layer chromatography, and paper electrophoresis. The enzyme reaction was continued for about 4 hr until 1 μmole of ammonia had been liberated per 1 μmole of substrate. Aliquots of this incubation mixture were subjected to thin-layer chromatography and paper electrophoresis. The product (product I) formed from Cbz-L-glutamine by peptidoglutaminase I action was located in a position identical with that of known Cbz-L-glutamic acid on both the thin-layer and paper electrophoresis system. Similarly, the product (product II) formed from Cbz-L-glutaminylglycine by peptidoglutaminase II action was located in a positions corresponding to that of Cbz-L-glutamylglycine in the thin-layer and paper electrophoresis systems. In neither of these experiments were free amino acids or other possible hydrolysis products of the substrates detectable.

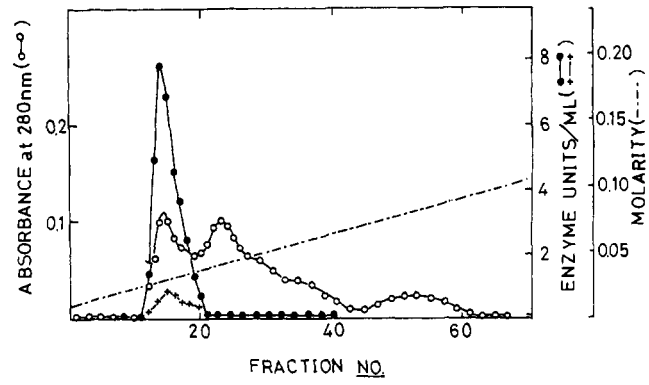

FIGURE 2: Hydroxylapatite chromatography of peptidoglutaminase I. Peptidoglutaminase I fractions from DEAE-Sephadex step (19 mg) were applied to a hydroxylapatite column (1.2 × 23 cm) as described in the text. Chromatography was carried out by the use of linear gradient of increasing phosphate concentration. The elution of protein was followed at 280 nm. Enzyme activity was measured with Cbz-L-glutamine (●—●) and L-glutamine (+—+).

The remaining incubation mixture was lyophilized, and residue dissolved in 1.0 ml of 80% methanol. This solution was hydrogenated for 3 hr in the presence of palladium black (25 mg) and 1 drop of glacial acetic acid. The catalyst was filtered, and solvents were removed *in vacuo*. The residue was dissolved in water, and aliquots were applied to thin-layer chromatography and paper electrophoresis. Product I gave L-glutamic acid on reduction. Product II gave α-L-glutamylglycine after catalytic decarbobenzoxylation. These results indicate that peptidoglutaminase I catalyzes the hydrolysis of γ-amide of Cbz-L-glutamine and peptidoglutaminase II catalyzes the hydrolysis of γ-amide of Cbz-L-glutamine and peptidoglutaminase II catalyzes the hydrolysis of γ-amide of Cbz-L-glutaminylglycine.

Substrate Specificity. The specificity of the two enzymes toward a number of substrates was tested (Table II). Peptidoglutaminase I was incapable or nearly incapable of catalyz-

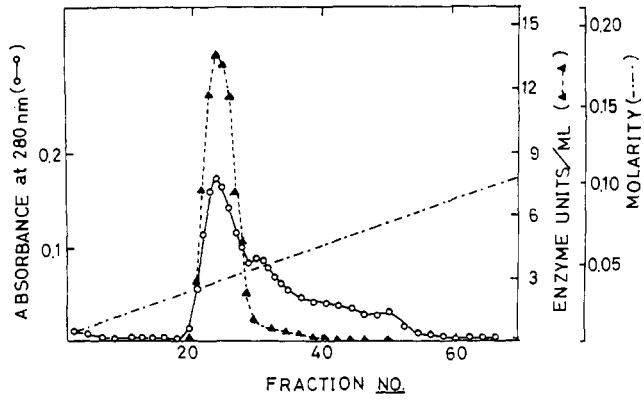

FIGURE 3: Hydroxylapatite chromatography of peptidoglutaminase II. Peptidoglutaminase-II fractions from DEAE-Sephadex step (19.6 mg) were applied to a hydroxylapatite column (1.5 × 23 cm) as described in the text. The elution of protein was followed at 280 nm. Enzyme activity was measured with Cbz-L-glutaminyl-L-proline (▲---▲).

