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NICKEL (II) COMPLEXES OF HISTIDYL-PEPTIDES AS FENTON-REACTION CATALYSTS

JEAN TORREILLES, MARIE-CHRISTINE GUERIN and AMAL SLAOUI-HASNAOUI

INSERM Unité 58-60 rue de Navacelles, 34090 Montpellier, France

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Addition of histidyl-peptides containing the glycyl-glycyl-L-histidyl sequence stimulated the catalysis of Ni(II) hydrogen peroxide reduction. Maximum bleaching of murexide or nitrosodimethylaniline was obtained with glycyl-glycyl-L-histidine. A decrease in the bleaching rates was observed upon addition of SOD or hydroxyl radical scavengers, showing that the hydrogen peroxide/Ni(II)/glycyl-glycyl-L-histidine system generated superoxide anions as well as hydroxyl radicals. In contrast, addition of glycyl-glycyl-L-histidine inhibited the Cu(II) hydrogen peroxide reduction.

When peptides or proteins were exposed to oxygen radicals produced by Ni(II)/glycyl-glycyl-L-histidine catalysis of hydrogen peroxide reduction, the observed effects were similar to those produced by oxygen radicals generated by water radiolysis or by Fe(II) or Cu(II) mediated Fenton-reactions: hydroxylation of phenylalanine, interchange of disulfides, destruction of tryptophans and dityrosine formation.

KEY WORDS: Nickel, histidyl peptide, Fenton-reaction, hydrogen peroxide, protein damage.

INTRODUCTION

Although nickel and its compounds would have toxic and carcinogenic activities in man and experimental animals,[1] nickel has not been reported to catalyse the generation of activated oxygen species in spite of its closeness to iron (nickel and iron have similar valence electronic structures[2] and partly share the same intestinal adsorptive mechanism).

Since a great variety of chelating agents enhances iron-driven oxygen radical generation[3] under physiological conditions, we examined whether there are any chelating peptides which stimulate, *in vitro*, the production of oxygen radicals by nickel-dependent reduction of hydrogen peroxide.

Among the 26 peptides tested, only peptides containing the glycyl-glycyl-L-histidyl sequence gave positive results with spectroscopic techniques for detection of oxygen radicals such as murexide bleaching,[4] nitrosodimethylaniline bleaching[5] and nitroblue tetrazolium coloration.[6]

To examine the possible generation of superoxide anions and hydroxyl radicals in the glycyl-glycyl-L-histidine/Ni(II) mediated reduction of hydrogen peroxide, we studied the effects of superoxide dismutase and hydroxyl radical scavengers such as ethanol, mannitol and dimethylsulfoxide.[7]

To test the reactivity of hydrogen peroxide/Ni(II)/glycyl-glycyl-L-histidine system toward proteins, we studied the efficiency of this system in the hydroxylation of phenylalanines,[8] in the interchange of disulfides,[9] in the destruction of tryptophans[10] and in the formation of dityrosine.[11]

MATERIALS AND METHODS

Nickel chloride and copper sulfate were purchased from Aldrich, all peptides were from Bachem Feinchemikalien AG, except glycyl-glycyl-L-histidine and derivated peptides which were synthetized by Neosystem Lab.. Superoxide dismutase (SOD 3000 Units/mg from bovine blood), bovine serum albumin, lactate dehydrogenase were from Sigma Chemical Co. All other enzymes were from Boehringer Mannheim.

A solution of 30% hydrogen peroxide was from Merck AG. All other reagents were of analytical grade.

Absorbances were recorded with a Cary 118C spectrometer and fluorescence spectra with a Perkin-Elmer MPF 3 recording spectrometer.

HPLC was carried out on LKB equipment (the column, mobile phase composition and flow rate are described in the Figure legends). The experiments were carried out at ambient temperature and detection by fluorescence was performed with a JASCO 820 FP detector. HPLC grade solvents were obtained from Prolabo SA Paris.

All aqueous solutions were prepared in deionized glass distilled water and stock peptides solutions were made "metal free" by treatment with chelex-100 according to Willard *et al.*[12]

The H_2O_2/Ni(II)/glycyl-glycyl-L-histidine system contained: 10^{-2} M H_2O_2, 10^{-4} M $NiCl_2$ and 10^{-4} M glycyl-glycyl-L-histidine.

RESULTS

Stimulation of the Reduction of Hydrogen Peroxide by Ni(II)/Histidyl-peptide Complexes

The stimulatory effects of histidyl-peptides containing the glycyl-glycyl-L-histidyl sequence for the nickel-reduction of hydrogen peroxide are shown in Table I: modifications in the absorbance of murexide, dimethylnitrosoaniline and nitroblue tetrazolium were observed only in the presence of compounds containing the glycyl-glycyl-L-histidyl sequence. When hydrogen peroxide, nickel ions or peptide were omitted, the solutions remained unaltered. Figure 1 shows the effect of pH on the reaction.

These results indicate that hydrogen peroxide, nickel ions and glycyl-glycyl-L-histidyl sequence containing peptides are required for the production of the oxidants responsible for absorbance modifications of the indicators of oxygen radical production.

When solutions were preincubated with superoxide dismutase before peptide addition, no solution absorbance modifications were observed. Ethanol, dimethylsulfoxide and butanol, which are effective HO$^{\cdot}$ scavenging agents,[13] all caused a decrease in the absorbance modification rates of the solutions.

