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POSSIBLE SIDE-REACTIONS WITH DIAZOCARBONYL DIPEPTIDE ESTERS AS PROTEIN MODIFYING REAGENTS

by

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The reaction of diazocarbonyl compounds with chymotrypsinogen and chymotrypsin in the presence of cupric ions was accompanied by the oxidative destruction of tryptophan and methionine residues and to a lesser extent of tyrosine and cystine residues of the proteins. Cu^{2+}/diazoacetyl-DL-norleucine methyl ester complex was found to promote the oxidation of cystine residues of the proteins while the similar complex with the L-isomer of the diazocarbonyl compound was ineffective. Inclusion of α-aminocarbonyl compounds in the reaction medium not only prevented the Cu^{2+} ion mediated decomposition responsible for the diminution in the potency of the diazocarbonyl compound as a modifying agent, but also permitted the reaction to be accomplished over the pH range of 4.5 to 7.0. The side reactions, namely the oxidative destruction of amino acids could be minimized by exclusion of oxygen from the reaction medium.

1. INTRODUCTION

Diazocarbonyl compounds have found wide application in enzymology. These compounds serve as photoaffinity labels in photochemical reactions (3, 5) and as modifiers of nucleophilic side chains of amino acid residues of proteins in dark reactions (15, 18). Thus, 6-diazo-5-oxo-L-norleucine (16) and azaserine (7) were found to interact with a cysteinyl sulfhydryl group of phosphoribosyl pyrophosphate amido trans-

Abbreviations: DAG = α-diazoacetylglycine ethyl ester; DL-DAN = α-diazoacetyl-DL-norleucine methyl ester; L-DAN = α-diazoacetyl-L-norleucine methyl ester; TLC = thin layer chromatography. All other abbreviations of amino acids, amino acid derivatives and peptides are according to the guidelines of the IUPAC-IUB Commission on Biochemical Nomenclature (for a compilation see ref. 44).

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ferase and 2-formamido-N-ribosylacetamide-5'-phosphate: L-glutamine amido ligase, respectively. Most of the published work, however, concerns the modification of carboxyl groups. For example, diazoacetylglycine amide has been employed to modify a reactive aspartic acid residue in ribonuclease (30). The reaction of diazoacetamide with carboxyl residues of chymotrypsinogen and those of model compounds (9), as well as the interaction of diphenyldiazomethane with carboxyl groups of chymotrypsin and its precursor have been documented (1). The reaction of diazocarbonyl compounds with the functional groups of amino acid side chains is normally slow, but is considerably enhanced in the presence of Cu^{2+} ions. These observations are consistent with the well documented catalytic effect of elemental copper and Cu^{2+} ions on the reactions of diazoketone-derived carbonyl carbenes (28, 46). The reaction of several diazocarbonyl compounds with pepsin in the presence of Cu^{2+} ions (6, 13) provided the basis for the implication of essential carboxyl groups in the active site of this acid protease. Some of these reagents possessed substrate-like structural features, e.g. 1-diazo-4-phenyl-2-butanone (14) and L-1-diazo-4-phenyl-3-tosylamido-butanone (8), rendering them specific to functional groups at the active site. Other reagents, such as diazoacetylglycine ethyl ester and diazoacetic acid ester (reviewed in ref. 37) were found to be much less specific, but nevertheless helped to furnish useful information pertaining to the functional groups involved in pepsin catalysis. The unique requirement of Cu^{2+} ions for reactions of these reagents with pepsin and other acid proteases (2, 8, 20, 23, 24, 25, 27, 29, 37–41) has been a recognized fact. Thus, inactivation by Cu^{2+}/diazocarbonyl compounds (in particular by Cu^{2+}/diazoacetyl-DL-norleucine methyl ester), as well as by 1,2-epoxy-3-(p-nitrophenoxy)-propane (40), has been used as a criterion for the classification of a hydrolytic enzyme as an acid protease. However, in view of the well known high general chemical reactivity of diazoketone-derived carbonyl carbenes (28, 46) it appeared important to elucidate any non-specific modifications that might occur as a result of the interaction of Cu^{2+}/diazocarbonyl complexes with proteins. The current study is concerned with the identification of such nonspecific reactions, and means to prevent them, using chymotrypsinogen and chymotrypsin as model proteins.

