References

Donovan, J. W. (1969), *J. Biol. Chem. 244*, 1961.
Edmondson, D. E., and Tollin, G. (1971a), *Biochemistry 10*, 113.
Edmondson, D. E., and Tollin, G. (1971b), *Biochemistry 10*, 124.
Edmondson, D. E., and Tollin, G. (1971c), *Biochemistry 10*, 133.
Eisinger, J. (1969a), *Biochemistry 8*, 3902.
Eisinger, J. (1969b), *Biochemistry 8*, 3908.
Eisinger, J. (1969c), *Photochem. Photobiol. 9*, 247.
Förster, Th. (1959), *Disc. Faraday Soc. 27*, 7.
Förster, Th. (1965), Modern Quantum Chemistry, Istanbul Lectures, New York, N. Y., Academic Press.
Herskovits, T. T., and Sorensen, Sr., M. (1968a), *Biochemistry 7*, 2523.
Herskovits, T. T., and Sorensen, Sr., M. (1968b), *Biochemistry 7*, 2533.
Hinkson, J. W. (1968), *Biochemistry 7*, 2666.
Hinkson, J. W., and Bulen, W. A. (1967), *J. Biol. Chem. 242*, 3345.
Longworth, J. W. (1968), *Photochem. Photobiol. 7*, 587.
MacKenzie, R. E., Föry, W., and McCormick, D. B. (1969), *Biochemistry 8*, 1839.
Massey, V., and Curti, B. (1966), *J. Biol. Chem. 241*, 3417.
Massey, V., and Palmer, G. (1966), *Biochemistry 5*, 3181.
McCormick, D. B. (1970), *Experientia 26*, 243.
Schecter, A. N., Chen, R. F., and Anfinsen, C. B. (1970), *Science 167*, 886.
Shethna, Y. I., Wilson, P. W., and Beinert, H. (1965), *Biochim. Biophys. Acta 113*, 225.
Teale, F. W. J. (1960), *Biochem. J. 76*, 381.
Teale, F. W. J., and Weber, G. (1957), *Biochem. J. 65*, 476.
Visser, J., and Veeger, C. (1970), *Biochim. Biophys. Acta 206*, 224.
Weber, G. (1961), *Nature (London) 190*, 27.
Weber, G., and Young, L. (1964), *J. Biol. Chem. 239*, 1424.
Weinryb, I., and Steiner, R. F. (1970), *Biochemistry 9*, 135.
Williams, J. W., Herskovits, T. T., Laskowski, Jr., M. (1965), *J. Biol. Chem. 240*, 3574.

# Specific Oxidation of Copper Binding Sites in Copper(II)–Oligopeptide Complexes*

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ABSTRACT: The specific oxidation of the copper(II) binding sites by chloroiridate in two peptide–copper complexes is described.

The octapeptide and the 20-peptide derived from the amino terminal of RNase were used to prepare the 1:1 copper complexes. In both cases the copper was found to bind to the $\alpha$-amino end of the molecule, using the chloroiridate oxidation technique. The possibility of using this or similar reactions for locating copper binding sites in proteins is discussed.

Recently it has been shown that $IrCl_6^{2-}$ oxidizes Cu(II)-tetrapeptide complexes *via* the formation of a Cu(III) intermediate (Levitzki *et al.*, 1967) the final step being the modification of two of the metal ligands. Thus it was of interest to investigate the interaction of $IrCl_6^{2-}$ with copper complexes of larger peptides in an attempt to find whether the chloroiridate oxidation can be used as a method for the location of copper in both synthetic copper–protein complexes and in natural occurring copper proteins.

In the present investigation the reaction of $IrCl_6^{2-}$ with the copper complexes of RNase fragments was undertaken.

## Experimental Section

*Materials.* The N-terminal octapeptide ($P_{1-8}$) and 20-peptide (S-peptide) were kindly donated by Mr. S. Levit from our department. The octapeptide was prepared by chymotryptic digestion of the 20-peptide obtained by Nagarse digestion of RNase (S. Levit and A. Berger, in preparation). DNP-amino acids were the product of Mann. $Na_2IrCl_6 \cdot H_2O$ was obtained from Alfa Inorganic Chemicals.

*Methods.* The electrophoresis at pH 6.5 and 1.4 was performed as described earlier (Levitzki *et al.*, 1967). The determination of $IrCl_6^{2-}$ and ketoacyl peptides, and total amino acid analyses were performed as described previously (Levitzki *et al.*, 1967).

