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Amino acid- and pterin-metal chemistry as an approach to biological functions

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Abstract: The aromatic rings of phenylalanine, tyrosine, and tryptophan (Trp) have been shown to be involved in aromatic ring stacking with other aromatic rings such as 2,2'-bipyridine (bpy) in ternary metal complexes as prototypes of the enzyme-metal-substrate intermediate in metalloenzyme catalysis. Stability enhancement primarily due to the intramolecular stacking in ternary Cu(II) complexes was concluded from the equilibrium constants for hypothetical equilibria involving formation of ternary complexes with stacked rings. X-ray crystal structure analysis of the ternary Cu(II) complexes revealed the stacking interaction in the solid state, supporting the conclusion from the solution studies. Theoretical calculation on $[Cu(Trp)(bpy)(H_2O)]^+$ afforded the fully optimized structure that is very similar to the one revealed by X-ray crystal structure analysis. The structures of the metal complexes with pterin derivatives and the redox reactions with tetrahydropterins have been revealed as a step toward understanding the mechanism of the hydroxylation of phenylalanine by phenylalanine hydroxylase in the presence of a pterin cofactor which is regarded as a functionalization of aromatic amino acids.

INTRODUCTION

Amino acid side chains of proteins and physiologically active peptides play essential roles in catalysis, molecular recognition, information transfer, and other biological functions. They are involved in binding with target molecules and achieve high efficiency and specificity by a combination of interacting groups (1). The active site of a metalloenzyme and the intermediate enzyme-substrate complex may be mimicked by binary and ternary metal complexes of low molecular weight, respectively, and are expected to give detailed information regarding the molecular arrangement and reaction mechanisms. We have been studying noncovalent ligand-ligand interactions in ternary metal complexes with amino acids, dipeptides, etc. and established hydrogen bonds and aromatic ring stacking interactions. Thus, electrostatic interactions or hydrogen bonds in Cu(II) complexes involving an acidic and a basic amino acid, such as Cu(L-Arg)(EDMA) (Arg = arginine; EDMA = ethylenediamine-N-monoacetate) (2), and aromatic ring stacking interactions as revealed for Cu(L-Trp)(bpy) (Trp = tryptophan; bpy = 2,2'-bipyridine) (3) have been characterized by potentiometric, spectroscopic, and crystallographic methods. The properties of the side groups of aromatic amino acids, phenylalanine (Phe), tyrosine (Tyr), and Trp in metal complexes are particularly interesting, because they can be involved in interactions with the central metal ion as well as other aromatic rings. In addition, these amino acids are important starting materials for the biosynthesis of neurotransmitters called catecholamines and serotonin, the initial step for which is the hydroxylation of the aromatic amino acids by hydroxylases such as phenylalanine hydroxylase (PAH) in the presence of iron or copper and a reduced pterin cofactor 5,6,7,8-tetrahydrobiopterin (4). This hydroxylation reaction may be regarded as a functionalization of aromatic amino acids. In spite of this, the mechanism of activation of dioxygen by the hydroxylases with the aid of the pterin cofactor is so far unknown. In view of the importance of pterin derivatives in various enzymatic reactions and lack of knowledge about metal-pterin interactions, we are investigating metal-pterin complex formation and the redox reactions between metal ions and reduced pterin derivatives (5). Receptor binding by weak interactions, side chain conformations, and site-specific hydroxylation reactions by PAH and a reduced pterin are closely interrelated in terms of functions of aromatic amino acids, and the studies on chemical models may give clues to clarifying them.

AROMATIC RING STACKING INVOLVING AMINO ACID SIDE CHAINS

Various types of noncovalent interactions involving amino acid side chains are possible around the central metal ion: (i) hydrophobic interactions between aliphatic nonpolar groups; (ii) electrostatic interactions between oppositely charged groups; (iii) hydrogen bonds between polar or charged groups; (iv) stacking

between aromatic rings; (v) interactions between an aromatic ring and the central metal ion. Since transition metal ions serve as a source of information arising from the structure and electronic transitions, these interactions are reflected on spectroscopic and thermodynamic properties of the complexes, and determination of the structure in the solid state provides structural details and evidence supporting the interactions in solution. We studied electrostatic interactions or hydrogen bonds in ternary metal complexes of charged amino acids M(A)(B), where M, A, and B refer to metal ion, acidic amino acid, and basic amino acid, respectively (2). Our recent studies are aimed at elucidating stacking interactions involving the aromatic side groups of amino acids or dipeptides (AA), especially the Tyr phenol group which has both hydrophobic and hydrophilic moieties, and coordinated diamines or diimines (DA) such as 2,2'-bipyridine (bpy) and 1,10-phenanthroline (phen) (3). Tyr constitutes an essential part of enkephalin and other opioid peptides and is concluded to be the coordinating group and source of the radical in the reaction of galactose oxidase (6).