TABLE II: Substrate Specificity of Peptidoglutaminases I and II.[a]

Substrate	Ammonia Liberation: Peptidoglutaminase I (Specific Activity)	Ammonia Liberation: Peptidoglutaminase II (Specific Activity)
L-Ile-Gln-Asn-Cys-Pro-Leu-Gly-*N*-acetate	0	0.33
Cbz-L-Gln-Gly	0	52.4
Cbz-L-Gln-L-Pro	0	73.0
t-AOC-L-Gln-L-Pro	0	39.2
L-Phe-L-Gln-Gly	0	33.5
L-Pyroglutamyl-L-Gln-L-Ala	0	27.8
L-Gln-Gly	0.9	27.8
Cbz-L-Gln	62.2	1.7
Gly-L-Gln	61.2	0.4
L-Leu-Gly-L-Gln	48.5	0.1
L-Tyr-L-Gln	61.0	0.6
L-Pro-L-Gln	44.1	0.1
N-Acetyl-L-Gln	53.8	0.7
Phthaloyl-DL-Gln	27.0	0.4
L-Gln	4.8	1.5
L-Asn	0	0
Cbz-L-Asn	0	0
Gly-L-Asn	0	0
L-Leu-amide	0	0
Gly-L-Phe-amide	0	0

[a] Incubation mixtures were composed of, phosphate buffer (pH 7.5), 20 μmoles; substrate, 5 μmoles; enzyme, 2–37 μg of protein, all in 0.5 ml. Incubations were carried out for 10 and 30 min at 30°.

ing the hydrolyzing γ-amide of glutamine if the carboxyl and α-amino groups participated in peptide-bond formation. However, if only the α-amino groups were substituted, rapid hydrolysis of the γ-amide occurred. Note that the rates of γ-amide hydrolysis varies with the group attached to the α-amino group of the L-glutamine. With the limited number of substrates tested, no generalizations concerning the effect of these substrates is apparent. Examining the rate of hydrolysis of L-glutamine by peptidoglutaminase I, it is apparent that though substitution at the α-amino position is not requisite, the lack of substitution at this α-amino group leads to a slow rate of γ-amido group hydrolysis.

Peptidoglutaminase II hydrolyzed the γ-amide of L-glutamine at very slow or negligible rates if the glutamine carboxyl were not involved in peptide-bond formation. If this carboxyl was involved in peptide-bond formation, then every example excepting one, a rapid hydrolysis of the γ-amide occurred. The L-Ile-Gln-Asn-Cys-Pro-Leu-Gly-*N*-acetate polypeptide appeared to be resistant to enzymic γ deamidation. This may not be a surprising result, as the substituent at either α-amino or carboxyl group had an effect on the hydrolysis rate. Again, insufficient numbers of peptides were available to come to any conclusions.

Neither enzyme was capable of hydrolyzing the β-amide of L-asparagine, or Cbz-L-asparagine or Gly-L-asparagine. Nor were these enzymes capable of hydrolyzing L-leucinamide or glycyl-L-phenylalaninamide. In order to ensure that no peptidase activity was occurring during these specificity experiments, the reaction mixtures were also examined by thin-layer chromatography. No indication of peptide-bond degradation was evident, even if the reaction mixtures were incubated for an additional 2 hr.

These results indicate that peptidoglutaminase I was specific for the hydrolysis of γ-amide of glutamine residues when located at the carboxyl-terminal end of a peptide and peptidoglutaminase II was specific for the hydrolysis of γ-amide of glutamine residue located at the amino-terminal or endo position on a peptide.

