*SPECIFIC INACTIVATION OF PEPSIN BY A DIAZO KETONE**

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Communicated October 24, 1966

During the course of our systematic examination of the possible utility of diazo alkanes as chemical reagents in the study of the role of protein carboxyl groups in enzymic catalysis, it was found[1] that diphenyldiazomethane (DDM) readily inactivates crystalline swine pepsin. Because of the relatively high chemical reactivity of DDM, however, the complete inactivation of pepsin was accompanied by the incorporation of more than one diphenylmethyl group per pepsin molecule, and both pepsinogen and alkali-denatured pepsin were found to be attacked by the reagent. More selective diazo compounds were therefore sought, and the results obtained with one of the test reagents merits detailed report at this time. As described in what follows, tosyl-L-phenylalanyldiazomethane (more accurately, L-1-diazo-4-phenyl-3-tosylamidobutanone, to be abbreviated L-DPTB) inactivates pepsin rapidly and completely with the incorporation of one phenylalanyl residue per pepsin molecule, and under the conditions of these studies L-DPTB does not react to a significant extent either with pepsinogen or with alkali-denatured pepsin.

Materials and Methods.—L-DPTB was prepared by adding tosyl-L-phenylalanylchloride[2] (2.0 gm, 6.6 mmoles) to a solution of diazomethane (20 mmoles) in ether (50 ml) at 0°. After the mixture had been kept at room temperature (in the dark) for 16 hr, the solvent was removed *in vacuo*, and the residue was recrystallized from 1-butanol-cyclohexane. [Yield, 0.8 gm (35%); m.p. 109–111° decomp.] A second recrystallization raised the melting point to 112–114° decomp. This material had an optical rotation of $[\alpha]_D^{25} = -122°$ (c 0.4, ethanol) and exhibited a strong infrared band at 4.71 μ, characteristic of an α-diazo ketone. Calculated for $C_{17}H_{17}N_3O_3S$ (343.4): C, 59.5; H, 5.0; N, 12.2. Found: C, 59.8; H, 5.2; N, 12.0. The preparation of L-DPTB has been described previously by Schoellmann and Shaw,[3] who reported a melting point of 94–96° and an infrared band at 4.68 μ.

For the preparation of C^{14}-labeled L-DPTB, uniformly labeled L-phenylalanine (New England Nuclear Corp.) was used as the starting material; the final product was recrystallized to constant specific radioactivity (12,700 cpm/μmole).

The preparation of D-DPTB followed the route for the L-isomer described above, starting with D-phenylalanine, and gave a product melting at 113–114° decomp. (mixed m.p. with L-isomer, 94–99°), and having an optical rotation of $[\alpha]_D^{25} = +129°$ (c 0.4, ethanol). Calculated for $C_{17}H_{17}N_3O_3S$ (343.4): C, 59.5; H, 5.0; N, 12.2. Found: C, 59.9; H, 5.1; N, 11.9.

The synthetic substrate benzyloxycarbonyl-L-histidyl-L-phenylalanyl-L-tryptophan ethyl ester was provided by Dr. K. Inouye, and its cleavage by pepsin was followed by means of the ninhydrin method in the manner described previously.[4]

Swine pepsin (twice crystallized) was obtained from the Worthington Biochemical Corporation (lot PM 708), and stock solutions were prepared by dissolving 50 mg in 30 ml of 6.25 mM acetate buffer (pH 5.4), and dialyzing the solution overnight at 2° against 1 liter of the buffer. The protein concentration was then adjusted to 1.25 mg/ml, as measured spectrophotometrically at 280 mμ (specific absorbance $E = 1.38$). The enzymic activity of this preparation was 3275 ± 100 units/mg, when assayed with hemoglobin as the substrate in the manner described previously.[1] This sample of pepsin will be designated pepsin-W.

A sample of pepsin also was prepared from crystalline swine pepsinogen (Worthington Biochemical Corp., lot PG 114) by activation at pH 2.0 and 14°, as in the procedure of Rajagopalan *et al.*[5] After passage at 2° through a SE-Sephadex column equilibrated with 0.4 *M* acetate buffer (pH 4.4), the protein-containing fractions of the effluent were pooled, concentrated by means of Aquacide II, and passed at 2° through a Sephadex G-25 column equilibrated with 6.25 mM acetate buffer (pH 5.4). The pepsin that emerged at the void volume of the column was collected,

and the protein concentration was adjusted to 1.25 mg/ml. The specific enzymic activity of this preparation was found to be 3380 ± 70 units/mg, with hemoglobin as the substrate. This sample of pepsin gave a single peak upon chromatography on hydroxylapatite,[5] and will be designated pepsin-P.