Stabilization of mixed ligand complexes due to ligand-ligand interactions is evaluated by the hypothetical equilibrium as shown for the system stabilized by stacking (Eq. 1; charges are omitted for clarity) (3,7):

$$Cu(AA)(DA') + Cu(AA')(DA) \overset{K}{\rightleftharpoons} Cu(AA)(DA) + Cu(AA')(DA') \quad (1)$$

where AA and DA denote ligands with aromatic groups and AA' and DA' denote ligands similar to AA and DA, respectively, but without aromatic groups. Intramolecular ligand-ligand interactions are possible only in Cu(AA)(DA), so that the equilibrium constant K serves as a measure of stabilization essentially due to the interactions. From the overall stability constants of complex species, $\beta_{Cu(AA)(DA)}$ etc., the log K value can be calculated according to Eq. 2:

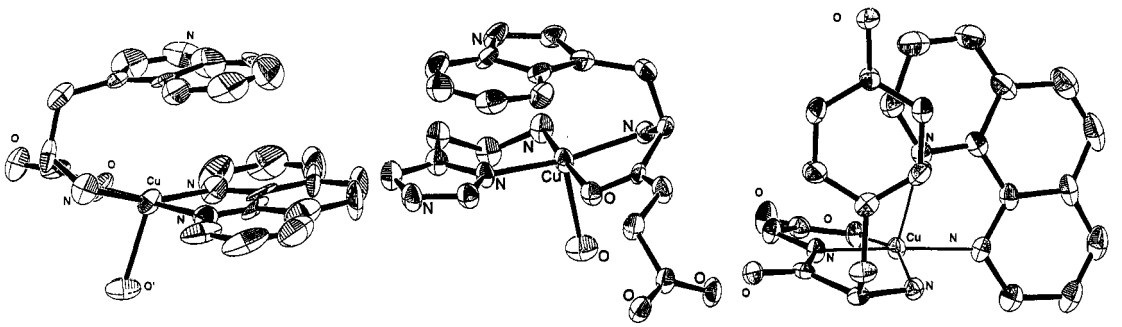
$$\log K = \log \beta_{Cu(AA)(DA)} + \log \beta_{Cu(AA')(DA')} - \log \beta_{Cu(AA)(DA')} - \log \beta_{Cu(AA')(DA)} \quad (2)$$

The log K values determined for AA = aromatic amino acids or peptides and DA = bpy, phen, or histamine (hista) at 25 °C and I = 0.1 mol dm^{-3} (KNO_3) are summarized in Table 1, where the values for AA = amino acids were calculated relative to Cu(L-Ala)(en) and those for AA = dipeptides to Cu(Gly·Gly)(en) (Gly·Gly = glycylglycine; en = ethylenediamine) without the peptide group deprotonation or Cu(Gly·Gly(H_{-1}))(en) with deprotonation (3a,7,8). Table 1 shows that the complexes with aromatic rings on both AA and DA are stabilized, the log K values being in the order of the size of the ring. Charges on the ring substituent destabilize the complex as seen from the values for deprotonated L-tyrosinate (L-$TyrO^-$). It is interesting to note in this connection that Cu(I_2TyrO^-)(DA) with a negative charge on the diiodophenol ring (I_2TyrO^- = 3,5-diiodotyrosinate (I_2Tyr) with the ionized phenol OH group) are less stable than Cu(L-I_2Tyr)(DA) but as stable as Cu(L-Trp)(DA) (DA = bpy or phen), indicating that stacking takes place in Cu(I_2TyrO^-)(DA). The stacking interaction concluded from the stability constants has been confirmed by the ^1H NMR spectra of Pd(dipeptide)(DA) complexes in D_2O (3b) and supported by crystal structure analysis of a number of ternary Cu(II) and Pd(II) complexes involving stacking between AA and DA.