Liberation of Ammonia from Partially Hydrolyzed Casein. As presented in Table III, the both peptidoglutaminases catalyzed the liberation of ammonia from casein partially hydrolyzed by purified *Aspergillus* alkaline proteinase, but not from the unhydrolyzed casein. The liberation of free glutamic acid from this hydrolysate after peptidoglutaminases treatment was not observed. Proteolytic activity was not detected by peptidoglutaminase-I or -II preparations when measured by a modification of Anson's method with casein as the substrate (Anson, 1938; Hayashi *et al.*, 1967a). These results indicate that the liberated ammonia from the partially hydrolyzed casein after treatment with peptidoglutaminases originated from some amide components on the peptides.

The extent of hydrolysis of γ-amide residues of glutamine in partially hydrolyzed casein by the peptidoglutaminases could not be accurately calculated because the content of glutamine residues in the partially hydrolyzed casein was unknown. The total amide content in a sample of protein may be determined by the estimation of the ammonia formed during acid catalyzed hydrolysis (Wilcox, 1967). When peptidoglutaminase I and II were incubated together with the partially hydrolyzed casein for 90 min at 37°, about 70% of the peptide-bound amide was hydrolyzed as compared to a value determined by the estimation of the ammonia formed during 1 N HCl hydrolysis for 2 hr at 100°.

Hydroxylamine Incorporation into Glutamine Derivatives. Meister *et al.* (1955) noted that *E. coli* glutaminase slowly catalyzed the synthesis of γ-glutamyl hydroxamate when glutamine was incubated in the presence of hydroxylamine. Folk and Cole (1965) also noted that transglutaminase catalyzed the synthesis of hydroxamate. Furthermore, it has been reported that glutamotransferase (GTF)[1] catalyzes the formation of hydroxamate from glutamine with hydroxylamine (Stumpf, 1955). As shown in Table IV, peptidoglutaminases also catalyze the incorporation of hydroxylamine into glutamine derivatives with the peptidoglutaminases showing the same substrate specificity that the corresponding enzymes had for ammonia liberation.

Difference from Transglutaminase. Folk and Cole (1965) discovered that transglutaminase obtained from guinea pig liver catalyzes both the release of ammonia from, and the incorporation of ethanolamine into Cbz-L-glutaminylglycine. Time course curves for release of ammonia from, and incorporation of [^{14}C]ethanolamine into Cbz-L-glutamine by peptidoglutaminase I are presented in Figure 4A. The incorporation of [^{14}C]ethanolamine into Cbz-L-glutamine was not observed using peptidoglutaminase I. Similar results were obtained when comparing ammonia formation and ethanolamine incorporation using Cbz-L-glutaminyglycine and peptidoglutaminase II (Figure 4B). On the contrary, the incorporation of [^{14}C]ethanolamine into Cbz-L-glutaminylglycine was clearly observed with transglutaminase. The presence of ethanol-

TABLE III: Liberation of Ammonia from Partially Hydrolyzed Casein.[a]

	Casein	Casein Hydrolysate		
		30-min Incubation		
	NH_3 Liberated (90 min)	NH_3 Liberated (μmoles)	Free Glu Liberated (μmole)	90-min Incubation NH_3 Liberated (μmoles)
Peptidoglutaminase I (20 μg)	Trace	3.5	<0.05	4.1
Peptidoglutaminase II (37 μg)	Trace	1.3	<0.05	5.1
Peptidoglutaminase (20 μg) + peptidoglutaminase II (37 μg)	Trace	4.7	<0.05	6.5
E. coli glutaminase (2.5 units)	0	0		

[a] Incubation mixtures contained: partially hydrolyzed casein (10 or 14 mg), peptidoglutaminase I (20 μg) or peptidoglutaminase II (37 μg), and phosphate buffer (140 μmoles, pH 7.0), all in 2.0 ml. Incubations were carried out for 30 or 90 min at 37°. After addition of 0.2 ml of 50% trichloroacetic acid, the samples were applied to ammonia determination and free glutamic acid content analysis.

amine has no effect on the rate of ammonia formation from these substrates by either of the peptidoglutaminases. The product Cbz-L-glutamic acid was identified as the product of peptidoglutaminase I action on Cbz-L-glutamine and Cbz-L-glutamylglycine as the product of peptidoglutaminase II action on Cbz-L-glutaminylglycine. No radioactive spot other than [^{14}C]ethanolamine was detected even when the incubation mixture was directly applied to a paper chromatography.

