Specific Oxidation of Peptides *via* Their Copper Complexes*

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ABSTRACT: The oxidation of the copper(II) complex of tetra-L-alanine with 2 equiv of chloroiridate at pH 6–9 yields equimolar quantities of di-L-alanine amide and pyruvoyl-L-alanine. Similarly, di-L-alanine amide and pyruvoyl-di-L-alanine are obtained from penta-L-alanine. The copper(II) complexes of other peptides showed similar fragmentation in which the third amino acid residue (from the amino end) is destroyed. The mechanism proposed for this reaction involves oxidation of the Cu^{II} to Cu^{III}, the intramolecular reduction of Cu^{III} to give a free-radical intermediate; in a subsequent step the Cu^{II} which remains bound to this radical is again oxidized to Cu^{III} by a second molecule of $IrCl_6^{2-}$ leading to oxidation of the peptide group and ultimately to the formation of a carbonylic group. The possibility of using this or similar reactions for locating complexed copper atoms in peptides and protein is discussed.

Enhancement of the radiolytic inactivation of enzymes by copper has been interpreted (Anbar and Levitzki, 1966) as due to formation of Cu^{III} during the irradiation (by the reaction $Cu^{II} + OH \rightarrow Cu^{III} + OH^-$) with subsequent oxidative damage to the amino acid residues acting as ligands in the protein–copper complex. This mechanism is supported by the observation (Levitzki and Anbar, 1967) that the copper-enhanced radiolytic inactivation of RNase is accompanied by increased loss of histidyl residues, which are assumed to be the ligands for the metal in the RNase–Cu^{II} complex (Saundry and Stein, 1966; Breslow and Girotti, 1966). The present work describes a chemical system in which a single electron-transfer oxidant (Na_2IrCl_6) oxidizes copper complexes of peptides *via* the formation of Cu^{III} as an intermediate. The oxidation specifically affects those amino acid residues which act as ligands of the copper. Owing to its specificity this reaction could be used for locating copper binding sites in natural copper proteins.

Materials and Methods

All solutions were prepared in triple-distilled water, obtained by redistillation over alkaline permanagnate and then over phosphoric acid. H·Ala_3·OH, H·Ala_4·OH, H·Ala_5·OH, H·Val·Ala_2·Phe·OH, Z·Ala_2·OH, Z·Ala_2·$NHNH_2$. H·Ala·OBz hydrochloride, and H·Gly·OBz hydrochloride were obtained from Yeda Research and Development Co., Rehovoth. H·Lys·Ala_3·OH was kindly supplied by Dr. I. Schechter from the Weizmann Institute of Science.

Sodium hexachloroiridate ($Na_2IrCl_6 \cdot 6H_2O$) was supplied by Alfa Inorganic Chemicals Inc., Mass. This and all other inorganic chemicals which were of analytical grade were used without further purification. Palladium (10%)-on-carbon powder was purchased from Baker Co., N. J. PdO_2 was obtained from Fluka (Puriss, p.a.). Triethylamine was redistilled before use. Pyruvic acid was distilled at 25 mm and the midfraction was used for the synthesis of pyruvoyl peptides. Tetrahydrofuran was distilled and dried over sodium before use. DCC[1] was Fluka Puriss. Dowex 1-AG-X4 (Bio-Rad) was converted to the acetate form by the method described by Dixon (1964). The Dowex A-1 chelating resin was regenerated by the following sequence: rinsing the resin with two bed volumes of 1 N HCl, five bed volumes of water, two bed volumes of 1 N NaOH, and five bed volumes of water. The pH of the resin was brought down to 6.0 with 0.5 M sodium acetate and the resin was washed with water.

Z·Ala₂O·HSI. Z·Ala_2·OH (10 mmoles) and *N*-hydroxysuccinimide (Anderson *et al.*, 1964; 10 mmoles) were dissolved in dioxane–ethyl acetate (3:1, v/v, 30 ml), and DCC (10 mmoles) was added at −5°. The reaction mixture was stirred for 2 hr at −5°, and for 12 hr at room temperature. After addition of a few drops of 50% acetic acid the DCU was filtered. The filtrate was evaporated to dryness, and the oily residue was crystallized from ethyl alcohol and dried over P_2O_5 at room temperature; yield 76%, mp 146°.

Anal. Calcd for $C_{18}H_{21}N_3O_7$ (391): N, 10.8. Found: N, 10.9.

Z·Ala₂·Ala·OH.* Z·Ala·O·HSI (1 mmole) and H·Ala*·OH (DL) (Johns and Whelan, 1966; 1 mmole) were mixed in water–dioxane (3:2, v/v, 30 ml) with $NaHCO_3$ (2 mmoles), at room temperature overnight.

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[1] Abbreviations used: Pyr, pyruvoyl; Z, benzyloxycarbonyl; Bz, benzyl; O·HSI, *N*-hydroxysuccinimide ester; NADH, reduced nicotine–adenine dinucleotide; DNPH, 2,4-dinitrophenylhydrazine; DCC, *N,N'*-dicyclohexylcarbodiimide; DCU, dicyclohexylurea; DMF, dimethylformamide; H·Ala*·OH, α-*C*-deuterioalanine; BAW, 1-butanol–acetic acid–water (4:1:1, v/v).

After evaporation, the residue was dissolved in water, precipitated with 1 N HCl, filtered, and washed with water. The blocked tripeptide was dried at 60° over P_2O_5.

Anal. Calcd for $C_{17}H_{23}N_3O_6$ (365): C, 56.9; H, 6.3; N, 11.5. Found: C, 56.9; H, 6.2; N, 11.6.

Z·Ala₂Ala·Ala·OBz.* Z·Ala₂Ala*·OH (1 mmole) and alanine benzyl ester (1 mmole) were dissolved in DMF (10 ml) and DCC (1 mmole) was added to the solution at −5°. The reaction mixture was kept for 2 hr at −5° and for 12 hr at room temperature. DCU was removed by filtration after concentrating the solution. The blocked peptide was precipitated with water, filtered, washed with water, and dried *in vacuo* over H_2SO_4 at 100°; yield 60%.

Anal. Calcd for $C_{27}H_{34}N_4O_7$ (526): C, 65.2; H, 5.9; N, 9.7. Found: C, 65.0; H, 5.9; N, 9.7.