TABLE 1. Stabilization of Ternary Cu(II) Complexes Due to Aromatic Ring Stacking.

AA	log K (25 °C; I = 0.1 mol dm^{-3} (KNO_3)) DA		
	bpy	phen	hista
L-Phe	0.60	0.64	0.26
L-Tyr	0.90	1.05	0.51
L-TyrO[a]	0.25		0.11
L-Ptyr[b]	-0.14	-0.02	-0.15
L-I_2Tyr	1.82	2.12	
L-I_2TyrO[a]	1.18	1.37	
L-Trp	1.19	1.39	0.60
L-Tyr·Gly	1.35	1.46	
L-Tyr·Gly(H_{-1})[c]	0.92	0.74	
L-Trp·Gly			1.31

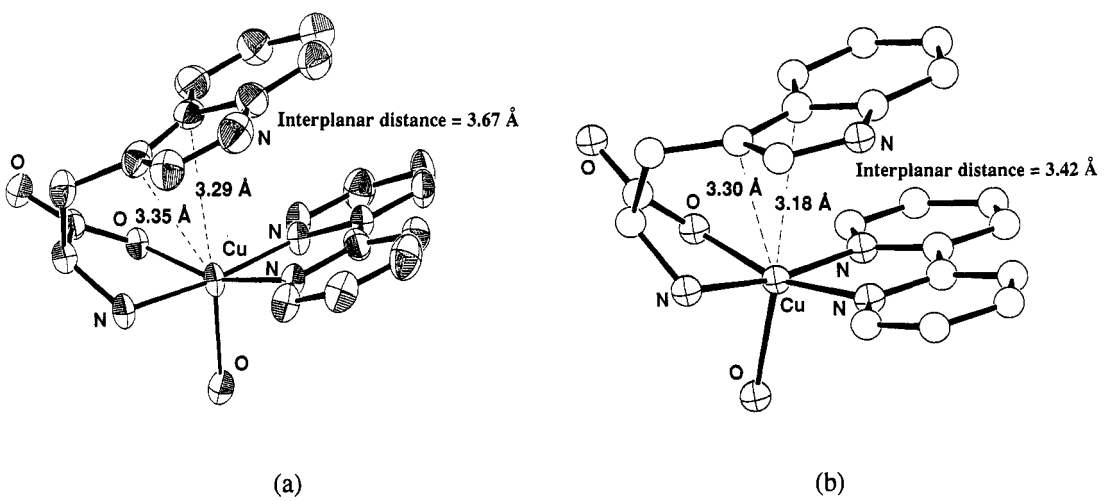
[a]The phenol OH group is deprotonated. [b]The fully deprotonated phosphoester of Tyr. [c]The peptide group is deprotonated.



(a) (b) (c)

Fig. 1. Molecular structures of $[Cu(L\text{-}Trp)(phen)]^+$ (a), $[Cu(L\text{-}Trp\cdot Gly)(hista)]^+$ (b), and [Cu(L-Tyr·Gly(H_{-1}))(phen)] (c).

Figure 1 shows the molecular structures of Cu(L-Trp)(phen) (9), Cu(L-Trp·Gly)(hista) (8), and Cu(L-Tyr·Gly(H_{-1}))(phen) (7), all of which have large positive log K values (Table 1). The stacking between the side chain of AA and coordinated rings of DA typically shown in Fig. 1(a) occurs with the average interplanar distance of 3.5-3.7 Å. In addition to the pyridine rings of bpy and phen, the imidazole ring of hista is also involved in stacking as in (b). It deviates from the Cu(II) coordination plane probably due to stacking in Cu(L-Trp·Gly)(hista) as well as in Cu(L-Phe or L-Tyr)(hista) (10,11). Aromatic rings of peptide side chains can be stacked in two ways as seen from (b) and (c), where the dipeptides are bidentate and terdentate, respectively, and stacking similar to (a) are possible with bidentate coordination. With effective terdentate coordination stacking of the Tyr·Gly side group in (c) occurs with phen coordinated perpendicularly, although stabilization due to stacking as viewed from log K (0.92 for DA = bpy) is less than that for bidentate coordination without deprotonation (1.35). Stacking over the coordination plane necessarily places the aromatic ring close to the central Cu(II) ion with the distance of 3.2-3.5 Å, which is shorter than the sum of the van der Waals radii and suggests the presence of bonding interactions between Cu(II) and the aromatic ring. The fact that the phenol group of Tyr in the binary Cu(II) complex, Cu(L-Tyr)$_2$ (12), and the aromatic side groups in some Cu(II) complexes are bent over the coordination plane supports such bonding interactions. Because of the weakness of the interactions, however, it has been difficult to present direct evidence for their existence by experimental methods. In order to understand the