Denatured pepsin was prepared by dissolving a sample of commercial crystalline pepsin in 0.1 *M* K_2HPO_4 (10 mg/ml; pH 7.8), and allowing the solution to stand for 1 hr at room temperature. Assay by the hemoglobin method showed no detectable pepsin activity. The solution was applied to a Sephadex G-25 column and eluted with water at room temperature. The protein solution that emerged at the void volume was lyophilized, and stock solutions of this preparation were obtained by dialysis, in the manner described above for pepsin-W.

When pepsinogen was employed in experiments in which DPTB acted directly on the zymogen, the requisite weight of the protein was dissolved in 6.25 mM acetate (pH 5.4) at 0°, and the solution was used immediately. The potential pepsin activity of the preparation was found to be 2470 ± 70 units/mg.

The experiments on the effect of L-DPTB or of D-DPTB on the above protein preparations were conducted at 15°. To 4.0 ml of the protein solution was added 0.5 ml of 0.01 *M* $CuCl_2$, followed 10 min later by 0.5 ml of a freshly prepared 1.43 mM solution of the reagent in ethanol. (In experiments in which the copper catalyst was omitted, the $CuCl_2$ solution was replaced by an equal volume of water.) At stated time intervals, 0.2-ml samples were withdrawn, diluted to 5.0 ml with 0.016 *M* HCl (pH 1.8), and assayed for pepsin activity with hemoglobin as the substrate. Control experiments, in which 0.5 ml of ethanol replaced the solution of the diazo ketone, were performed for all tests with active pepsin. In all cases it was found that the enzymic activity of the control solutions, both in the presence and absence of $CuCl_2$, remained constant throughout the period of the experiments (up to 90 min). Furthermore, exposure of pepsin to $CuCl_2$, under the conditions of these studies, had no observable effect on the specific enzymic activity.

In the experiments on the incorporation of C^{14} from labeled L-DPTB, the composition of the reaction mixtures was the same as that given above, and at stated time intervals the reaction was terminated by the addition of 0.5 ml 5 *M* trichloroacetic acid. After being kept for a few minutes at 50°, the suspension was filtered on 24-mm disks of filter paper (Schleicher and Schuell no. 507) held in a 25-mm Millipore filter holder. The precipitates were washed successively with 2% trichloroacetic acid in 20% aqueous acetone (50 ml), acetone (50 ml), and dioxane (50 ml), dried in air, and dissolved in 88% formic acid (5 ml). Samples (1.0 ml) of the solutions were plated in triplicate, and the radioactivity of the dried material (*ca.* 1 mg) was determined by means of a Nuclear-Chicago gas flow counter and a Nuclear-Chicago model 183B scaler. The protein concentration of the solutions was determined spectrophotometrically at 280 mμ; separate experiments showed that, in the range of protein concentration encountered in these experiments, the absorbance of the solutions was proportional to the concentration of trichloroacetic acid-precipitated protein. To determine the extent of residual contamination in the washing procedure described above, C^{14}-DPTB was added after the pepsin had been precipitated with trichloroacetic acid, and it was found that the protein sample retained 15 ± 1 cpm/mg; this value was used as a correction factor throughout.

Results and Discussion.—The data in Table 1 show that, at pH 5.3 and 15°, L-DPTB rapidly inactivates pepsin in the presence of $CuCl_2$, but has no measurable effect in the absence of the copper salt. The catalytic action of metallic copper, cuprous salts, and cupric salts on the reactions of diazo ketones has long been known,[6] the nature of the products depending on the structure of the diazo ketone and the solvent.[7] The effect of $CuCl_2$ in promoting the reaction of L-DPTB with pepsin is therefore consistent with a large body of organic-chemical evidence. A similar catalytic effect of cupric ion has been noted in the inactivation of pepsin by diazoacetyl-DL-norleucine methyl ester.[8] The mechanism whereby the copper catalysts exert their action in the reactions of diazo ketones does not appear to have been elucidated; among the products of the decomposition of α-diazo ketones are the strongly reducing enediols,[9] which may be expected to reduce Cu(II) to Cu (I). The effective catalyst in the reaction of L-DPTB may therefore be the cuprous ion.