When Cbz-L-glutaminylglycine was incubated with [^{14}C]-ethanolamine in the presence of transglutaminase (Figure 4B), a radioactive spot of a Cbz-peptide (R_F value, 0.81) that was not ethanolamine (R_F value, 0.42) was detected on a paper chromatogram, using a solvent system composed of 1-butanol–acetic acid–2.8% (w/v) ammonia water (4:1:1, v/v). The material eluted from the radioactive area of Cbz-peptide gave only radioactive ethanolamine and nonradioactive glutamic acid and glycine after acid hydrolysis as identified by paper electrophoresis.

These results indicate that peptidoglutaminases I and II did not catalyze transglutamination as was shown by transglutaminase action on ethanolamine and Cbz-peptide but catalyze the hydrolysis of γ-amide of glutaminyl peptides.

Discussion

Two new glutaminases have been partially purified from a *Bacillus circulans* species. These have been named peptidoglutaminases because of their specificity toward peptide-bound L-glutamine and their negligible action on free L-glutamine. Peptidoglutaminase I was found to be specific for catalyzing the hydrolysis of the γ-amide of L-glutamine with the carboxyl group free but the α-amino group substituted. Peptidoglutaminase II was specific for hydrolysis of the γ-amide group of L-glutamine that was substituted at the carboxyl position but the α-amino group could be either free or substituted. The reaction rate for both enzymes were affected by the substituent or substituents forming the peptide bond (Table II).

With peptidoglutaminase I, reaction rates were maximal when the amino group was substituted with Cbz, Gly, or L-Tyr and was less rapid when substituted with acetyl, Pro, or L-Leu-Gly. This last example would suggest that not only the direct attachment, but also the second substituent can affect the reaction rate.

With peptidoglutaminase II, the added possibilities for substitution made the problem of deciphering substituent effects even more difficult due to lack of sufficient substrates. The only example with a free amino group, L-Gln-Gly, had a relatively low reaction rate as compared to Cbz-L-Gln-

TABLE IV: Hydroxamate Formation from Glutamine Derivatives by Peptidoglutaminase I and II.[a]

	Absorbance at 525 mμ	
Derivatives	Peptidoglutaminase I	Peptidoglutaminase II
Cbz-L-Gln-Gly	0	0.820
Cbz-L-Gln-L-Pro	0.012	0.800
t-AOC-L-Gln-L-Pro	0	0.610
L-Phe-L-Gln-Gly	0	0.660
L-Pyroglutamyl-L-Gln-L-Ala	0.008	0.540
L-Gln-Gly	0.002	0.125
Cbz-L-Gln	0.400	0
Gly-L-Gln	0.420	0
L-Leu-Gly-L-Gln	0.345	0.013
L-Tyr-L-Gln	0.220	0
L-Pro-L-Gln	0.290	0
N-Acetyl-L-Gln	0.338	0
Phthaloyl-DL-Gln	0.370	0.193
L-Gln	0.006	0.002
L-Asn	0	0
Cbz-L-Asn	0	0
Gly-L-Asn	0	0

[a] Incubation mixtures contained: phosphate buffer (pH 7.5, 40 μmoles, substrate (10 μmoles), hydroxylamine (100 μmoles), peptidoglutaminase I (2.0 μg of protein), or peptidoglutaminase II (3.7 μg of protein), all in 1.0 ml. Incubations were carried out for 30 min at 30°.