2. MATERIALS AND METHODS

2.1. Materials

Glycine, Z-glycine, glycine ethyl ester hydrochlorid, glycyl-DL-norleucine, L-norleucine were purchased from Sigma, St. Louis, U.S.A. Dicyclohexylcarbodiimide was obtained from Fluka AG, Switzerland, N-acetyl-L-tyrosine ethyl ester from Mann Research Laboratories, New York, U.S.A., proflavin from Nutritional Biochemicals, Corp. Cleveland, Ohio, U.S.A., Aquacide III (flake polyethylene glycol) from Calbiochem, San Diego, California, U.S.A., and hydrogen bromide from Matheson of Canada. Chymotrypsinogen A and chymotrypsin Aα were products of Worthington Biochemicals, Freehold, N.J., U.S.A. Other reagents were of high commercially available purity (analytical grade) and solvents were purified and/or dried according to standard procedures.

2.2. Synthesis of α-diazocarbonyl compounds

2.2.1. Diazoacetylglycine ethylester (DAG)

Glycylglycine ethyl ester was prepared from Z-glycine and glycine ethyl ester hydrochloride by the mixed anhydride method, followed by catalytic hydrogenolysis, as described by HOFFMAN and TILAK (19). The diazotization of the free dipeptide ester was performed by the biphasic method (30, 35, 43) yielding a yellow powder which was dried in vacuo over KOH pellets. Yield: 40%; m.p. range 96–102 °C; $\lambda_{max}^{CH_3OH}$ = 250 nm (ε = 19950 $M^{-1}cm^{-1}$).

2.2.2. Diazoacetyl-DL-norleucine methyl ester (DL-DAN)

Commercial glycyl-DL-norleucine was esterified as described by RAJAGOPALAN et al. (27). Diazotization of the dipeptide ester was performed by the biphasic method (30, 35, 43). In a typical experiment, glycyl-DL-norleucine methyl ester hydrochloride (125 mg) was dissolved in 0.45 ml cold 2 M-sodium acetate. To this solution were added solid sodium nitrite (55.4 mg) and one milliliter of methylene chloride. The biphasic mixture was acidified with glacial acetic

acid (25 µl) and thoroughly mixed. After standing for one hour at approx. 4 °C, the layers were separated and the aqueous phase was washed twice with one milliliter methylene chloride. The combined organic phase was then washed twice with one milliliter cold sodium bicarbonate solution (5%), dried with anhydrous Na_2SO_4, filtered and taken to dryness under vacuo at room temperature. The diazocarbonyl compound was obtained as a yellow oil which solidified at −20 °C. Yield: 40 mg (38%). The spectral characteristics are as follows. IR ($CHCl_3$): 2110 cm^{-1} (diazo group), 1745 cm^{-1} (carbonyl in ester), 1640 cm^{-1} (carbonyl in diazo ketone). UV: $\lambda_{max}^{CH_3OH}$ = 250 nm (ε = 18200 M^{-1} cm^{-1}); ^{1}H-NMR ($CDCl_3$): δ = 0.9–1.8(9) m, 3.75(3)s, 4.9(1)s, 6.2(1) broad singlet. The UV and IR data correspond well to those reported for other α-diazocarbonyl compounds (12). This diazotization procedure is described in detail because it was noted that the product obtained by direct diazotization in aqueous solution (27) was invariably contaminated by educt (glycyl-DL-norleucine methyl ester) and/or α-hydroxyacetyl-DL-norleucine methyl ester as reflected by a low value of $\varepsilon_{250\ nm}$ for the isolated product. Furthermore, we observed that mixing of Cu^{2+} ions and pure DL-DAN leads to a rapid yellow precipitate, while no such reaction occurs when Cu^{2+} ions were mixed with contaminated DL-DAN prepared by diazotization in aqueous solution. Consequently, it was found that addition of educt to the pure DL-DAN prevents the precipitation from occurring (cf. sections III and IV for further details pertaining to these observations).