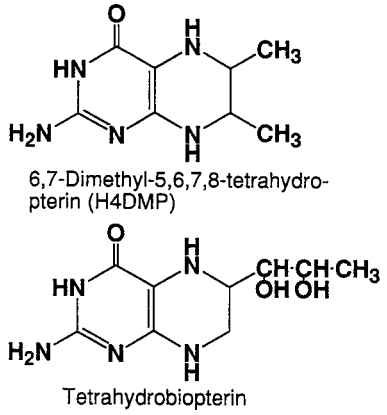
(a) (b)

Fig. 2. Molecular structure of $[Cu(Trp)(bpy)]^+$ (a) and fully optimized structure of $[Cu(Trp)(bpy)(H_2O)]^+$ (b) where the axially coordinated Trp carboxylate group is replaced by a water molecule.

stacking interactions in ternary complexes of the type Cu(AA)(DA) we performed a theoretical calculation on $[Cu(L\text{-}Trp)(bpy)(H_2O)]^+$ starting from the experimental structure (Fig. 2(a)) by the *ab initio* density functional method with the use of program DGauss (13). The fully optimized structure attained by this procedure involves the intramolecular stacking between the aromatic rings of L-Trp and bpy with the average distance of 3.42 Å, which is close to the value of 3.67 Å obtained by X-ray analysis (Fig. 2(b)) (14). The small difference may be interpreted as due to intermolecular interactions in the solid state and in the isolated state. Examination of the molecular orbitals revealed the formation of bonding orbitals between the six-membered ring of the indole nucleus and the pyridine ring of coordinated bpy and, in addition, demonstrated the bonding orbitals between Cu(II) and the five-membered ring of indole, which is to our knowledge the first computational evidence for the molecular orbital formation between stacked rings and between a metal ion and an aromatic ring.

COPPER-PTERIN COMPLEXES

Tyr is synthesized by hydroxylation of Phe by PAH in the presence of biopterin in the tetrahydro form. This reaction is the first step of the synthetic route to the neurotransmitters dopamine, norepinephrine, etc., in which tyrosine hydroxylase, dopamine β-hydroxylase, and tryptophan hydroxylase are also involved. The hydroxylases from mammals require nonheme iron and biopterin for activity, and PAH from *Chromobacterium violaceum* is reported to involve copper in place of iron (4), although there is some conflicting results on the role of copper (15). The aromatic ring of the aromatic amino acids is hydroxylated by activated O_2 in the presence of tetrahydrobiopterin, but neither the iron site structures nor the reaction mechanisms are established yet. Electron spin-echo envelope modulation and X-ray absorption spectra indicated that the copper site of Cu-containing PAH has two coordinated histidine imidazole groups (16,17), and the tetrahydro form of 6,7-dimethylpterin (H_4DMP) was found to react with the bound Cu(II) by ESR studies (18). Since tetrahydrobiopterin is required as a cofactor and is oxidized to quinonoid dihydropterin which is then reduced by NADH to the tetrahydro form in the enzymatic reaction, information



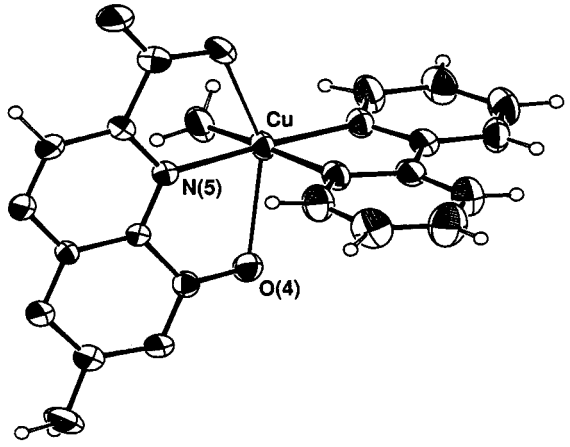


Fig. 3. Structure of $[Cu(bpy)(PC)(H_2O)]$ with a replaceable water molecule in the coordination plane (19).