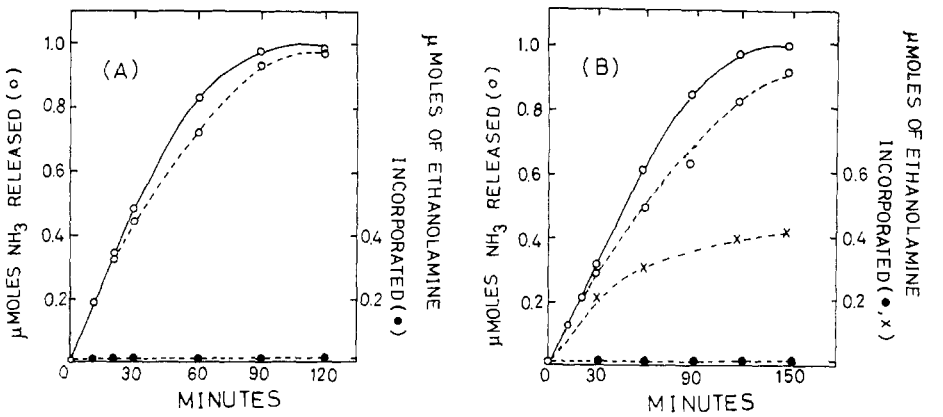

FIGURE 4: Tests for transglutaminase activity in peptidoglutaminases. (A) The complete system contained Cbz-L-glutamine (30 μmoles), phosphate buffer, pH 7.6 (120 μmoles), peptidoglutaminase I (10 μg), and [^{14}C]ethanolamine (300 μmoles, 0.002 μCi/μmole) in a total volume of 3 ml. The control flasks contained the complete system minus ethanolamine. (O---O) Ammonia (micromoles/micromole of substrate) produced during incubation of complete system. (●---●) Ethanolamine incorporation (micromoles/micromole of substrate) during incubation of complete system. (O—O) Ammonia formation (micromole/micromole of substrate) in control flasks. (B) The complete system contained Cbz-L-glutaminylglycine (30 μmoles), phosphate buffer, pH 7.6 (120 μmoles), peptidoglutaminase II (8.5 μg), and [^{14}C]ethanolamine (300 μmoles, 0.002 μCi/μmole). (O---O) Ammonia formation (micromoles/micromole of substrate); (●---●) ethanolamine incorporation (micromoles/micromole of substrate). Control flask contained the complete system minus ethanolamine. (O—O) Ammonia formation (micromoles/micromole of substrate) from control. The complete system for transglutaminase contained Cbz-L-glutaminylglycine (25 μmoles), Tris buffer, pH 8.0 (200 μmoles), transglutaminase (30 mg), $CaCl_2$ (25 μmoles), glutathione (50 μmoles), and [^{14}C] ethanolamine (250 μmoles, 0.002 μCi/μmole). (×---×) Ethanolamine incorporation (micromoles/micromole of substrate). Peptidoglutaminases incubated at 30° and transglutaminase at 37° for time period indicated. Determination of ethanolamine incorporation as per Methods section.

Gly, with L-Phe-L-Gln-Gly intermediate. Similarly, Cbz-L-Gln-L-Pro had a fast reaction rate, whereas *t*-AOC-L-Gln-L-Pro was relatively slow. While it is obvious that substituents do have an effect on hydrolysis rate, there were not sufficient numbers of peptides available to reach any valid conclusions.

To summarize some of our other findings, we found that L-glutamine was poorly hydrolyzed by either peptidoglutaminase, that the pH optimum was broad and at about pH 7.5, that a number of divalent cations had little or no effect on the deamidation rate, and could participate in hydroxamate formation with this reaction having the same specificity as the peptidoglutaminases had for deamidation.

A number of glutaminases which appear to have certain similarities in their catalytic action as compared to peptidoglutaminases have been reported from several laboratories. The ubiquitous occurrence of glutaminase (L-glutamine amidohydrolase) in plants, in microorganisms, and in animal and the properties of the enzyme have been reviewed recently (Roberts, 1960). Glutaminase has been used often to refer to any enzyme acitvity which can split off the γ-amide of L-glutamine as ammonia. On the basis of the limited data on the specificity of glutaminase from kidney and brain, it appears that glutamine derivatives substituted in the α-amino group (benzoylglutamine) or α-carboxyl group (glutaminylglycine) are not deamidated (Krebs, 1935). On the other hand, extracts of rat liver are able to deamidate chloroacetylglutamine to a slight degree (Carter and Greenstein, 1947). The enzymes from *Escherichia coli* hydrolyzed L-glutamine and α-methyl-DL-glutamine but it was without activity on *N*-acetylglutamine, L-isoglutamine, and a number of compounds related to glutamine at a measurable rate (Meister *et al.*, 1955; Hartman, 1968). Thus, the specificity of previously reported glutaminases are restricted to free glutamine or a few of its derivatives.