H·Ala₂·Ala·Ala·OH.* The blocked tetrapeptide was dissolved in DMF, and water was added (just enough not to cause precipitation). The blocking groups were removed by hydrogenation over PdO_2 overnight. The catalyst was removed and the solvent was evaporated to dryness. The tetrapeptide was dissolved in water, precipitated with acetone, and dried over H_2SO_4 *in vacuo;* yield, 95%. The compound was electrophoretically pure having the same electrophoretic mobility as H·Ala₄·OH at pH 1.5.

Anal. Calcd for $C_{12}H_{22}N_4O_5$-(302): C, 47.6; H, 7.3; N, 18.6. Found: C, 47.5; H, 7.3; N, 18.7.

Aminoisobutyryl·Ala·OBz· Benzyloxycarbonylaminoisobutyric acid (10 mmoles) and H·Ala·OBz·HCl (10 mmoles) were dissolved in $CHCl_3$ (30 ml) and triethylamine (10 mmoles) was added. DCC (10 mmoles) was added to the chloroform solution at −5° and the reaction mixture was kept for 2 hr at −5° and for 12 hr at room temperature. A drop of acetic acid was added to the reaction mixture and the DCU was removed by filtration. The chloroform was removed and the oily residue was dissolved in ethyl acetate. The ethyl acetate solution was washed with 1 N HCl, 5% $NaHCO_3$, and water, dried over Na_2SO_4, filtered, and evaporated. The oily residue was dissolved in 12 ml of 30% (w/v) HBr in acetic acid and kept at room temperature for 15 min. Anhydrous ether (500 ml) was added and the precipitate formed was washed twice with dry ether. The hygroscopic aminoisobutyrylalanine benzyl ester hydrobromide was kept in a desiccator over P_2O_5 *in vacuo.*

Z·Ala₂·aminoisobutyryl·Ala·OBz. The aminoisobutyrylalanine benzyl ester hydrobromide was coupled to Z·Ala₂·$NHNH_2$ *via* the azide (*cf.* Greenstein and Winitz, 1961). The blocked tetrapeptide was homogeneous on thin layer chromatography using BAW as the solvent system; yield 60%.

Anal. Calcd for $C_{28}H_{36}N_4O_7$ (540): C, 62.1; H, 6.6; N, 10.4. Found: C, 62.2; H, 6.7; N, 10.4.

H·Ala₂·aminoisobutyryl·Ala·OH. The blocked tetrapeptide was dissolved in methanol–water. Blocking groups were removed by hydrogenation overnight using PdO_2 as the catalyst. The catalyst was filtered, the methanol was removed by evaporation, and the tetrapeptide was isolated by precipitation with acetone. The product was electrophoretically pure with an electrophoretic mobility slightly less than that of H·Ala₄·OH, at pH 1.5.

Anal. Calcd for $C_{13}H_{24}N_4O_5$ (316): C, 49.4; H, 7.7; N, 25.3. Found: C, 49.4; H, 7.7; N, 25.2.

Pyr·Gly₂·OBz. Pyruvoylglycine (1.0 mmole) (Wieland *et al.*, 1958), glycine benzyl ester hydrochloride (1.0 mmole), and triethylamine (1.0 mmole) were dissolved in 3.0 ml of $CHCl_3$. DCC (1.1 mmoles) in 1.0 ml of $CHCl_3$ was added to the mixture at −5°. After stirring at room temperature for 3 hr, a few drops of 50% acetic acid were added to the reaction mixture, and the DCU was removed by filtration. The filtrate was washed with water, 1 N H_2SO_4, 1 N $KHCO_3$, and again with water. The solvent was evaporated and the residue was dissolved in ethyl acetate and crystallized by adding petroleum ether (bp 30–60°). The product was dried *in vacuo* over H_2SO_4 and solid wax; yield 60%, mp 104–106°. The substance gave one DNPH-positive spot on thin layer chromatography (R_F 0.80 using the BAW solvent).

Anal. Calcd for $C_{14}H_{15}N_2O_5$ (292): C, 57.9; H, 5.5; N, 9.7. Found: C, 58.2; H, 5.7; N, 9.8.

Pyr·Gly₂·OH. The pyruvoyl·Gly_2·OBz was dissolved in CO_3OH and hydrogenated over 10% Pd–C in a closed system where the hydrogen uptake could be measured. When the calculated amount of hydrogen was consumed, the hydrogenation was stopped in order to avoid the reduction of the pyruvoyl group (Wieland *et al.*, 1958). The catalyst was removed by filtration, the CH_3OH was evaporated, and the residue was dissolved in a minimal amount of ethyl acetate. On addition of petroleum ether the substance crystallized and was collected after standing in the cold overnight. The peptide was dried *in vacuo* over H_2SO_4 and solid wax; yield 96%, mp 150–152° dec. The substance gave one DNPH-positive spot on thin layer chromatography (R_F 0.50, BAW).

Anal. Calcd for $C_7H_{10}N_2O_5$ (202): C, 41.7; H, 5.0; N, 13.9. Found: C, 42.1; H, 4.9; N, 13.8.

Pyr·Ala·OH was prepared by the nonenzymatic transamination procedure (Dixon, 1964) from H·Ala_2·OH (1 mmole) and glyoxylic acid (10 mmoles). After separation of the pyruvoylalanine on Dowex 1-AG-X4 (acetate form) the fractions containing the Pyr·Ala·OH were collected, lyophilized, redissolved in 2 N HCl, and extracted three times with ethyl acetate. The organic phase was dried over Na_2SO_4, filtered, and the peptide was precipitated with petroleum ether, and dried *in vacuo* over H_2SO_4 and solid wax; yield 25%, mp 151°. The substance gave a single spot on thin layer chromatography (R_F 0.75, BAW).

Anal. Calcd for $C_6H_9NO_4$ (159): C, 45.3; H, 5.7; N, 8.8. Found: C, 45.2; H, 5.7; N, 8.8.

Pyr·Ala₂·OH was prepared from H·Ala_3·OH as above; yield 20%, mp 157–160° dec. The substance gave a single spot on thin layer chromatography (R_F 0.70, BAW).