2.2.3. Diazoacetyl-L-norleucine methyl ester (L-DAN)

This compound was prepared from HBr · H-Gly-L-Nle-OMe by the diazotization method described above. Its spectral properties were identical with those of DL-DAN.

HBr · H-Gly-L-Nle-OMe was synthesized from Z-glycine and L-norleucine according to standard methods of peptide synthesis (44). Overall yield was 15% (relative to Z-glycine). M.p. 145–148 °C (crystallized from ether/methanol; approx. 9:1). TLC on Silica Gel in n-butanol/glacial acetic acid/water/5% ammonia/acetone (9:2:4:2:3) revealed a strong yellow spot (hydrobromide, R_f = 0.69) and a weak pink spot (free amine, R_f = 0.80). Spots were located using the ninhydrin reagent.

2.3. General methods

Melting points are uncorrected and were determined on a Fischer-Johns melting point apparatus. Ultraviolet spectra were recorded on a Cary 14, infrared spectra on a Perkin-Elmer IR-10 and nuclear magnetic resonance spectra on a Varian T-60 spectrometer.

Amino acid analysis were performed according to the method of Spackman et al. (36), with the aid of a Beckman-Spinco 120B amino acid analyzer. Samples were hydrolyzed with 5.7 N-HCl for 36 hours under vacuum at 105 °C. TLC plates were developed with ninhydrin or Rydon/Smith reagent (32).

2.4. Assay for enzymatic activity

The concentration of chymotrypsin in solution was determined spectrophotometrically using $E_{280nm}^{0.1\%}$ = 2.0 as cited by EDELHOCH (11). Chymotryptic activity was measured with N-acetyl-L-tyrosine ethyl ester as substrate following either the titrimetric (21) or spectrophotometric (34) procedure using a Radiometer pH-stat titrator assembly and a Cary 14, respectively. Tryptic activation of chymotrypsinogen was achieved according to the published procedure (10).

2.5. Chemical modification of enzyme

Modification experiments were performed on two different scales using 0.15 M stock solutions of diazocarbonyl reagent (in methanol) and copper(II)acetate (in water). In small scale experiments, one milliliter of a solution of chymotrypsin (1 mg · ml^{-1}) in either phosphate or acetate buffer (50 mM) of the desired pH was treated with small volumes of the reagent stock solutions which were added either in sequence or as a mixture. Thus, to achieve an enzyme:Cu^{2+}:DAN ratio of 1:75:75, 20 µl each of copper(II)acetate and DAN were used. In some cases, reagent solutions were added repeatedly. At various time intervals, 20 µl aliquots were

removed and assayed spectrophotometrically for chymotryptic activity.

When the modified protein was to be characterized by amino acid analysis and kinetic measurements, a larger quantity of protein was modified and isolated as follows. A suitable excess of reagents (as a mixture, or in sequence) was added to 20 ml of a solution of chymotrypsin or chymotrypsinogen (1 mg · ml^{-1}) in a 50 mM buffer of the desired pH. The enzyme solution was precooled to 15 °C and the reaction was performed in the dark at that temperature. Enzyme activity was tested on 20µl aliquots as described above. After the reaction had progressed to the desired stage of inactivation, the solution was adjusted to pH 4.0 by the addition of 0.015 M-HCl (to destroy the unreacted reagent) and concentrated to a volume of 2–3 ml using dialysis tubing and aquacide III. The concentrate was then applied on a Sephadex G-15 column (1.5 × 30 cm) previously equilibrated with 1mM-HCl, which was also used for elution. The fractions containing the protein were pooled and lyophilized. In some cases the samples were rechromatographed on Sephadex G-15 in order to ensure the complete removal of all low molecular norleucine containing compounds. On the average, 70–80% of the protein initially used in the reaction with the modifying reagents could be recovered under these conditions.