It was found that Ca^{2+}-dependent transglutaminase from guinea pig liver catalyzes both the replacement and hydrolysis reactions of protein- or peptide-bound glutamine (Clarke *et al.*, 1959; Folk and Cole, 1965). Thus, there appears to be similarity between the specificity of peptidoglutaminases reported here and that of transglutaminase. In order to check on the identity of these two enzyme types experiments using [^{14}C]-ethanolamine incorporation into Cbz-peptides were run. Peptidoglutaminases could not catalyze the replacement reaction between γ-amide of substrates and ethanolamine, but could catalyzed the hydrolysis reaction, whereas, transglutaminase could catalyze this transamidation. Thus the mode of catalytic action of peptidoglutaminases are different from that of transglutaminase. Furthermore, it has been shown that peptidoglutaminases normally catalyze the hydrolysis reaction in the absence of Ca^{2+}, whereas transglutaminase showed an absolute requirement for Ca^{2+}.

These two major differences between peptidoglutaminases and transglutaminase would clearly indicate the differences between these enzymes types.

Glutamotransferase (GTF) had been discovered in bacteria, plant and mammaliam tissues which exchange the amide group of glutamine with hydroxylamine and other amines (Stumpf, 1955; Waelsch, 1952). Bacterial GTF was activated by Cu^{2+}, and GTF from plant, *Neurospora*, or mammalian tissue had an absolute requirement for Mn^{2+}. It has been shown that bacterial GTF did not catalyze the formation of hydroxamate from acetylglutamine, glycylglutamine, and glutaminylglycine in the enzymic test. Peptidoglutaminases catalyzed the formation of hydroxamate from these glutamine derivatives; was not affected by Mn^{2+} and was slightly inhibited by Cu^{2+} when tested at 10 mM concentrations.

All these enzymes described above differ from peptidoglutaminases in their ability to act on glutaminyl peptides and in their requirement for metal ions. It seems reasonable to conclude that peptidoglutaminases I and II are new types of amidases.

Application of specific enzyme preparations such as L-glutamic acid decarboxylase, L-glutaminase, L-aspartic acid decarboxylase, and L-asparaginase after complete enzymatic digestion of protein and peptides could provide a means for evaluating the content of asparaginyl, glutaminyl, aspartyl,

and glutamyl residues (Tower *et al.*, 1962). The availability of these peptidoglutaminases which selectively remove γ-amide of peptide-bound glutamine should be a distinct advantage in the characterization of peptides. Peptidoglutaminases may be able to eliminate a complete hydrolysis of protein or peptide for the determination of its glutamine content because of its specificity toward deamidation of peptide-bound glutamine.

Acknowledgments

We are very indebted to Professor S. Sakakibara for advice and for synthesizing peptides that were indispensable for this work. We are very grateful to Professors M. Yamazaki, Y. Ikeda, and K. Arima for valuable advice. The assistance of Mr. H. Sakurai and Mr. H. Mori in this work is gratefully acknowledged.