*Dinitrophenylation.* The copper-free peptide solution was brought to 0.2 M $NaHCO_3$ (pH 8.0) and an excess of FDNB[1] was added. The reaction mixture was stirred overnight at room temperature. The excess FDNB was extracted with ether several times and then the aqueous solution was lyophilized; 1.0 ml of 6 N HCl was added to the lyophilized material in a hydrolysis tube which was vacuum sealed and incubated at 120° for 12 hr. After hydrolysis the HCl was removed over KOH *in vacuo*. The residue was dissolved in DMF and the mixture was analyzed for DNP-amino acids on thin-layer

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[1] Abbreviations used are: FDNB, 1-fluoro-2,4-dinitrobenzene; DNPH, 2,4-dinitrophenylhydrazine; DMF, dimethylformamide.

chromatography with 1-propanol–26% $NH_4OH$ (7:3, v/v) or $CHCl_3$–$CH_3OH$–$CH_3CO_2H$ (95:1:1, v/v) as solvent systems.

## Results

*Oxidation of the Octapeptide.* $P_{1-8}$ and Cu(II) in 1:1 and 1:2 molar ratios were incubated at pH 8 with increasing amounts of $IrCl_6^{2-}$ as described in Table I. It can be seen that the ratios $IrCl_6^{2-}$:Cu(II):$P_{1-8}$ = 2:1:1 are sufficient to yield the maximal oxidation of threonine, the other amino acid residues being untouched even in the presence of higher amounts of copper and chloroiridate. As in the case of the oxidation of Cu(II)–tetrapeptide complexes (Levitzki *et al.*, 1967) the reaction is complete within 40 min. From Table I it is apparent that only the 1:1 Cu(II)–$P_{1-8}$ complex is formed and the additional amounts of copper do not bind to the peptide.

Upon subjecting the reaction mixture to electrophoresis at pH 1.4, two ninhydrin-positive products were found, one being identical in the electrophoretic mobility with synthetic H-Lys-Glu-OH (25 cm in 2 hr). Also, the electrophoretic mobility of the dipeptide fragment extracted from the pH 1.4 electrophoreogram was found to be identical with synthetic H-Lys-Glu-OH when subjected to electrophoresis at pH 6.5. Furthermore, upon total amino acid analysis only lysine and glutamic acid were found, in ratios Lys:Glu = 1.00:0.98. The slower moving spot (18 cm at pH 1.4, 2 hr) was found to have the composition: Ala:Lys:Phe = 3.00:1.10:1.02. In both products threonine was absent. Thus the oxidation of Cu(II)–$P_{1-8}$ can be written as

$$\text{H-Lys-Glu-Thr-Ala}_3\text{-Lys-Phe} \xrightarrow[\text{Cu(II)}]{2IrCl_6^{2-}} \text{H-Lys-Glu-OH} + \text{X-Ala}_3\text{-Lys-Phe-OH}$$

the threonine being the only amino acid oxidized. In order to investigate the nature of X and to discover whether the pentapeptide fragment is really blocked, the latter was extracted from the pH 1.4 electrophoreogram and dinitrophenylated with FDNP at pH 8.0 (0.2 M $NaHCO_3$), as described under Materials and Methods. The only DNP derivative identified after total acid hydrolysis (105°, 22 hr) was found to be $\epsilon$-DNP-Lys. Thus it seems that the $\alpha$-$NH_2$ in the pentapeptide fragment is blocked by the oxidation product of threonine. Upon reaction of the blocked pentapeptide with DNPH, 0.55 mole of carbonyl/mole of fragment was found. In conclusion, Cu(II)–$P_{1-8}$ is oxidized by $IrCl_6^{2-}$ as given by equation 1 to yield a dipeptide and a blocked pentapeptide, the third amino acid from the N terminal being oxidized. It seems, therefore, that the pattern of $IrCl_6^{2-}$ oxidation of Cu(II)–tetrapeptide complexes (Levitzki *et al.*, 1967), is preserved and does not depend on the nature and length of the Cu(II)–oligopeptide complex. In order to find whether this last statement is valid for 1:1 Cu(II) complexes with high molecular weight peptides, the oxidation of the copper complex of S-peptide was studied.

*Oxidation of S-peptide.* The oxidation of 1 mole of 1:1 Cu(II)–S-peptide complex by 2 moles of $IrCl_6^{2-}$ was studied under identical conditions described for the oxidation of $P_{1-8}$. Electrophoresis at pH 6.5 of the reaction mixture after the removal of $Cu^{2+}$ revealed the formation of two reaction products. One is identical in electrophoretic mobility with H-Lys-Glu-OH (5.0 cm, 25 min, pH 6.5), the slower one (2.5 cm, 25 min, pH 6.5) shows, in addition to the ninhydrin reaction the Pauli test (Cramer, 1955) typical of the histidyl residue. Upon extraction and total amino acid analysis the two peptide fragments showed the following composition. Fast-moving peptide had Lys:Glu = 1.02:1.00 and the slow-moving peptide had the composition: Lys, 1.02; His, 1.01; Arg, 1.00; Thr, 0.97; Ser, 2.95; Glu, 3.01; Ala, 5.00; Met, 0.89; Phe, 0.99. Thus one can write the equation for the S-peptide oxidation

$$\text{H-Lys-Glu-Thr-Ala}_3\text{-Lys....} \longrightarrow \text{H-Lys-Glu-OH} + \text{XAla}_3\text{-Lys....}$$