on the modes of complex formation and redox reactions with the iron or copper ion are vital for understanding the mechanism of the hydroxylation by PAH. Previous solution and synthetic studies on the metal-binding properties of oxidized pterin (= 2-amino-4-hydroxypteridine) derivatives and lumazine indicated that the pterin ring coordinates to Cu(II) through its 4-oxo oxygen (O(4)) and 5-nitrogen (N(5)) to form a five-membered chelate ring in a manner similar to 8-hydroxyquinoline. We found that folate reacts with $Cu(bpy)^{2+}$ to form $[Cu(bpy)(PC)(H_2O)]$ (PC = pterin-6-carboxylate), which has a unique structure with PC bound through N(5) in the equatorial and O(4) and 6-carboxylate at the axial positions (Fig. 3) (19). The Cu(II) complex of 2-(N-dimethyl)-6,7-dimethylpterin (DMDMP), $[Cu(DMDMP)_2(NO_3)_2]$ has the structure with two pterin rings coordinated in the equatorial positions through O(4) and N(5) in the *trans* form (20). The Fe(II) complex of 2-(N-dimethyl)pterin (NDMP) was found to have the same structure. Cu(II) and Fe(III) can undergo redox reactions with a tetrahydropterin such as H_4DMP by a one-electron process. With these metal ions, we may expect formation of Cu(I) and Fe(II) and the trihydropterin radical $H_3DMP^{\bullet}$, but there has not been reported any radical formation during the enzymatic turnover. The aim of

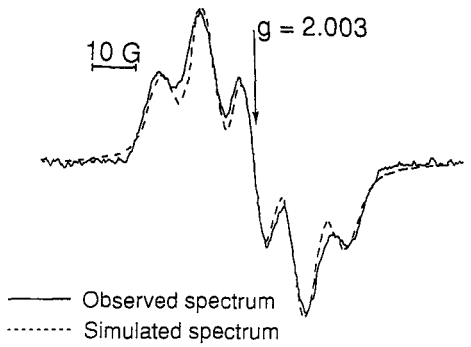


Fig. 4. Observed and simulated ESR spectra of $H4DMP^{\bullet+}$ (5b).

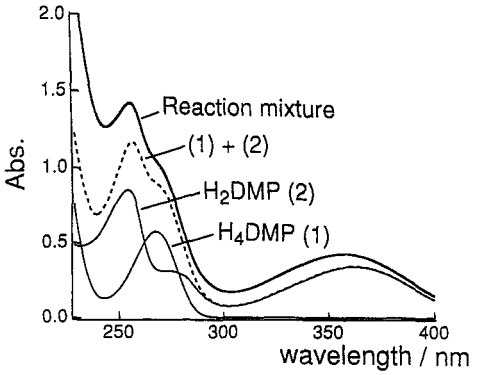


Fig. 5. Absorption spectra of the redox reaction mixture and its possible components.

the metal-pterin chemistry is to reveal the mechanism of tetrahydrobiopterin-metal ion interaction leading to the hydroxylation by PAH by structural and redox chemical investigations. The reaction of Cu(II) with H4DMP which has the cofactor activity was studied by ESR and absorption spectra (5b). A colorless reaction mixture obtained by mixing equimolar amounts of $Cu(NO_3)_2$ and H4DMP·2HCl in 50 v/v% CH_3OH-CH_3CN under N_2 exhibited the ESR spectrum of a radical species (Fig. 4), which was very similar to the one obtained by oxidation of H4DMP with H_2O_2 in CH_3OH-CF_3COOH and assinged to the protonated trihydropterin radical $H4DMP^{\bullet+}$ (21,22). The spectrum was satisfactorily simulated by considering the highest spin density at N(5) indicated by EHMO calculations (Fig. 4). The yields of the radical species based on the ESR intensity of 4-hydroxy-TEMPO were as high as 43 % with $Cu(CH_3COO)_2$ at 10 ms after mixing the reactants (23). The ^1H NMR spectrum of the reaction mixture clearly demonstrated the presence of H4DMP and H2DMP (the dihydro form of DMP) in equal amounts and the absence of Cu(II) ion left. The absorption spectrum obtained by mixing the $Cu(NO_3)_2$ and H4DMP solutions in 50 v/v% CH_3OH-CH_3CN showed that two peaks appeared at *ca.* 250 and 360 nm with a shoulder at *ca.* 270 nm (Fig. 5). It can be nearly reproduced by a 1:1 mixture of protonated H4DMP and H2DMP, indicating that formation of $H3DMP^{\bullet}$ is immediately followed by disproportionation according to Eq. 3 (protonation of the pterin ring is omitted):