Anal. Calcd for $C_9H_{14}N_2O_5$ (230): C, 47.0; H, 6.1; N, 12.2. Found: C, 46.5; H, 6.1; N, 12.1.

Pyr·Phe·OH was prepared from H·Ala·Phe·OH as above; yield 32%.

Anal. Calcd for $C_{12}H_{13}NO_7$ (235): C, 61.4; H, 5.5; N, 6.0. Found: C, 61.0; H, 5.5; N, 5.9.

Determination of Pyruvoyl Peptides. The sample containing 0.05–0.35 μmole of substance was diluted to 2.0 ml with 5% trichloroacetic acid and 1.0 ml of DNPH (0.1% in 2 N HCl) was added. After 15 min, 2.0 ml of ethyl acetate was added and the phases were mixed for 20 sec with the aid of a Cyclo-Mixer. Then 3.0 ml of 10% Na_2CO_3 was added and the phases were mixed again. After complete phase separation 5.0 ml of the lower aqueous phase was transferred to another test tube and 4.0 ml of 2 N NaOH was added. After 15 min the absorbance was read at 440 mμ. All pyruvoyl peptides described above gave the same calibration curve (Figure 1).

Quantitative high-voltage paper electrophoresis at pH 1.4 was carried out on Whatman No. 1 paper (105 × 46 cm) as described by Schechter and Berger (1966).

High-Voltage Paper Electrophoresis at pH 6.5. Separation of the peptides, peptide-amides, and ketoacyl peptides was best obtained by electrophoresis at pH 6.5 (pyridine–acetate buffer, 1.2 M in pyridine–0.07 M in acetic acid). Electrophoresis was carried out for 20 min at 60 v/cm using either Whatman No. 1- or 3MM paper. Samples of 10–30 μl containing up to 0.3 μmole of peptide were applied to the middle of the paper (25 × 50 cm). When quantitative runs were performed the electrophoresis sheet was treated as described for runs at pH 1.4. For isolation of the peptide-amides formed during the oxidation reaction, the reaction mixture containing up to 4 μmoles of peptide-amide was applied as a streak across the paper. After electrophoresis the area containing the amide was cut from the paper, eluted with distilled water, and the eluate was lyophilized.

Enzymatic Degradation of Peptide-Amides by Leucine Aminopeptidase. Leucine aminopeptidase was obtained as a lyophilized powder from Sigma Chemical Co., St. Louis (type II, C_1 = approximately 10; lot 14B-0300). Stock solutions of the enzyme (1 mg/ml in 0.05 M Veronal buffer (pH 8.0)–0.01 M $MnSO_4$) were prepared. Incubation mixtures contained the peptide-amide (about 5 μmoles), 50 μg of enzyme, 5×10^{-3} M $MnSO_4$, and 0.01 M Veronal buffer (pH 8.0). Samples of 20–30 μl were taken at suitable intervals and subjected to electrophoresis at pH 1.4 and 6.5.

Amino Acid Analysis of Unknown Peptide-Amides. After elution of the peptide-amides from the paper, about 0.1 μmole of the amide was hydrolyzed in 6 N HCl for 22 hr at 110°, lyophilized, and the mixture was analyzed in a Beckman-Spinco Model 120 amino acid analyzer. A peptide-free piece of the electrophoresis paper was run as a blank and the amount of NH_3 obtained was subtracted from that found in the amide.

Electron Paramagnetic Resonance Measurements. The measurements of the Cu^{II} signals were carried out in solution. The instrument was a Varian Model V-4512 electron paramagnetic resonance spectrometer equipped with a flow system.

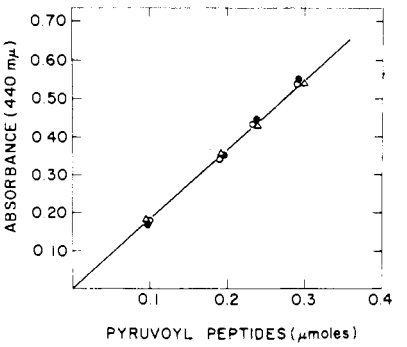


FIGURE 1: Calibration curve for the determination of pyruvoyl peptides. All experimental details are given under Materials and Methods. Blank values were 0.06–0.07 absorbance unit. (Δ–Δ–Δ) Pyr·Gly·OH, (O–O–O) Pyr·Ala·OH, and (●–●–●) Pyr·Ala_2·OH.

Oxygen Concentration Measurements. Oxygen concentration was measured using the Oxygraph (Gilson Medical Electronics, Middleton, Wis.). When it was desired to check if oxygen is consumed, the reactions were performed in the Oxygraph in a closed system and the concentration of O_2 during the reaction was recorded.

Na_2IrCl_6 Determination. The concentration of $IrCl_6^{2-}$ was determined by measuring the absorption at 484 mμ using 3200 M^{-1} cm^{-1} as the molar extinction coefficient (Joergensen, 1957). Reaction was stopped at desired times by adding 0.1-ml aliquots to 0.9 ml of 1 N HCl.

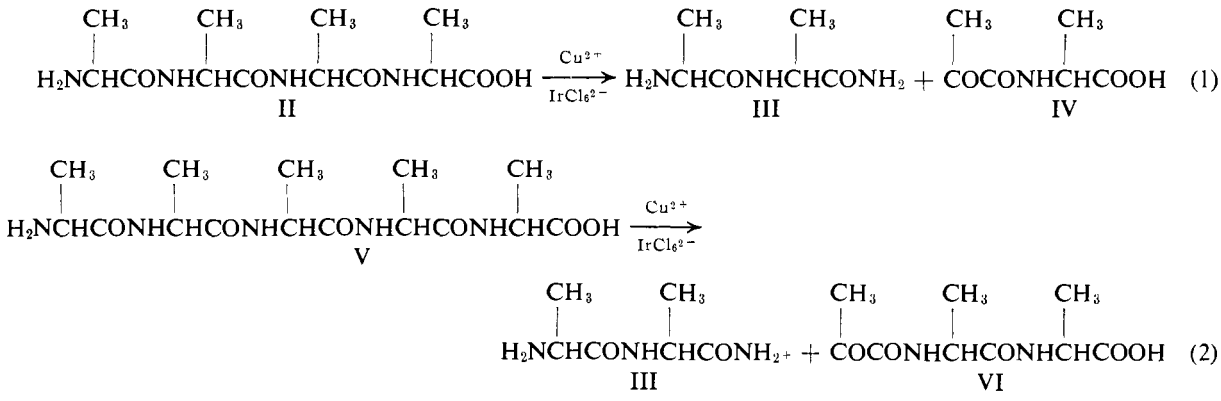
Preparation of Biuret-Cu^{III} Complex. Biuret (10 mmoles) and $CuSO_4 \cdot 5H_2O$ (5 mmoles) were mixed in water (30 ml) and the pH was raised to 7 by adding 2 N NaOH. Then $Na_2IrCl_6 \cdot 6H_2O$ (10 mmoles) was added to the resulting suspensions and the mixture was stirred for 3 hr. The brown precipitate formed was filtered, washed thoroughly with distilled water, and dried over H_2SO_4 *in vacuo* at 100°.