TABLE 1

INACTIVATION OF PEPSIN BY 1-DIAZO-4-PHENYL-3-TOSYLAMIDOBUTANONE (DPTB)

Reaction components	Pepsin Activity Lost (%) 5 Min	10 Min	15 Min	45 Min	Phe Incorporated (moles/mole protein) 5 Min	10 Min	15 Min	45 Min
Pepsin-W + L-DPTB	0	0	0	0	0	0.01	0.02	0.01
Pepsin-W + L-DPTB + $CuCl_2$	52	81	88	94	0.47	0.80	0.84	0.99
Pepsin-P + L-DPTB + $CuCl_2$	51	80	86	91	0.48	0.84	0.97	1.04
Pepsin-P + D-DPTB + $CuCl_2$	0	6	20	61	—	—	—	—
Denatured pepsin + L-DPTB + $CuCl_2$	—	—	—	—	0.03	0.02	0.10	0.10
Pepsinogen + L-DPTB + $CuCl_2$	0	0	0	0	0.01	0.01	0.01	0.02

Each incubation mixture (5 ml) contained 5 mg of protein, except in the experiment with pepsinogen, where 6 mg of protein was present, to give a protein concentration of 0.0286 mM in all cases. The concentration of diazo ketone was 0.143 mM, and that of $CuCl_2$ was 1.0 mM, giving a ratio of protein/reagent/$CuCl_2$ = 1:5:35. The pH was maintained at 5.3–5.4 with 5 mM acetate buffer containing 10% ethanol; temperature, 15°. The calculated loss in pepsin activity (hemoglobin assay) is based on control values for pepsin-W of 3275 units/mg and for pepsin-P of 3380 units/mg; the control value for the potential pepsin activity of pepsinogen was 2470 units/mg. The calculated extent of incorporation of Phe is based on the value of 363 cpm/0.0286 μmole, after correction of the measured radioactivity of the sample for background and residual contamination (see *Materials and Methods*). The molecular weights of pepsin and pepsinogen were assumed to be 35,000 and 42,000, respectively.

The rapid inactivation of pepsin by L-DPTB labeled with C^{14} in the phenylalanyl (Phe) residue is accompanied by a parallel incorporation of C^{14}-Phe into the protein. Under the conditions of the experiments reported in Table 1, the loss of 52 per cent of the enzymic activity involved the incorporation of 0.47 mole of Phe per mole of pepsin. This incorporation ceases when 1 mole of Phe has been introduced per mole of protein, and the enzyme has been completely inactivated. Table 1 records data up to 45 minutes of reaction time, but experiments of longer duration (up to 90 min) gave the same results as at 45 minutes for the reaction of pepsin-W with L-DPTB in the presence of $CuCl_2$. That this cessation of incorporation is not a consequence of the complete destruction of the reagent during the initial 45 minutes of reaction is shown by the results with D-DPTB, whose susceptibility to decomposition by the solvent should be the same as that of the L-isomer. As will be seen in Table 1, the inactivation of pepsin by D-DPTB is much slower; during the reaction period between 45 and 90 minutes there is significant further inactivation of pepsin (up to 80% loss of activity), indicating that some of the reagent is still available for reaction. Therefore, it may be concluded that L-DPTB inactivates pepsin with the introduction of a single L-phenylalanyl residue at a reactive group essential for catalytic activity.

Rajagopalan *et al.*[5] have shown that commercial preparations of crystalline swine pepsin are inhomogeneous as judged by column chromatography and by analysis for amino-terminal and carboxyl-terminal amino acid residues, whereas pepsin carefully prepared by activation of pepsinogen behaves as a homogeneous protein by these criteria. For this reason, both pepsin-W (a commercial preparation) and pepsin-P (derived from pepsinogen) were subjected to the action of L-DPTB in the presence of $CuCl_2$. It will be seen that the results for the two pepsin preparations are essentially the same, both as regards the rate of inactivation and the extent of incorporation of the L-phenylalanyl residue of the reagent.