3. RESULTS

3.1. Effects of modifications on enzymatic activity and amino acid composition

The effect of some α-diazocarbonyl compounds (DAG, L-DAN and DL-DAN) on chymotrypsinogen and chymotrypsin was investigated under a variety of experimental conditions such as inclusion or exclusion of Cu^{2+} ions in the reaction medium, substitution of Cu^{2+} ions by Ag^{+} ions and variations in the concentration of the reagents. Following recovery of the protein from the reaction mixture, its catalytic activity (or in the case of zymogen, its ability to yield an active enzyme upon activation with trypsin), as well as the amino acid composition were determined. These data are recorded in Table I. Cu^{2+} ions, either in the presence or absence of glycyl-DL-norleucine methyl ester, exerted very little effect on the aspects of the protein mentioned above. Likewise, the diazocarbonyl compounds in the absence of Cu^{2+} ions had no significant influence on the activity and amino acid composition of the proteins. However, in the presence of Cu^{2+} ions these compounds were found to cause strong inactivation of chymotrypsin while, interestingly, in the case of chymotrypsinogen they effected only a slight decrease in the activity attainable upon tryptic activation. Examination of the protein samples following their reaction with a mixture of Cu^{2+} ions and diazocarbonyl compounds revealed considerable modification of tryptophan, methionine and to a lesser extent of half-cystine and tyrosine residues. Other amino acids remained unaffected. In most cases there were only traces of norleucine incorporated, indicating negligible modification of carboxylic functions under the conditions employed in these experiments. At low reagent concentration, the loss of activity noted with chymotrypsin (or of potential activity in the case of zymogen) was found to be at least partially due to an alteration in the Km of the enzyme for the substrate N-acetyl-L-tyrosine ethyl ester. The Km-value determined for modified enzyme was 1.5 mM relative to a value of 0.7 mM for unmodified chymotrypsin (45). These observations are consistent with the increase in Km of the enzyme accompanying the oxidation of tryptophan and methionine residues (33, 42).

DAG effected rapid and complete inactivation of chymotrypsin. The L-isomer of DAN was more potent than the racemic mixture of the compound in its reaction with chymotrypsin. This phenomenon does not appear to be related to the stereospecific interaction of the L-isomer with the active site of the enzyme, since proflavin, known to bind at this region (4), failed to prevent the Cu^{2+}/L-DAN mediated inactivation of the enzyme. Thus, the apparent inactivation of the enzyme by this reagent appears to be mostly due to interactions with functional groups other than those at the active site of the protein. Silver ions were much less effective than Cu^{2+} ions in promoting inactivation of the enzyme in the presence of diazocarbonyl compounds. This is in agreement with their known ability to catalyze the Wolff rearrangement,

Table I.

Reaction of α-diazocarbonyl compounds with chymotrypsin and chymotrypsinogen[a)]

Reagents in reaction medium[b)]	Incubation time (min.)	Per cent loss of activity[c)]	A280[d)]/A260	Residues (mole per mole protein)[e)]				
				Tyr	Cys	$CysSO_3$	Met	Nle
Chymotrypsin:	60		3.10	4.0	10.0	0	2.0	0
+ Cu^{2+}(150)	45	0	3.10	3.7	8.7	0	1.5	0
+ Cu^{2+}(75) + H-Gly-DL-Nle-OMe(75)	45	0	3.10	3.9	8.2	0	1.5	0
+ DL-DAN	60	10	3.10	3.8	8.2	0	1.5	0
+ Cu^{2+}(75) + DL-DAN(75)	30	70	1.90	3.1	0	7.2	0.3	0.6
+ Cu^{2+}(75) + DL-DAN(75) + 4mM $Na_2S_2O_4$	30	80	2.00	3.5	0	3.5	1.1	1.2
+ Cu^{2+}(75) + DL-DAN(75) + H-Gly-DL-Nle-OMe(75)[g)]	15	100	2.54	3.4	6.0	1.2	0	0.2
+ Cu^{2+}(75) + L-DAN(75)	30	100	1.70	3.6	8.0	0	0	traces
+ Cu^{2+}(37)-L-DAN(37)	60	100	1.30	4.1	10.5	0	0.8	traces
+ Ag^{+}(75) + DL-DAN(75)	30	12	–	–	–	–	–	–
+ Cu^{2+}(75) + DAG(75)	15	100	–	–	–	–	–	–
Chymotrypsinogen:	60		2.90	4.0	10.0	0	2.0	0
+ DL-DAN(75)	45	0	2.90	4.1	7.3	0	1.4	0
+ Cu^{2+}(75) + DL-DAN(75)	60	0	2.00	4.0	9.6	0	1.6	0.1
+ Cu^{2+}(75) + L-DAN(75)[f)]	60	28	1.85	3.7	7.8	1.0	0.8	0.1