References

Anson, M. L. (1938), *J. Gen. Physiol. 22*, 79.
Bray, G. A. (1960), *Anal. Biochem. 1*, 279.
Breed, R. S., Murray, E. G. D., and Smith, N. R. (1957), Bergey's Manual of Determinative Bacteriology, 7th ed, Baltimore, Md., Williams & Wilkins, p 628.
Cedrangolo, F., Salvatore, F., Cimino, F., and Zappia, V. (1965), *Enzymologia 29*, 143.
Clarke, D. D., Mycek, M. J., Neidle, A., and Waelsch, H. (1959), *Arch. Biochem. Biophys. 79*, 338.
Folk, J. E., and Cole, P. W. (1965), *J. Biol. Chem. 240*, 2951.
Gale, E. F. (1944), *Biochem. J. 39*, 46.
Greenstein, J. P., and Winitz, M. (1961), Chemistry of the Amino Acids, Vol. II, New York, N. Y., Wiley, p 935.
Hartman, S. C. (1968), *J. Biol. Chem. 243*, 853.
Hayashi, K., Fukushima, D., and Mogi, K. (1967a), *Agr. Biol. Chem. 31*, 1237.
Hayashi, K., Fukushima, D., and Mogi, K. (1967b), *Agr. Biol. Chem. 31*, 642.
Layne, E. (1957a), *Methods Enzymol. 3*, 450.
Layne, E. (1957b), *Methods Enzymol. 3*, 451.
Lipmann, F., and Tuttle, L. C. (1954), *J. Biol. Chem. 159*, 21.
Meister, A. (1955), *Methods Enzymol. 2*, 380.
Meister, A., Levintow, L., Greenfield, R. E., and Abendschein, P. (1955), *J. Biol. Chem. 215*, 441.
Miller, H. K., and Waelsch, H. (1952), *J. Amer. Chem. Soc. 74*, 1092.
Mycek, M. J., and Waelsch, H. (1960), *J. Biol. Chem. 235*, 3513.
Pelczar, M. J., Jr. (1957), Manual of Microbiological Methods by the Society of American Bacteriologists, New York, N. Y., McGraw-Hill.
Roberts, E. (1960), *Enzymes 4*, 285.
Schellenberg, P. (1962), *Angew. Chem. 74*, 118.
Stumpf, P. K. (1955), *Methods Enzymol. 2*, 263.
Tower, D. B., Peter, E. L., and Wherrett, J. R. (1962), *J. Biol. Chem. 237*, 1861.
Waelsch, H. (1952), *Advan. Enzymol. 13*, 289.
Wilcox, P. E. (1967), *Methods Enzymol. 11*, 63.
Woodward, R. B., Olofson, R. A., and Mayer, H. (1961), *J. Amer. Chem. Soc. 83*, 1010.

Isotopic Effects and Inhibition of Polysaccharide Phosphorylase by 1,5-Gluconolactone. Relationship to the Catalytic Mechanism*

Jan-I Tu, Gary R. Jacobson,† and Donald J. Graves‡

ABSTRACT: Reaction rates of acid hydrolysis and hydrolysis by alkaline phosphatase were compared to rates obtained with rabbit muscle phosphorylase *b* and *Escherichia coli* maltodextrin phosphorylase by using reaction mixtures containing 1-deuterio-α-D-glucose 1-phosphate or the normal isotope. Secondary isotope effects were observed consistent with a mechanism involving a transition state with considerable positive charge similar to that expected for a carbonium ion. Inhibition studies were reported with 1,5-gluconolactone, a compound that possesses a half-chair conformation similar to the oxonium ion. The inhibition is highly specific and competitive with glucose 1-phosphate and noncompetitive with respect to glycogen in the direction of synthesis. For glycogen degradation, 1,5-gluconolactone is noncompetitive either with respect to arsenate or with respect to glycogen. These data have been interpreted to mean that 1,5-gluconolactone competes for the glucosyl transfer site of polysaccharide phosphorylase and that the transition state involves formation of an enzyme glucosyl complex in which the glucosyl residue is in the half-chair conformation.

1,5-Gluconolactone affects the properties of the pyridoxal 5′-phosphate binding site. The possible interaction of 1,5-gluconolactone with pyridoxal 5′-phosphate at the active site is discussed.

It has been clearly established that in the phosphorolysis of sucrose by sucrose phosphorylase an intermediate enzyme glucosyl complex is formed (Doudoroff *et al.*, 1947; Silverstein *et al.*, 1967; Voet and Abeles, 1970). No evidence has yet been given for such an intermediate in the polysaccharide phosphorylase reaction. Early studies with polysaccharide

* From the Department of Biochemistry and Biophysics, Iowa State University, Ames, Iowa 50010. *Received August 25, 1970.* Journal Paper J-6704 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa, Project No. 1843. This research was supported by a research grant (GM-09587) from the National Institutes of Health, U. S. Public Health Service.

† National Science Foundation undergraduate research participant.

‡ Recipient of a research career development award of the U. S. Public Health Service (Grant GM6753); to whom to address correspondence.