The copper-catalyzed inactivation of pepsin by L-DPTB leads to parallel loss of enzymic activity toward hemoglobin and benzyloxycarbonyl-L-histidyl-L-phenylalanyl-L-tryptophan ethyl ester (Z-His-Phe-Trp-OEt), one of the most sensitive of the

currently available synthetic substrates for pepsin.[4] A sample of pepsin-W that had lost, after treatment with C^{14}-L-DPTB and $CuCl_2$, 39 per cent of its original activity toward hemoglobin, hydrolyzed Z-His-Phe-Trp-OEt at the Phe-Trp linkage at an initial rate of 0.013 μmole $\times$ ml^{-1} $\times$ min^{-1} (0.5 mM substrate, 0.04 mg enzyme/ml; pH 4.0; 37°). This rate, when compared with the control value of 0.021 μmole $\times$ ml^{-1} $\times$ min^{-1} for the untreated enzyme, indicates a loss of 38 per cent of the original activity toward the synthetic substrate. The partially inactivated enzyme was found to contain 0.41 mole C^{14}-Phe per mole of protein.

The clearest evidence of the specific nature of the $CuCl_2$-catalyzed reaction of the diazo ketone with pepsin comes from the results with alkali-denatured pepsin and with pepsinogen (Table 1). Under conditions where pepsin is rapidly labeled by C^{14}-L-DPTB, the incorporation of C^{14}-Phe is very slight, indicating that a reactive group essential for catalytic activity has been rendered unreactive upon denaturation of the protein at pH 7.8. Furthermore, the failure of the reagent to cause any detectable loss in the potential enzymic activity of pepsinogen, and to introduce significant amounts of C^{14} into the zymogen, gives further support for the view that L-DPTB reacts specifically with a group that is essential for the catalytic action of pepsin. This group, if present in pepsinogen, appears to acquire sufficient reactivity toward the diazo ketone only upon activation of the zymogen.

The choice of DPTB for test as a reagent for the specific inactivation of pepsin was influenced by the known preference of the enzyme for peptide bonds involving aromatic amino acids.[4] Furthermore, the stereochemical specificity of pepsin is pronounced with respect to the configuration of the amino acid residues on either side of a sensitive peptide bond. Thus, whereas benzyloxycarbonyl-L-histidyl-L-phenylalanyl-L-phenylalanine ethyl ester is cleaved rapidly by pepsin at the bond between the two aromatic amino acid residues, the replacement of either of the two L-phenylalanyl residues by the D-antipode renders the peptide completely resistant to hydrolysis by pepsin.[10] It was of interest, therefore, to find a large difference in the rates at which the L- and D-isomers of DPTB inactivate the enzyme (Table 1), since this difference reflects to some degree the known stereochemical specificity of pepsin. It should be added that the configuration about the α-carbon atom of L- or D-phenylalanine is retained in the diazo ketone, as the reaction of the acid chloride with diazomethane does not involve the asymmetric center.

Although α-diazo ketones, like other diazo alkanes, may be expected to esterify carboxyl groups, a decision as to the nature of the group in pepsin that is specifically attacked by L-DPTB must await further experimental study. In particular, the isolation and identification of a labeled peptide from partial digests of pepsin treated with C^{14}-labeled L-DPTB is essential, and efforts in this direction are in progress. Furthermore, a systematic examination of the copper-catalyzed reaction of α-diazo ketones with model peptides in various solvents may be expected to throw light on the relative reactivity of various side-chain groups, and to contribute to an understanding of the mechanism of the reaction.

In addition to earlier evidence for the participation of carboxyl groups in the catalytic action of pepsin,[1, 11] recent work on the inactivation of pepsin by *p*-bromophenacyl bromide has led to the suggestion that the β-carboxyl group of an aspartyl residue is involved in the active center of the enzyme.[12, 13] If the group attacked by L-DPTB is a side-chain carboxyl group, the question may be raised whether the

slower rate of inactivation by the D-isomer is a consequence of steric difficulty in a reaction with the carboxyl group that is attacked by the L-compound, or whether the interaction of pepsin with D-DPTB leads to a reaction with a different carboxyl group, also essential for catalytic action. This possibility merits consideration in view of the suggestion[11] that pepsin catalysis may involve the cooperative action of two carboxyl groups in the active site.

Among the other side-chain groups that must be considered as possible sites of attack in the $CuCl_2$-catalyzed reaction of L-DPTB with pepsin is the phenolic hydroxyl group of tyrosine, whose importance for the catalytic action of pepsin has been noted in earlier work.[14] One of the known routes of decomposition of an α-diazo ketone leads to a ketene,[6] which at pH 5 may be expected to acylate preferentially a phenolic group.