a) Chymotrypsin or chymotrypsinogen (1 mg · ml^{-1}) in 50 mM acetate buffer, pH 5.0 was incubated with the reagents as shown. Reagents were free from educt and were added sequentially, with copper(II)acetate before α-diazocarbonyl compounds. With the exception of one experiment where $Na_2S_2O_4$ was added, no precautions against dissolved oxygen were taken.

b) Numbers in parenthesis denote the molar excess of the reagent(s) over that of protein.

c) In the case of chymotrypsinogen, loss of activity refers to the loss of ability to undergo activation by trypsin.

d) A280:A260 ratio is used as a measure of the extent of oxidation of tryptophan residues. Diminution in A280:A260 ratio indicates oxidation of the indole moiety of tryptophan (42).

e) Data for the amino acid residues found susceptible to modification are shown.

f) The extent of modification varied from one commercial sample of zymogen to another. The extent of nonspecific modification was always significantly lower with chymotrypsinogen than with chymotrypsin.

g) The reagents were premixed.

converting diazocarbonyl-derived carbenes into ketenes (28, 46).

The data recorded in Table I also reveal yet another significant difference in the effects mediated by the L- and the racemic mixture of DAN. DL-DAN in the presence of Cu^{2+} ions promoted extensive oxidation of cystine residues as indicated by the presence of cysteic acid (with little or no half-cystine residues) in the HCl hydrolysates of the modified protein. In contrast to these findings, L-DAN and Cu^{2+} ions failed to produce such oxidation of cystine residues, the amino acid being recovered intact in the HCl hydrolysates of the modified protein. The differences in the stereochemical features of the complexes of L- and D-isomers of DAN with Cu^{2+} ions appear responsible for the above observations.

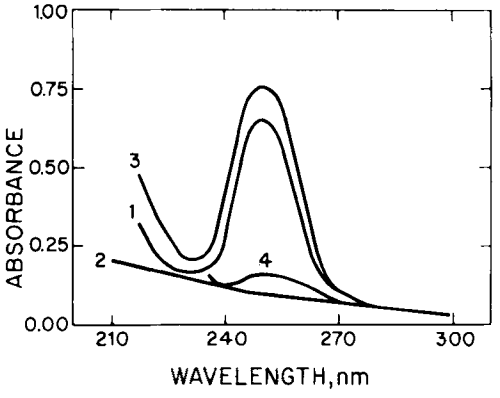


Figure 1. Absorption spectrum of L-DAN at pH 5 in 50mM-phosphate buffer.

1 = L-DAN (30 μM); 2 = L-DAN (30 μM) in the presence of copper(II)acetate (30 μM); 3 = L-DAN (30 μM) in the presence of copper(II)acetate and glycyl-DL-norleucine methyl ester (30 μM each); 4 = copper(II)acetate and glycyl-DL-norleucine methyl ester (30 μM).

3.2. The role of Cu^{2+} ions

The results recorded above as well as those available from the literature demonstrate a vital need for the presence of Cu^{2+} ions to achieve maximal modification of proteins by diazocarbonyl compounds. A review of the literature revealed a lack of concensus concerning procedure (premixing of, and/or sequence of addition of reagents) needed to achieve maximal modification. This situation, as well as the observation mentioned in section 2.2. concerning the methods of diazotization and the purity and properties of the resulting products, prompted further investigations into the role played by Cu^{2+} ions in the reaction. The absorption spectra of DAN in the presence and absence of Cu^{2+} ions and glycyl-DL-norleucine methyl ester are shown in Figure 1. The spectrum for DAN (curve 1) is typical of that of an α-diazocarbonyl compound and is characterized by a λ_{max} of 250 nm (28, 46). The band at 250 nm is virtually abolished in the presence of Cu^{2+} ions (curve 2). This is consistent with the formation of a Cu^{2+}-complexed carbene or with the Cu^{2+} ions catalyzed decomposition of α-diazocarbonyl compounds to α-hydroxy acids and other compounds (17, 28, 46). Inclusion of glycyl-DL-norleucine methyl ester effectively prevented the Cu^{2+} ion mediated disappearence of the absorption spectra of the α-diazocarbonyl compound (curve 3).