$$2H3DMP^{\bullet} \overset{K_{dis}}{\rightleftharpoons} H4DMP + H2DMP \qquad (3)$$

where K_{dis} was calculated to be 7.7×10^2. The absorption spectral changes obtained for 1:1 $((C_2H_5)_4N)_2[CuCl_4]$–H4DMP·2HCl in the first 20 msec in 50 v/v % CH_3OH-CH_3CN initially showed a broad peak at *ca.* 325 nm (Fig. 6). The 1:1 $Cu(CH_3COO)_2$–H4DMP·2HCl system exhibited an isosbestic point at 280 nm with a decreasing peak at *ca.* 320 nm. The peaks at 320-325 nm are in accord with the maximum at *ca.* 320 nm observed upon oxidation of H4DMP by $N_3^{\bullet}$ at pH 7 and are therefore assigned to the radical species (24). Similar radical formation was also detected for the reaction of Fe(III). From these observations we may conclude that Cu(II) and Fe(III) react with H4DMP through one-electron redox processes to give $H3DMP^{\bullet}$.

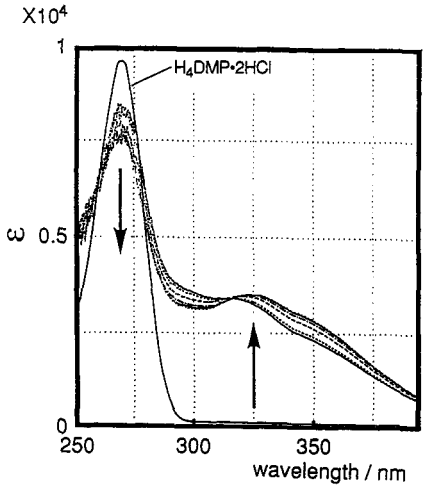


Fig. 6. Absorption spectral changes for 1:1 $((C_2H_5)_4N)_2[CuCl_4]$–H4DMP·2HCl (5b).

On the other hand, the Cu(II) complexes of dipeptides have been found to react with H4DMP slowly. The ESR spectra obtained by the rapid-mixing-freezing technique indicated that Cu(Gly·Gly) reacted with H4DMP at pH 7 to give a ternary complex Cu^{II}(Gly·Gly(H_{-1}))(H4DMP) without redox reactions. This suggests that at an intermediate step the active site of Cu-containing PAH may coordinate tetrahydropterins without electron transfer, which corresponds with the observation by Pember et al. (18). The coordination mode of the ternary complex as an intermediate Cu^{II}(Gly·Gly(H_{-1}))(H4DMP) is inferred to be similar to

that revealed for Cu(Gly·Gly(H_{-1}))(PC), where PC is bound to Cu(II) as in Fig. 3, by structural considerations and comparison of the ESR parameters. Considering that the aromatic ring of phenylalanine tends to occupy a position above the Cu coordination plane and to be stacked with the coordinated imidazole rings or the pterin ring as suggested by the CD spectra of Cu(Phe·Gly)(DMP), the structure of the intermediate of phenylalanine hydroxylation may be considered as a quaternary complex comprising a metal ion, bpy or other ligands, a pterin cofactor, and O_2 or substrate phenylalanine. Biological evidence showing that a quaternary intermediate is involved during the enzyme reaction (25) and the solution equilibrium studies on quaternary complex formation strongly suggest that there is formed a quaternary intermediate, where metal-aromatic, aromatic-aromatic, and other related weak interactions are important for effective and specific enzymatic reactions.