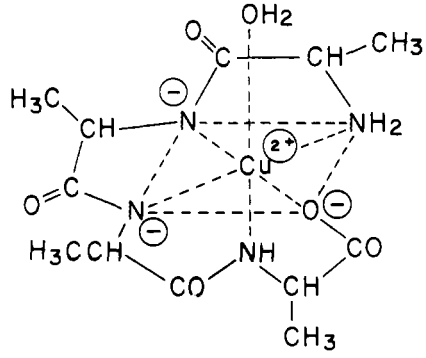
Anal. Calcd for $NaCu(C_2H_3N_3O_2)_2$ (289): Cu, 22.2; N 29.2. Found: Cu, 22.2; N, 28.8.

Copper (1 g-atom) in the biuret complex yielded, upon reaction with acidified potassium iodide solution, 2 equiv of I_2, thus establishing the trivalency of the copper within the compound. Alternatively, $NaCu^{III}$-$C_2H_3N_3O_2^{2-}$ was precipitated instantaneously upon mixing a solution of $Cu^{II}(C_2H_3N_3O_2^{2-})_2$ at pH 11.0 with 1 equiv of aqueous Na_2IrCl_6 at pH 5.0.

Results

At neutral pH values, tripeptides and larger oligopeptides form stable 1:1 copper complexes (Dobbie *et al.*, 1955), with the removal of three protons, one from the terminal ammonium group and one from each of the next two peptide nitrogens. The structure of the 1:1 complex of Cu^{II}–H·Ala_4·OH (I) is probably as shown below (*cf.* Kim and Martell, 1966). The tetra-





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and pentapeptides of alanine were chosen as the simplest models for studying the oxidation of long-chain peptides.

The Oxidation of Cu^{II}–H·Ala_4·OH and Cu^{II}–H·Ala_5·OH by Na_2IrCl_6. The reaction between Cu^{II}–H·Ala_4·OH (II) and Na_2IrCl_6 was studied at concentrations of about 10^{-3} M. When aqueous chloroiridate (2 moles) was added to the Cu^{II}–peptide solution (1 mole) at pH 6–8, instantaneous reduction of 1 mole of the oxidant occurred, as seen by the decrease in intensity of the characteristic absorption of the $IrCl_6^{2-}$ ion at 484 mμ to half its initial value. The remaining absorption then decreased with a half-time of about 7 min. In control experiments ($IrCl_6^{2-}$ plus peptide without Cu^{2+}) insignificant diminution of the chloroiridate absorption took place.

In order to identify the main products of the oxidation the reaction mixture was analyzed at time infinity (30 min). Copper was removed by the addition of Dowex A-1 chelating resin and aliquots of the mixture were subjected to electrophoresis at pH 6.5. Two products appeared on the electrophoreogram: one ninhydrin positive, identified as alanylalanine amide (H·Ala_2·NH_2 (III)), and the other giving a yellow spot with the DNPH reagent, identified as pyrovolyalanine (Pyr·Ala·OH (IV)). The reaction thus proceeds according to eq 1. Under the same conditions, Cu^{II}–H·Ala_5·OH (V) and $IrCl_6^{2-}$ gave H·Ala_2·NH_2 (III) and Pyr·Ala_2·OH (VI) (eq 2).

Identification of Products. H·ALA_2·NH_2. The ninhydrin-positive material had the same electrophoretic mobility toward the anode as authentic H·Ala_2·NH_2, namely 10.8 cm in 20 min at pH 6.5 and 61 cm in 4 hr at pH 1.4. After total acid hydrolysis (6 N HCl. 22 hr, 105°) it yielded alanine as the only amino acid. On enzymatic digestion for 24 hr with leucine aminopeptidase, the amide eluted from the electrophoreogram gave alanine and alanine amide as the sole products (half-time of the reaction was 1 hr). These products were identified by comparison with authentic markers (electrophoresis at pH 1.4 and 6.5). The completeness of the digestion showed that no racemization of the alanine residues occurred during the chloroiridate oxidation.

PYR·ALA·OH AND PYR·ALA_2·OH. The DNPH-positive materials formed from H·Ala_4·OH and H·Ala_5·OH were identified as Pyr·Ala·OH and Pyr·Ala_2·OH, respectively. They had the same electrophoretic mobilities as the authentic compounds, namely 10 and 8.5 cm, respectively (pH 6.5, 20 min), and the same R_F values on thin layer chromatography (0.75 and 0.70, respectively). Furthermore they had the appropriate pyruvic acid content as was shown by hydrolyzing the material under mild acid conditions (105°, 1 hr) in evacuated sealed tubes, and estimating the pyruvate using the lactic dehydrogenase–NADH system (Shifrin *et al.*, 1959).

When the oxidation reaction was carried out in tritium-enriched water (200 mc/ml), a scan of the electrophoreogram (pH 6.5) in a Vanguard strip counter showed that none of the oxidation products had incorporated tritium. This seems to exclude the formation of dehydroalanine as a precursor of the pyruvoyl group.