Treatment of pure DAN or DAG with Cu^{2+} ions resulted in the rapid appearance of a brownish yellow precipitate, a phenomenon that was found to be partially abolished if all solvents and buffers were repeatedly deaerated and flushed with nitrogen prior to mixing the reagents. Turbid, and/or partially precipitated mixtures of Cu^{2+} ions and DAN or DAG were found to be only partially effective in the inactivation of chymotrypsin, with 50% or less loss of activity being obtained in contrast to the total inactivation achieved with a clear mixture of the reagents. The formation of the precipitate was not observed with DAN or DAG preparations that were either contaminated with the parent dipeptide ester or admixed with glycyl-glycine methyl ester or glycyl-DL-norleucine methyl ester, presumably due to the formation of a stable trimeric complex. These observations provide a possible explanation for the discrepancy with respect to the procedure capable of producing maximum modification of acid proteases. Thus, with inhomogeneous preparations (contaminated with the parent dipeptide esters) of DAN or DAG, premixing with Cu^{2+} ions has little effect on the extent of modification of protein. On the other hand, with homogenous preparations of DAN or DAG, premixing will

Table II.

Effect of α-aminocarbonyl compounds on Cu^{2+} ion induced decomposition of L-DAN.[a)b)]

α-Aminocarbonyl compound	Decomposition of L-DAN[c)]	% Inactivation of chymotrypsin[d)]
Alanine	+	0
Z-alanine	+	0
N-acetyl-L-tyrosine ethyl ester	+	0
Urea	+	0
Glycyl-glycine	−	100
Glycyl-DL-norleucine methyl ester	−	100
Glycine amide	−	100
Glycine ethyl ester	−	100
Phenylalanine ethyl ester	−	100
Hydroxylamine	+ [e)]	80

a) These preparations were free of contamination by glycyl-L-norleucine methyl ester.
b) Similar observations were made with DAG.
c) As evidenced by the formation of a brown precipitate upon mixing of the reagents. + = precipitate; − = no precipitate.
d) Equimolar mixtures (75-fold molar excess over chymotrypsin) of copper(II)acetate, α-aminocarbonyl and L-DAN (in that order) were prepared and added to solutions of chymotrypsin (1 mg · ml^{-1}) in 50 mM-phosphate buffer pH 5.0. Activity was assayed as described in section 2.
e) Weak white precipitate was formed.

result in formation of precipitate and a consequent decrease in the concentration of the reactive species. With pure preparations sequential addition of the reagents, Cu^{2+} ions followed by the α-diazocarbonyl compound appears desirable. Several substances (amino acids and their derivatives) were examined for their ability to prevent the Cu^{2+} ion induced decomposition of diazocarbonyl compounds. Results of these experiments are shown in Table II. These studies established the minimum structural features required for the prevention of Cu^{2+} ion mediated decomposition of DAN of DAG. For a substance to serve as an efficient protector, it should possess an unblocked amino group and a -CO-R function (R ≠ OH) separated from each other by at least a single methylene group.

3.3. pH-range of inactivation

Complete inactivation of chymotrypsin (as well as acid proteases reported in the literature), besides being dependent on relatively large excess of α-diazocarbonyl compounds and Cu^{2+} ions, occurs only over a narrow pH-range. Maximum inactivation could only be achieved between pH

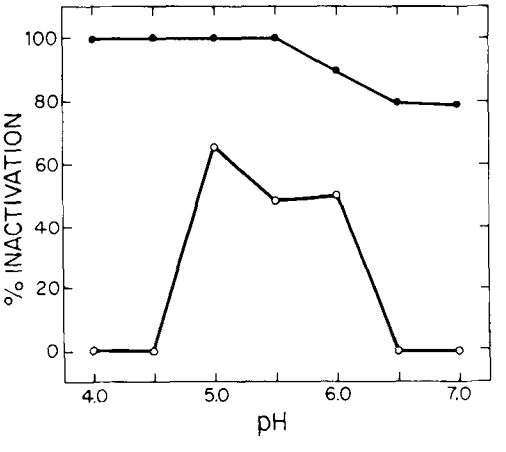


Figure 2. The effect of pH on the inactivation of chymotrypsin by DL-DAN in the presence of Cu^{2+} ions and glycyl-glycine ethyl ester.