As an approach to the identification of the group attacked in the $CuCl_2$-catalyzed reaction of C^{14}-L-DPTB with pepsin, samples of the enzyme that had been inactivated (100 units/mg, corresponding to 3% of the original activity toward hemoglobin; 0.93 mole C^{14}-Phe/mole protein) were treated with several nucleophilic reagents, and the radioactivity thereby released from the protein (0.5 mg/ml) was determined. Upon treatment with 1 *M* hydroxylamine at pH 11.0 for 22 hours at 30°, 87 per cent of the C^{14} previously bound to protein was rendered soluble in 0.8 *M* trichloroacetic acid. It was of special interest to find that treatment with 0.15 *M* KI at pH 5.0 (0.5 *M* acetate) for 22 hours at 30° led to the release of 52 per cent of the bound C^{14}-Phe, and to the appearance of significantly increased proteolytic activity toward hemoglobin (555 units/mg). In a control experiment (no KI; pH 5.0; 0.5 *M* acetate) the C^{14}-Phe released amounted to 7.5 per cent, and there was no measurable increase in proteolytic activity.

During the course of our systematic study of the effect of various diazo alkanes on pepsin, the $CuCl_2$-catalyzed action of the diazoacetyl derivatives of glycine ethyl ester (DAGE), of L-phenylalanine ethyl ester (DA-L-PE), and of D-phenylalanine ethyl ester (DA-D-PE) was examined. Analytically pure samples of these reagents were made by Miss I. Voynick from the corresponding glycylamino acid ethyl ester hydrochlorides according to the procedure of Curtius and Darapsky.[15] The detailed results will be reported in a separate communication, but for comparison with the findings recorded above for DPTB, it may be noted that at pH 5.1 (5 mM acetate, 10% ethanol), a pepsin-W concentration of 0.0286 mM, and a molar ratio of pepsin/diazo compound/$CuCl_2$ = 1:5:35, the extent of pepsin inactivation in five minutes at 15° was as follows: DAGE, 52 per cent; DA-L-PE, 75 per cent; DA-D-PE, 89 per cent. With all three reagents, the extent of inactivation exceeded 90 per cent in 15 minutes. It is clear that these diazoacetamido compounds are extremely effective inhibitors of pepsin, but that the stereochemical discrimination found with the L- and D-isomers of DPTB is not evident. This finding suggests the possibility that the mode of the copper-catalyzed interaction of pepsin with the diazoacetamido compounds mentioned above, as well as with the analogous diazoacetyl-DL-norleucine methyl ester,[8] may be different from that operative with α-diazo ketones such as DPTB. Studies on this question are in progress.

Although the copper-catalyzed action of diazo ketones and diazoacetamido compounds on pepsin and other enzymes offers considerable promise and merits intensive study, the direct reaction of diazo alkanes such as diphenyldiazomethane may be

useful in special cases, as shown in recent work demonstrating the role of a carboxylate group in the maintenance of the catalytic activity of chymotrypsin.[16]

Summary.—The diazo ketone L-1-diazo-4-phenyl-3-tosylamidobutanone (L-DPTB), in the presence of cupric ion, rapidly inactivates crystalline swine pepsin at pH 5.3 with parallel loss of enzymic activity toward hemoglobin and the synthetic substrate benzyloxycarbonyl-L-histidyl-L-phenylalanyl-L-tryptophan ethyl ester. Complete inactivation is accompanied by the incorporation of one L-phenylalanyl residue (from the reagent) per pepsin molecule. Under comparable experimental conditions, L-DPTB does not react appreciably with pepsinogen or with alkali-denatured pepsin, indicating that the reagent specifically affects a group that is essential for the catalytic action of the enzyme. The D-isomer of the diazo ketone reacts with pepsin much more slowly than does the L-form. The inactivation of pepsin by the isomeric forms of DPTB is compared with the effect of L- and D-forms of diazoacetylphenylalanine ethyl ester, which in the presence of cupric ion rapidly inactivate pepsin but do not appear to exhibit the stereochemical discrimination found with the diazo ketone.

The able technical assistance of Warren A. Carlson is gratefully acknowledged.

* This study was aided by grants from the U.S. Public Health Service (GM-06452) and from the National Science Foundation (GB-5212X).

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