Stoichiometry of the Reaction under Different Conditions. The amounts of products formed at different peptide-to-oxidant ratios are shown in Figure 2. The stoichiometry of the reaction suggests the following equation:

$$2IrCl_6^{2-} + Cu^{II}\text{–H·}Ala_3\text{·OH} \longrightarrow 2IrCl_6^{3-} + Cu^{II}\text{–H·}Ala_2\text{·}NH_2 + \text{Pyr·Ala·OH} + 2H^+ \quad (3)$$

In the same manner, it was shown that the copper–pentaalanine complex undergoes a similar reaction:

$$2IrCl_6^{2-} + Cu^{II}\text{–H·}Ala_5\text{·OH} \longrightarrow 2IrCl_6^{3-} + Cu^{II}\text{–H·Ala·}NH_2 + \text{Pyr·}Ala_2\text{·OH} + 2H^+ \quad (4)$$

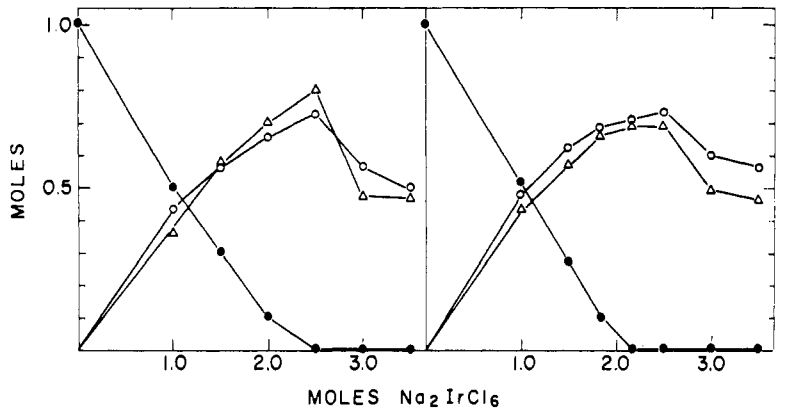


FIGURE 2: Stoichiometry of the Cu^{II}–H·Ala_4·OH + $IrCl_6^{2-}$ and Cu^{II}–H·Ala_5·OH + $IrCl_6^{2-}$ reactions. Left drawing: The reaction mixture contained Cu^{II}–H·Ala_4·OH (1 μmole/ml) sodium tetraborate buffer (pH 8.0, 20 μmoles/ml), and Na_2IrCl_6. Incubation time was 30 min at room temperature. (●–●–●) H·Ala_4·OH disappearing, (○–○–○) H·Ala_2·NH_2 production, and (△–△–△) Pyr·Ala·OH production. Right drawing: The reaction mixture contained Cu^{II}–H·Ala_5·OH (1 μmole/ml), sodium tetraborate buffer (pH 8.0, 20 μmoles/ml), and Na_2IrCl_6. Incubation time was 30 min at room temperature. (●–●–●) H·Ala_5·OH disappearing, (△–△–△) H·Ala_2·NH_2 production, and (○–○–○) Pyr·Ala_2·OH production.

TABLE I: Destruction of H·Ala_2·NH_2, Pyr·Gly_2·OH, and Pyruvic Acid by $IrCl_6^{2-}$.[a]

	H·Ala_2·NH_2 Remaining (μmoles)		Pyr·Gly_2·OH Remaining (μmoles)		Pyruvic Acid Remaining (μmoles)	
Na_2IrCl_6 (μmoles)	With Cu^{2+}	Without Cu^{2+}	With Cu^{2+}	Without Cu^{2+}	With Cu^{2+}	Without Cu^{2+}
0.50	0.90	0.97	1.00	1.00	0.60	0.70
0.75	0.60	0.83	0.82	1.00	0.40	0.45
1.00	0.50	0.82	0.61	1.00	0.20	0.25

[a] H·Ala_2·NH_2 (1.0 μmole), Pyr·Gly_2·OH (1.0 μmole), or pyruvic acid (1.0 μmole) in 1.0 ml of 0.02 M tetraborate buffer (pH 8.0) in the presence and absence of Cu^{2+} was incubated with increasing amounts of Na_2IrCl_6 as specified in the table. Incubation time was 30 min at room temperature.

The oxidation of H·Ala_3·OH in the presence of copper also yielded H·Ala_2·NH_2. In this case, however, the yields of free pyruvic acid were low since it is extensively destroyed by chloroiridate (Table I).

Side Reactions. From Figure 2 it can be seen that the yields of H·Ala_2·NH_2 and Pyr·Ala·OH pass a maximum value (about 80%) at about 2.3 moles of oxidant/mole of peptide. The drop in the yields after the maximum indicates destruction of the products by chloroiridate during the reaction. This side reaction was studied with each of the products separately (Table I). The amount of reaction observed can account for the diminished yields observed beyond the optimal oxidant:peptide ratio.

Optimal Reaction Conditions. OXIDANT:Cu^{II}–PEPTIDE RATIO. The highest yields are obtained at a ratio of 2.3 to 1.0 (Figure 2).

pH. The amount of products formed is independent of pH in the range 6.0–9.0. At higher pH values $IrCl_6^{2-}$ is hydrolyzed and nonspecific oxidations occur which do not depend on the presence of copper. At pH 5, the $IrCl_6^{2-}$ is not reduced.

TIME. The disappearance of the peptide and the appearance of products with time is given in Figure 3. It can be seen that the reaction is complete after 30 min.

pH CONTROL. In unbuffered systems the pH decreases during the reaction. The only buffer found to be suitable was borate. Phosphate causes precipitation of the copper and organic buffers are undesirable because of possible side reactions.

Degradation of H·Lys·Ala·Ala·OH and H·Val·Ala·Ala·Phe·OH. Chloroiridate reacted with the copper complexes of H·Lys·Ala_3·OH and H·Val·Ala_2·Phe·OH under the conditions described above, according to eq 5 and 6, respectively.

Electrophoresis at pH 6.5 revealed that the pyruvoyl compounds formed were identical with synthetic pyru-

$$Cu^{II}\text{-}H\cdot Lys\cdot Ala_3\cdot OH + 2IrCl_6^{2-} \longrightarrow Cu^{II}\text{-}H\cdot Lys\cdot Ala\cdot NH_2 + Pyr\cdot Ala\cdot OH \quad (5)$$

$$Cu^{II}\text{-}H\cdot Val\cdot Ala_2\cdot Phe\cdot OH + 2IrCl_6^{2-} \longrightarrow Cu^{II}\text{-}H\cdot Val\cdot Ala\cdot NH_2 + Pyr\cdot Phe\cdot OH \quad (6)$$

voylalanine and pyruvoylphenylalanine, respectively. Their identity was also confirmed by thin layer chromatography as described above. The amides formed were eluted from the pH 6.5 electrophoreogram and subjected to amino acid analysis. $H\cdot Lys\cdot Ala\cdot NH_2$ gave $Lys:Ala:NH_2 = 1.0:1.0:0.93$ and $H\cdot Val\cdot Ala\cdot NH_2$ gave $Val:Ala:NH_2 = 1.0:1.0:1.2$.