Sodium acetate and phosphate buffers (50 mM) were used. O—O, activity at 30 min after the addition of a 75-fold molar excess each of Cu^{2+} and DL-DAN (in that order) to the enzyme solution (1 mg · ml^{-1}). ●—●, activity at 15 min after the addition of a solution prepared by mixing equal volumes of copper(II)acetate, glycyl-glycine ethyl ester and DL-DAN (in that order) to the enzyme. The molar excess of each reagent over the enzyme was 75-fold.

5.0 and 6.0 (Fig. 2). The instability of α-diazoketones below pH 5.0 and the tendency of $Cu(OH)_2$ to precipitate above pH 6.0 dictate the pH range over which modifications can be performed. In view of the ability of certain α-aminocarbonyl compounds to prevent the Cu^{2+} ion mediated decomposition of DAN (cf. Table II), and to form a reactive trimeric complex, their effect on the pH range of inactivation was investigated. As shown in Fig. 2, inclusion of a dipeptide ester in the reaction medium results in an expansion of the pH-range over which the enzyme could be modified. Similar results were obtained with other α-aminocarbonyl compounds listed in Table II. Thus, in the presence of such Cu^{2+} chelators, the Cu^{2+}/α-diazocarbonyl reagent can be employed over the range of pH 4 to 7. This could possibly widen the scope of this reagent for chemical modification of proteins.

3.4. Exclusion of oxygen

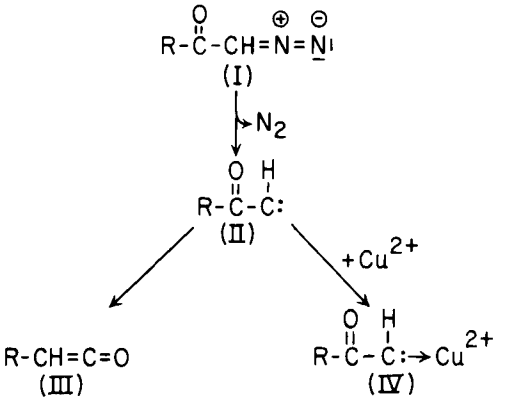
The reaction of the Cu^{2+}/α-diazocarbonyl reagents with chymotrypsin or chymotrypsinogen was always accompanied by the oxidation of methionine, tryptophan and, to a lesser extent, of tyrosine and cystine residues, suggesting the participation of oxygen in the process. Furthermore, oxygen appears to be involved in the precipitation noted in solutions containing Cu^{2+} ions and homogeneous diazoketones. In light of these observations the effect of sodiumdithionite, $Na_2S_2O_4$, an effective oxygen scavenger (26), on the Cu^{2+}/DAN induced modification of protein was investigated. The reaction of a 75-fold excess of Cu^{2+}/DL-DAN reagent with chymotrypsin (1 mg · ml^{-1}) at pH 5 in the presence of $Na_2S_2O_4$ (4mM) did not result in the usual formation of brownish precipitate (at concentrations lower than 4mM, $Na_2S_2O_4$ was ineffective in preventing such decomposition of reagent). Under these conditions, nearly 80% af activity was lost after 30 minutes of reaction and the isolated protein exhibited an optical density 280nm:260nm ratio of 2.0 (3.1 for native chymotrypsin) indicating some destruction of tryptophan. Amino acid analysis of the modified protein revealed a diminished oxidation of methionine and cystine residues and incorporation of 1.2 norleucines per mole of protein. However, the recovery of cystine residues in the HCl hydrolysates of the modified protein was low. It thus appears that Cu^{2+}/DL-DAN reacts with disulfide bonds (28, 46) in an oxygen-independent reaction which can partially be prevented by addition of the educt, i.e. glycyl-DL-norleucine methyl ester. The exact nature of this modification is subject of further investigations. Our observations indicate that exclusion of oxygen in modification reactions with Cu^{2+}/α-diazocarbonyl reagents is advantageous in so far as it reduces the nonspecific oxidative destruction of amino acid residues in the proteins. Exclusion of oxygen also prevents destruction of the reagent per se, a feature that would allow the modification reactions to be performed at lower molar excesses of reagents over protein.