It is seen also that slight nonspecific oxidation of the methionine residue occurs. In a separate experiment it was possible to show that slow oxidation of the methionine residue of the S-peptide occurs in the absence of $Cu^{2+}$. In conclusion it is seen that in the 1:1 Cu(II)–S-peptide complex the third threonine residue from the N-terminal end is oxidized as in the case of the Cu(II)–$P_{1-8}$ oxidation. It seems also that at low ratios of $IrCl_6^{2-}$ to copper complex, the Cu(II) chromophore is oxidized much faster than the methionine residue thus yielding low nonspecific oxidation of the methionine residue.

TABLE I: Oxidation of Cu(II)-$P_{1-8}$ by $IrCl_6^{2-}$.[a]

| $IrCl_6^{2-}/P_{1-8}$: | 1 | 2 | 4 | 4 | 4 | |
|---|---|---|---|---|---|---|
| Cu(II)/$P_{1-8}$: | 1 | 1 | 1 | 2 | 0 | Native Peptide |
| Lys | 2.20 | 2.20 | 2.18 | 2.20 | 2.18 | 2.18 |
| Thr | 0.46 | 0.00 | 0.00 | 0.00 | 0.21 | 0.98 |
| Glu | 0.99 | 0.98 | 0.99 | 0.98 | 0.99 | 0.99 |
| Ala | 3.00 | 3.00 | 3.00 | 3.00 | 3.00 | 3.00 |
| Phe | 1.03 | 1.02 | 1.07 | 1.06 | 1.03 | 1.04 |

[a] All the reaction mixtures contained 0.25 micromoles $P_{1-8}$, $CuSO_4$ in amounts given in the table, 10 micromoles of sodium tetraborate buffer (pH 8.0), and $Na_2IrCl_6$, in a final volume of 0.35 ml. After 40 min of incubation at room temperature few particles of Dowex A-1 were added to the solution for the removal of $Cu^{2+}$. The residual amounts of $IrCl_6^{2-}$ were determined spectrophotometrically and reduced by equivalent amounts of $Na_2SO_3$. The samples were lyophilized and then dissolved in 0.01N HCl. Samples were taken for total amino acid analysis. The results given in the table are calculated with respect to alanine. Identical results are obtained when glutamic acid is assumed to be quantitatively recovered.

## Discussion

In a previous publication (Levitzki *et al.*, 1967) it was shown that $Cu^{2+}$ complexes of model peptides, in which the metal atom is bound to the $\alpha$-nitrogen atoms of the four N-terminal amino acid residues of a chain can be oxidized by $IrCl_6^{2-}$. The reaction leads to the destruction of one of the ligands, accompanied by cleavage of the peptide chain.

In the present paper we show that the copper binding site in an 8 peptide and in a 20 peptide can be specifically modified by oxidation by $IrCl_6^{2-}$. In both the Cu(II)–$P_{1-8}$ and Cu(II)–S-peptide complexes the $IrCl_6^{2-}$ yields fragmentation at the amino terminal end and oxidation of the third amino acid residue (threonine) from the $\alpha$-amino end. Thus in both cases as in the case of Cu(II)–tetrapeptides (Levitzki *et al.*, 1967) the

Cu(II) binding site is composed of the four N-terminal amino acids.

The binding of Cu(II) to the $\alpha$-$NH_2$ end of the S-peptide and not to the histidyl residue (residue 12) is interesting in view of the fact that histidyl residues in proteins are known to have high affinity for $Cu^{2+}$ (Breslow, 1964; Breslow and Girotti, 1966), and are often assumed to be the ligands in both synthetic and natural copper–protein complexes. It is interesting to note that recently it has been found (Breslow and Girotti, 1970) that the dominant Cu(II) binding site on RNase at pH 7 is the $\alpha$-$NH_2$ terminus, in support of our findings. In the case of the bovine serum albumin–Cu(II) complex where Cu(II) was found to be bound to the $\alpha$-$NH_2$ end (Peters, 1960; Peters and Blumenstock, 1967; Shearer *et al.*, 1967), the histidyl residue is part of the copper binding site since it occupies position 3 from the N terminal.