OXIDATION OF H·ALA·ALA·AMINOISOBUTYRYL·ALA·OH. Chloroiridate reacted with the 1:1 copper complex of $H\cdot Ala_2\cdot$aminoisobutyryl$\cdot Ala\cdot OH$ under the conditions described above. Although the $IrCl_6^{2-}$ was reduced and the tetrapeptide oxidized, neither $H\cdot Ala\cdot Ala\cdot NH_2$ nor a carbonyl-containing product could be found among the products.

Kinetics of the Oxidation of Cu^{II}–Peptides by $IrCl_6^{2-}$. Figure 3 shows the rate of disappearance of $IrCl_6^{2-}$ and of the peptide (tetraalanine), and the rate of production of pyruvoylalanine and alanyl·alanine amide. It is seen that while the consumption of the first mole of $IrCl_6^{2-}$ is instantaneous, the consumption of the second mole is first order with a rate constant of $k = 0.1$ min^{-1}. The disappearance of $H\cdot Ala_4\cdot OH$ and the appearance of both $Pyr\cdot Ala$ and $H\cdot Ala_2\cdot NH_2$ are also first order and have the same rate constant. The rate constant was found to be independent of the initial concentration of the Cu^{II}–peptide complex. These results suggest a mechanism by which one electron is removed from the copper complex by a very fast process, followed by a second slower step. It was demonstrated by measurement of the electron paramagnetic resonance in a flow cell that the signal of the bivalent copper disappears with a rate constant $k > 10^5$ M^{-1} sec^{-1} and reappears at a rate comparable with the rate of product formation. No signal due to an organic radical was observed as an intermediate.

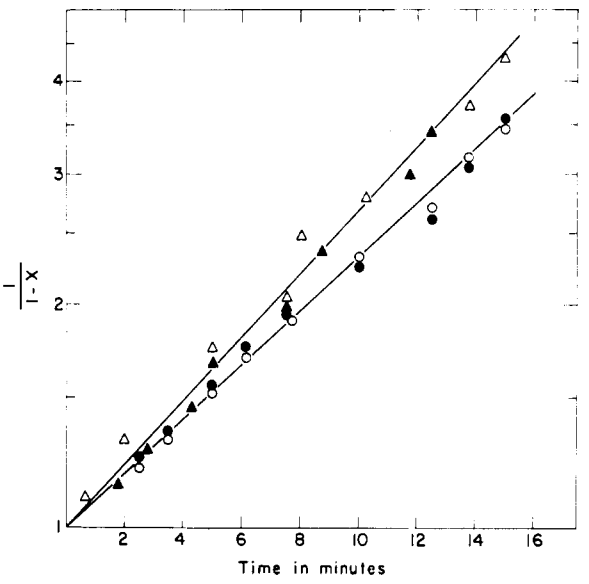


FIGURE 3: Time course of degradation of Cu^{II}–$H\cdot Ala_4\cdot OH$ by Na_2IrCl_6. The reaction mixture was of the same composition as described under Figure 1. The reaction was stopped at desired intervals by taking 0.1-ml samples and adding to 0.9 ml of 1 N HCl. X denotes the extent of reaction. (O–O–O) Peptide destruction, (●–●–●) $IrCl_6^{2-}$ (second mole) consumption, (△–△–△) $H\cdot Ala_2\cdot NH_2$ production, and (▲–▲–▲) $Pyr\cdot Ala\cdot OH$ production.

The possibility of a hydride-transfer mechanism for the reduction of the intermediate Cu^{3+} was examined by conducting kinetic runs with $H\cdot Ala\cdot Ala\cdot Ala^*\cdot Ala\cdot OH$, where Ala* stands for $HNCD(CH_3)CO$. It was assumed that cleavage of the CH bond at the α-carbon of Ala* would show a pronounced isotope effect. The deuterated tetrapeptide underwent oxidation at the same rate as the nondeuterated.

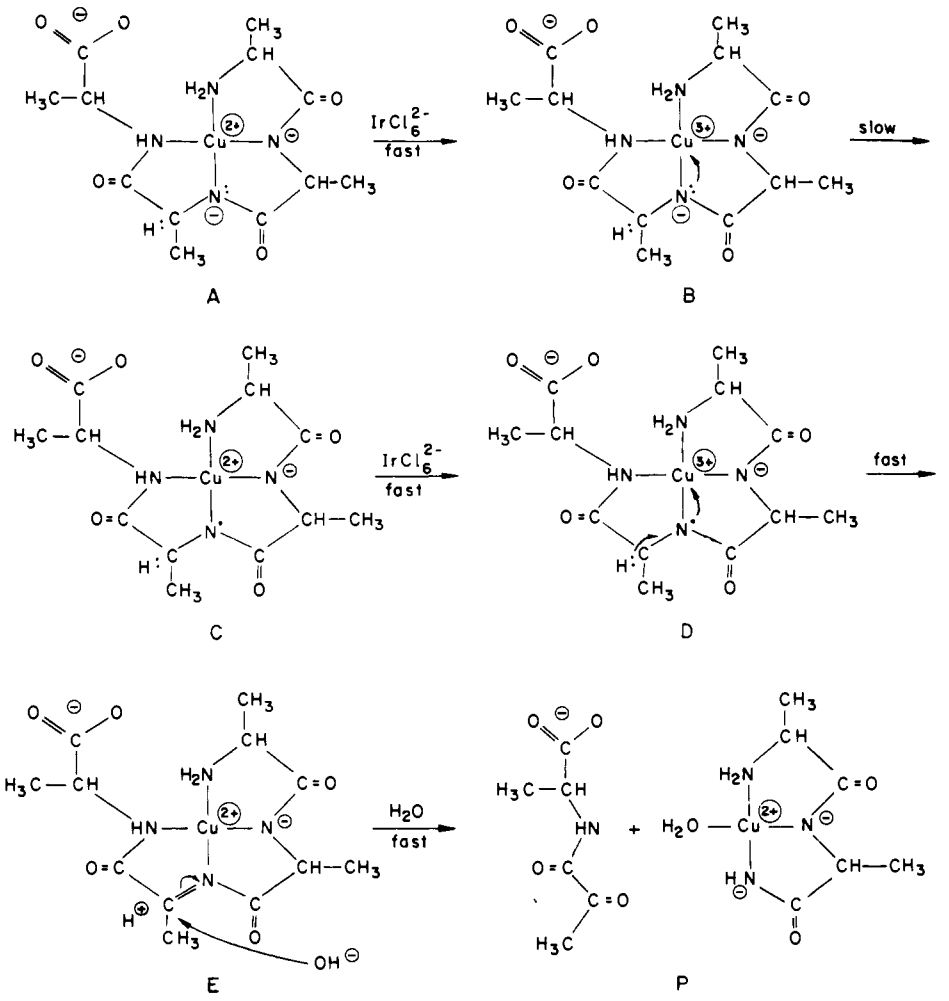
In order to check whether monovalent copper is present in any appreciable quantity during the oxidative degradation reaction, 2,2-biquinoline in glacial acetic acid (one volume, 0.05%) was added to aliquots of reaction mixtures containing either 1 or 2 moles of $IrCl_6^{2-}/Cu^{II}$–peptide. This was done immediately after addition of the chloroiridate as well as after 1–5 min. In no case was the characteristic violet color of the Cu(I)–biquinoline complex (Felsenfeld, 1960) observed.