4. DISCUSSION

α-diazocarbonyl compounds in conjunction with Cu^{2+} ions have been widely employed to selectively modify carboxyl function(s) of the active site of acid proteases (2, 8, 20, 23, 24, 25, 27, 29, 37–41). Relatively large molar excess of the reagent over that of the protein to be modified are normally employed. While the lability of the α-diazocarbonyl compounds in the presence of Cu^{2+} ions, especially at low pH, may necessitate the use of large excess of reagents, such conditions could also result in a nonspecific modification of catalytically unessential functional groups and consequent perturbation of the active conformation of the protein. The current study was undertaken: 1) to assess the nature of possible nonspecific modifications caused by Cu^{2+}/diazocarbonyl reagents, 2) to find conditions for the minimization of any such nonspecific modifications and 3) to establish conditions capable of preventing Cu^{2+} ion induced decomposition of diazocarbonyl compounds. Chymotrypsinogen and chymotrypsin were selected as model proteins in view of the extensive information available concerning their physico-chemical properties.

Neither an α-diazocarbonyl compound (L-DAN,DL-DAN or DAG) nor Cu^{2+} ions alone showed any effect on the activity (potential activity in the case of zymogen) of these two proteins. However, exposure of chymotrypsin to a 75-fold molar excess of a Cu^{2+}/DAN mixture resulted in the oxidative destruction of trypto-

phan, methionine, tyrosine and, to a lesser extent, of cystine residues with concomitant loss of catalytic activity. The requirement for Cu^{2+} ions to achieve the modification of proteins has been attributed (25) to the ability of these ions to complex, and thus stabilize, the reactive carbene (II) (cf. Scheme 1) and prevent its conversion to the ketene (III) via Wolf rearrangement. Indeed, Ag^{+} ions which promote this rearrangement (28, 46) were found to be much less effective than Cu^{2+} ions in the current as well as in other studies (2, 8, 20, 23, 24, 25, 27, 29, 37–41). Only trace amounts of norleucine were incorporated in the presence of Ag^{+} ions.



Scheme 1. Formation of α-diazocarbonyl derived carbenes(II) and their conversion to ketenes (III) (Wolff rearrangement) or Cu^{2+}-complexed carbenes (IV).

In the course of these modifications the appearance of turbidity, followed by a brown precipitate, indicated considerable reagent destruction rendering it difficult to assess the effective in situ concentration of the active reagent. It is also likely that certain transient intermediates resulting from the reagent destruction are actually responsible for oxidative side reactions. The fact that such precipitate formation is not always observed has led to ambiguities concerning the experimental procedure (premixing, order of sequential addition) for the use of Cu^{2+}/α-diazocarbonyl reagents, Some of our observations indicate that the basis for these discrepancies lies in the purity of the α-diazocarbonyl compounds employed. In our hands, these side reactions always occurred when pure diazocarbonyls were used. No such precipitate was observed with inhomogeneous preparations (contaminated with starting dipeptide ester) or with pure preparations to which the parent dipeptide ester had been added. However, even when, advertently or inadvertently, employing such trimeric mixtures, the possibility for oxidative side reactions is not eliminated since the ability of a combination of Cu^{2+} ions, oxygen and a primary amine to act as powerful oxidant is well established (22, 31). However, such oxidative destruction of amino acid residues can be minimized by exclusion of oxygen from the reaction medium, a condition that can be achieved by thorough deaeration of all solvent and addition of oxygen scavengers such as $Na_2S_2O_4$.

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