It seems that the $IrCl_6^{2-}$ method can be applied to locate Cu(II) binding sites in proteins. Thus it may be possible to apply the $IrCl_6^{2-}$ to natural copper-containing proteins and thereby locate the copper binding sites, a task not accomplished by the available physical techniques. This type of "affinity labeling" may also help X-ray crystallographers to locate the amino acid residues involved in Cu(II) binding when the crystallography of copper proteins is undertaken. It is however not yet clear whether the $IrCl_6^{2-}$ method will pick up binding sites not involving peptide nitrogens as the immediate ligands of the copper atom.

References

Bradshaw, R. A., Shearer, W. T., and Gurd, F. R. N. (1968), *J. Biol. Chem. 243*, 3817.

Breslow, E. (1964), *J. Biol. Chem. 239*, 3252.

Breslow, E., and Girotti, A. W. (1966), *J. Biol. Chem. 241*, 5651.

Cramer, F. (1955), Paper Chromatography, New York, N. Y., Macmillan and Co., p 80.

Levitzki, A., Anbar, M., and Berger, A. (1967), *Biochemistry 6*, 3757.

Peters, T., Jr. (1960), *Biochim. Biophys. Acta 39*, 546.

Peters, T., Jr., and Blumenstock, F. A. (1967), *J. Biol. Chem. 242*, 1574.

Shearer, W. T., Bradshaw, R. A., and Gurd, F. R. N. (1967), *J. Biol. Chem. 242*, 5451.

# Estrogen Biosynthesis. Stereospecific Distribution of Tritium in Testosterone-1$\alpha$,2$\alpha$-$t_2$*

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ABSTRACT: The stereospecific distribution of tritium in testosterone-1$\alpha$,2$\alpha$-$t_2$ produced by tris(triphenylphosphine)rhodium chloride catalytic reduction of 17$\beta$-hydroxy-1,4-androstadien-3-one with tritium was determined to be 43% at C-1$\alpha$, 7% at C-1$\beta$, 43% at C-2$\alpha$, and 6% at C-2$\beta$. The chemical determination was made by mild and drastic alkali treatment; chloranil followed by 2,3-dichloro-5,6-dicyanobenzoquinone dehydrogenation; and dienone–phenol rearrangement. Biochemical aromatization with human placental microsomes gave 17$\beta$-estradiol retaining 87% of the original tritium. Bromination of the 17$\beta$-estradiol afforded 2,4-dibromo-17$\beta$-estradiol with retention of 44% of the original tritium. The results showing removal of the 1$\beta$-equatorial and the 2$\beta$-axial hydrogens are compatible with previous work carried out using human placental preparations, but appear not to agree with work done using ovarian tissue. The advantages of using testosterone-1$\alpha$,2$\alpha$-$t_2$ in place of testosterone-1$\beta$,2$\beta$-$t_2$ for metabolic studies are discussed.

The interpretation of the results of investigations into the metabolism of testosterone[1] labeled with tritium is dependent upon the stereochemical distribution of the tritium in the steroid. The testosterone-1,2-$t_2$ previously used has been produced by palladium–charcoal catalytic reduction of 17$\beta$-hydroxy-1,4-androstadien-3-one with tritium (Osinski and Vanderhaeghe, 1960). The tritium label has been reported to be distributed 40–60% at C-1 and 60–40% at C-2. Of the tritium at C-1, 75–83% is $\beta$ oriented, but of the tritium at C-2, only 58% is $\beta$ (Brodie *et al.*, 1962, 1969a; Brodie, 1967). Placental aromatization of androgens removes the 1$\beta$ hydrogen (Brodie *et al.*, 1968; Townsley and Brodie, 1968; Morato *et al.*, 1962) and the 2$\beta$ hydrogen (Fishman *et al.*, 1969; Fishman and Guzik, 1969; Brodie *et al.*, 1969b). A loss of 82% of tritium of testosterone-1$\beta$,2$\beta$-$t_2$ was reported in this sequence (Fishman *et al.*, 1969) whereas previously reported data (Brodie *et al.*, 1969a) indicated that 69% of the tritium should have been lost. Therefore, it appears that there is an inconsistency of the distribution of tritium among testosterone-1$\beta$,2$\beta$-$t_2$ preparations. Reports that catalytic hydrogenation with tris(triphenylphosphine)rhodium chloride is a cis process (Biellmann and Jung, 1968; Osborn *et al.*, 1966; Jardine *et al.*, 1967) occurring at the $\alpha$ face of 1,4-androstadiene-3,17-dione (Djerassi and Gutzeviller, 1966) sug-

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[1] Abbreviations used are: testosterone, 17$\beta$-hydroxy-4-androsten-3-one; $E_2$ (17$\beta$-estradiol), 1,3,5(10)-estratriene-3,17$\beta$-diol; chloranil, 2,3,5,6-tetrachlorobenzoquinone; DDQ, 2,3-dichloro-5,6-dicyanobenzoquinone; diglyme, bis(2-methoxyethyl)ether.