The possibility that a milder oxidant might be able to replace the $IrCl_6^{2-}$ in the second, slow stage of the reaction was investigated. Reaction mixtures containing Cu^{II}–peptide and $IrCl_6^{2-}$ in equivalent quantities, and $Fe(CN)_6^{3-}$ up to 40-fold molar excess gave only 0.5 mole of products, the same as in the absence of $Fe(CN)_6^{2-}$. Similarly, molecular oxygen at a pressure of 100 atm (about 0.1 M, *i.e.*, 100-fold molar excess) at room temperature failed to increase the yield of products above 0.5 mole/mole of $IrCl_6^{2-}$.

Discussion

The mechanism for the oxidation described above must account for the following experimental results: (a) stoichiometry of two $IrCl_6^{2-}$ to one Cu^{II}–peptide, as summarized in eq 3; (b) rapid reduction of the first mole of $IrCl_6^{2-}$ (shown spectrophotometrically), accompanied by disappearance of Cu^{II} (demonstrated by electron paramagnetic resonance); (c) reduction of the second mole of $IrCl_6^{2-}$, disappearance of the peptide, and appearance of products including appearance of the electron paramagnetic resonance signal of the bound Cu^{II} by a relatively slow step. All these processes follow first-order kinetics (Figure 3), with identical rate constants; (d) reduction of 1 mole of Cu^{II}–peptide with 1 mole of $IrCl_6^{2-}$ yields 0.5 mole of products; (e) the absence of H:D isotope effect when the oxidized amino acid residue is deuteriosubstituted;

SCHEME I



(f) the formation of pyruvoyl derivatives without incorporation of hydrogen atoms from the solvent; (g) the absence of Cu(I) as an intermediate product; and (h) failure of the aminoisobutyryl peptide to cleave.

The mechanism suggested is summarized in Scheme I. The first mole of $IrCl_6^{2-}$ reacts rapidly with the copper–peptide complex A producing a transient trivalent copper–peptide complex B. In the second step, which is rate determining, an electron is transferred from the peptide chain to the bound Cu^{III}, yielding C. The Cu^{II} within C is again oxidized to Cu^{III} by a second molecule of $IrCl_6^{2-}$, leading to the formation of D. The latter undergoes intramolecular electron transfer to an intermediate E, which undergoes hydrolysis, resulting in complete degradation of the tetraalanine to P, namely, $H \cdot Ala_2 \cdot NH_2$ and $Pyr \cdot Ala \cdot OH$. Intermediate C may also be oxidized by B, as is the case when the ratio $IrCl_6^{2-}$:Cu^{II}–peptide is less than 2. The oxidation of C cannot be accomplished by milder oxidants such as oxygen or $Fe(CN)_6^{3-}$ which have lower redox potentials than $IrCl_6^{2-}$. This scheme is consistent with the experimental findings and can be expressed kinetically by the following set of reactions:

$$\underset{A}{Cu^{II}\text{–Pep}} + IrCl_6^{2-} \xrightarrow{k_1} \underset{B}{Cu^{III}\text{–Pep}} + IrCl_6^{2-} \quad (7)$$

$$\underset{B}{Cu^{III}\text{–Pep}} \xrightarrow{k_2} \underset{C}{Cu^{II}\text{–Pep*}} \quad (8)$$

$$\underset{C}{Cu^{II}\text{–Pep}} + IrCl_6^{2-} \xrightarrow{k_3} \underset{D}{Cu^{III}\text{–Pep}} + IrCl_6^{3-} \quad (9a)$$

$$\underset{C}{Cu^{II}\text{–Pep*}} + \underset{B}{Cu^{III}\text{–Pep}} \xrightarrow{k_3} \underset{D}{Cu^{III}\text{–Pep*}} + \underset{A}{Cu^{II}\text{–Pep}} \quad (9b)$$

$$\underset{D}{Cu^{III}\text{–Pep}} \xrightarrow{k_4} \underset{E}{Cu^{II}\text{–X}} \quad (10)$$

$$\underset{E}{Cu^{II}\text{-}X} \xrightarrow{k_5} \underset{P}{\text{products}} \qquad (11)$$

Assuming that k_2 is very much smaller than k_1, k_3', k_4, and k_5, one may apply the steady-state approximation (*i.e.*, d[C]/dt = 0, d[D]/dt = 0, and d[E]/dt = 0) and easily derive that (see Appendix)

$$\frac{d[B]}{dt} = \frac{d[P]}{dt} = k_2[B] \qquad (12)$$

When $[Ir]_0 \leqslant [A]_0$, eq 13, holds

$$\frac{d[Ir]}{dt} = k_1[A][Ir] \qquad (13)$$

However, although d[Ir]/dt is very large, it can easily be derived that the rate of product formation is still given by eq 12. When $[Ir]_0 = 2[A]_0$ then the first mole of $IrCl_6^{2-}$ disappears fast according to eq 13, and the second mole according to eq 14

$$\frac{d[Ir]}{dt} = \frac{d[P]}{dt} = k_2[B] \qquad (14)$$

i.e., at the rate at which the products are formed. This is in accord with experiment.