Spin trapping experiments by 5,5-dimethylpyrroline N-oxide (DMPO) indicated that the reaction mixture of 1:1 $Cu(bpy)^{2+}$-H_4DMP (2 mmol dm^{-3}, pH ~7) gave HO• upon bubbling O_2. This suggests that an intermediate complex which is capable of reacting with O_2 is formed from Cu(II) and H_4DMP. Phenylalanine ethylester was converted to *o*-, *m*-, and *p*-tyrosines by this system in small yields <4 % (Fig.7), whereas in the absence of $Cu(bpy)^{2+}$ no appreciable amounts of tyrosines were produced. Recently the role of copper in PAH from *Chromobacterium violaceum* has been questioned on the basis of the enzyme activity in the absence of copper and its inhibitory effect (15), but the above difference in reactivity indicates that copper is an essential component of the reaction at least in chemical model systems.

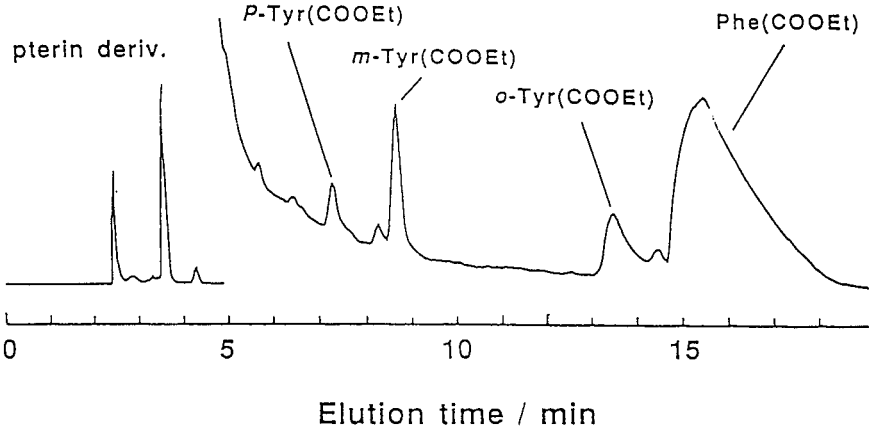


Fig. 7. Chromatogram of the hydroxylation reaction products of the 1:1:10 $Cu(bpy)^{2+}$–H_4DMP–phenylalanine ethylester system. Phe(COOEt) = phenylalanine ethylester; Tyr(COOEt) = tyrosine ethylester. Conditions: $Cu(bpy)^{2+}$, 2 mmol dm^{-3}; pH 7.0; O_2 bubbling, 2 min.

CONCLUSION

Aromatic rings of aromatic amino acids are involved in a variety of biological functions, some of which are dependent on the ring substituents. Phe is hydroxylated to Tyr to acquire functions such as the essential N-terminal amino acid residue of opioid peptides, a metal binding site, and molecular recognition through hydrogen bonding. Tyr is hydroxylated and finally converted to neurotransmitters catecholamines. On the other hand, modifications of the Tyr residue give rise to various functions of proteins. While formation of the tyrosine radical is recongized as an important step in the function of galactose oxidase (6) and ribonucleotide reductase (26,27), phosphorylation of Tyr is an important biological step in information transfer and control of enzyme activity, *etc.* Tyr phosphorylation occurs less frequently than serine and threonine phosphorylation but plays a specific role in the cell and tumor growth. In spite of the importance of protein phosphorylation, the mechanism of functionalization by Tyr phosphorylation is unknown. Table 1 shows that the ternary Cu(II) complexes stabilized by stacking of Tyr with bpy and phen are destabilized by Tyr phosphorylation, *i.e.*, the log *K* values are negative or close to zero for Cu(L-Ptyr)(DA), which means that stacking is cleaved by phosphorylation. Since intramolecular electrostatic interactions in Cu(L-Arg)(EDMA) *etc.* stabilize ternary complexes involving them, the effect of Tyr phosphorylation may be regarded as conversion of aromatic ring stacking to electrostatic or hydrogen bonding between the negatively charged phosphoester group and nearby positive groups such as the guanidinium group of Arg. On the basis of these considerations we proposed the conformational change due to Tyr phosphorylation

(2e). Aromatic amino acids have potential functions by modification of the rings, which will be an attractive source of complexing abilities and functions.

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