The assumption that k_1 and k_3 are very large is supported by the finding that the electron-transfer reaction involving the oxidation of tris(4,7-dimethyl-1,10-phenanthroline)–iron(II) by $IrCl_6^{2-}$ appears to be close to the diffusion-controlled limit (Halpern *et al.*, 1963). It might thus well be that the reactions of Cu^{II} complexes such as A and C with $IrCl_6^{2-}$ are also close to diffusion controlled. The absence of an electron paramagnetic resonance signal from the radicals C and D is probably due to their low steady-state concentrations as a result of the fact that k_3, k_3', and k_4 are large. k_5 must be much larger than k_2, otherwise the rate of product formation could not be equal to the rate of peptide disappearance and to the rate of disappearance of the second mole of $IrCl_6^{2-}$, as experimentally found.

The assumption that the intermediate E formed prior to hydrolysis has the structure suggested in Scheme I is supported by the following evidence. The reaction of $IrCl_6^{2-}$ with the copper complex of tetraalanine deuterated at the α-carbon of the third alanyl residue (counting from the N terminal) proceeds with the same rate as with the nondeuterated compound. This implies that cleavage of the C–H bond is not rate determining. The rate of the reaction seems thus to be determined by k_2, the rate of transfer of the first electron from the peptide nitrogen to Cu^{III}. Also, the formation of dehydroalanine as an intermediate can be excluded since, when the reaction was carried out in tritiated water, no incorporation of tritium into the methyl group of the pyruvoyl residue was observed. Addition of water to an intermediate dehydroalanyl double bond should have led to a tritiated product. The evidence (kinetic and electron paramagnetic resonance) for the existence of trivalent copper in our system is supported by the fact that we were able to prepare the stable complex of trivalent copper with biuret (Bour and Steggerda, 1966) by oxidation of Cu^{II} with $IrCl_6^{2-}$ at pH 7 in the presence of biuret, yielding a precipitate of $NaCu(C_2H_3N_3O_2^{2-})_2$.

$$Cu^{2+} + IrCl_6^{2-} + 2(C_2H_3N_3O_2^{2-})H_2 + 4OH^- \longrightarrow Cu^{3+}(C_2H_3N_3O_2^{2-})_2 + IrCl_6^{3-} + 4H_2O$$

Since no information on the steric conformation and the electronic structure of the Cu^{II}–peptide complex is available, no definite explanation for the specific reactivity of the α-hydrogen on the third amino acid residue can be offered. One might speculate however that because of the nonionized state of the third peptide nitrogen the five-membered ring which is cleaved is the least stable of the three.

The location of the binding sites of metal atoms in metalloproteins is of great interest, especially in enzymes where the metal atom participates in the catalytic process. Knowledge of the binding site may also be of considerable help in the interpretation of X-ray data obtained with native metalloproteins or metal derivatives of proteins. Limited information on the metal binding sites may be obtained from spectroscopic data, but this technique can only indicate the nature of the ligands involved and cannot establish their location within the molecule.

The present work explores the use of the bound metal to cause *local destruction* of the molecule (in our case by oxidative alteration of the ligands) after which the site of binding as well as the nature of the ligands can be determined by structural analysis of the fragments. The transition metals, which can attain transient unstable states of oxidation, are particularly suited for this purpose. Cases of ligand destruction by such a mechanism are known (Anbar, 1965; Levitzki and Anbar, 1967; Erlenmeyer *et al.*, 1965). Copper is especially useful in this respect, since (a) copper is present in a number of biologically active proteins, (b) other metals in biologically active proteins can be specifically replaced by copper, and (c) copper can be bound to a large number of proteins in stoichiometric amounts, probably at specific binding sites.

We chose the recently discovered reaction involving oxidation by chloroiridate with copper as catalyst (Anbar, 1965). As we have shown, the trivalent copper formed as the intermediate can indeed destroy at least one of its ligands. As a first step we used the 1:1 complexes of divalent copper with oligopeptides as model compounds. It was mentioned earlier that the copper atom is complexed by the terminal amino group and the two adjacent peptide nitrogens. The results obtained show that when this complex is oxidized by 2 moles of chloroiridate ($IrCl_6^{2-}$), the third amino acid residue from the N-terminal end is destroyed.

Preliminary experiments with a large number of

peptides (including an 8 peptide and a 20 peptide) showed similar oxidation of the third amino acid residue (A. Levitzki, M. Anbar, and A. Berger, to be published). It may be possible to utilize these findings for analytical purposes, since the dipeptide fragment originating from the N-terminal end can often be separated from the residual peptide.

Although the present model does not yet reflect the possibilities of locating copper atoms at specific binding sites, it seems that the approach discussed here (the local destruction of ligands) provides a means of attacking this problem. Work in this direction, using model peptides capable of additional interactions with copper, is now in progress.

Appendix

From eq 5 to 9, applying the steady-state approximation for [C], [D], and [E], one gets the rate of disappearance of B

$$-\frac{d[B]}{dt} = k_1[A][Ir] + k_2[B]\left(1 + \frac{[B]}{k_3[Ir] + k_3[B]}\right) \quad (15)$$

since

$$\frac{k_3[Ir]}{[B]} + k_3' \gg 1$$

eq 15 reduces to

$$-\frac{d[B]}{dt} = k_1[A][Ir] + k_2[B] \quad (16)$$

As the reaction of A with $IrCl_6^{2-}$ is close to diffusion controlled, the kinetic runs described in this paper result only from the *second* term and so eq 16 reduced to eq 12. Also, in the case when $[Ir]_0 = [A]_0$ then

$$k_3[Ir] \ll k_3'[B] \quad (17)$$

and the rate of disappearance of B is given again by eq 16.

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