Modification of Proteins by Hydrogen Peroxide/Ni(II)/Glycyl-glycyl-L-histidine Peptide.

a) Hydroxylation of phenylalanines The HO$^{\cdot}$ radicals react with benzene rings predominantly by addition to the ring and not by interaction with the substituents. Due to their electrophilic character, the HO$^{\cdot}$ radicals attack positions on the ring

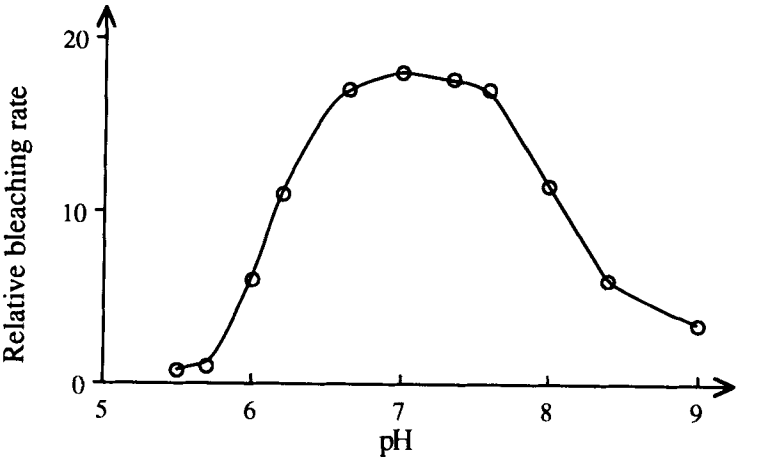

FIGURE 1 pH profiles for bleaching of murexide by oxygen radicals generated from the H_2O_2/Ni(II)/glycyl-glycyl-L-histidine system.

which are activated by electron donating substituents, i.e., ortho and para positions. There are a number of assays[14,15] for oxygen radical production which take advantage of the ability of $HO^{\cdot}$ to attack aromatic rings. The substrate we used was a simple peptide: glycyl-L-phenylalanine.

Figure 2 compares the HPLC chromatograms of a solution of glycyl-phenylalanine when, the hydrogen peroxide/Ni(II)/glycyl-glycyl-L-histidine system was added and, then when, the system ascorbic acid/Cu(II) was added. Detection was by fluorescence at the excitation and emission wavelength of tyrosine.

b) Interchange of disulfides Figure 3 compares the interchange reaction of disulfides: L-cystinyl-bis-L-phenylalanine and L-cystinyl-bis-L-tyrosine, caused by the Cu(II)/ascorbic acid system and also by the hydrogen peroxide/Ni(II)/glycyl-glycyl-L-histidine system. In both cases, the incubation of a mixture of the symmetric disulfides resulted in the formation of an asymmetric disulfide characterized as the L-cystinyl-L-phenylalanine-L-tyrosine.

c) Destruction of tryptophans Exposure of proteins to oxygen radicals produced by hydrogen peroxide/Ni(II)/glycyl-glycyl-L-histidine peptide system caused a loss of their native tryptophane fluorescence (Table II). Since the observed fluorescence emissions with native proteins result from the emissions of individual amino acids contained in the protein backbone and from complex energy transfers related to the protein conformation,[16] we considered that the destruction of tryptophan could produce significant protein structure modifications and, we did not attempt to correlate fluorescence intensities to number of tryptophan residues.

d) Formation of bityrosines 3,3′-dityrosine is a covalently bound biphenol formed from tyrosine in aqueous solutions by oxidation, the mechanism of the reaction can be formulated as phenolic coupling of two phenoxy radicals of tyrosine. In proteins, dityrosine may more likely be formed by intermolecular binding of tyrosines from two protein molecules, than by intramolecular binding within a single protein molecule.

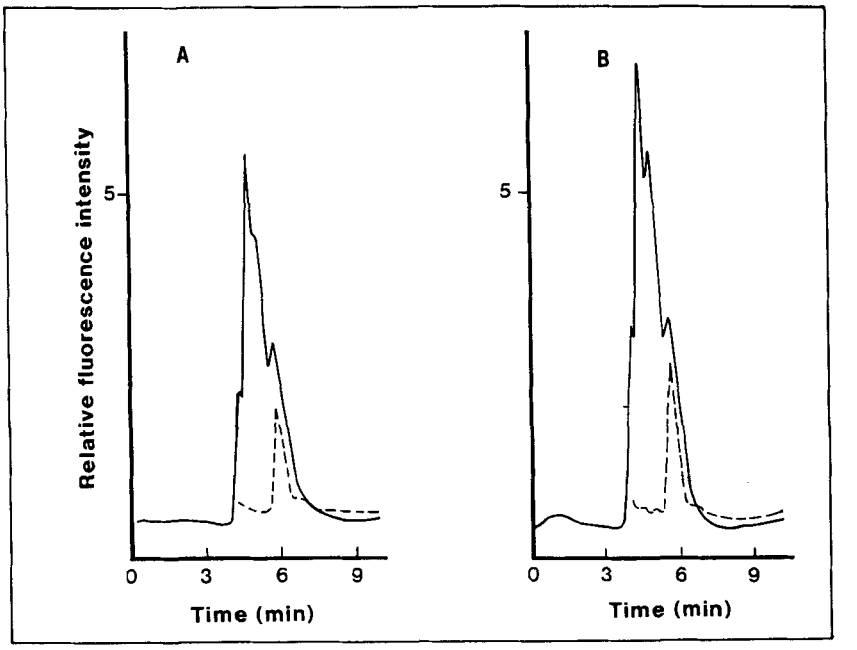

FIGURE 2 Identification of tyrosine isomers produced by exposure of glycyl-L-phenylalanine (10^{-3} M) to the oxygen radicals generated from the H_2O_2/Ni(II)/glycyl-glycyl-L-histidine system (part A). HPLC was carried out on a Bondapak C 18 column (4.6 × 250 mm), eluted with 10 mM potassium dihydrogenphosphate (0.7 mL/min). Tyrosyl-peptides were detected by fluorescence emission intensity at 300 nm (excitation at 280 nm). Dotten lines were recorded at $t_0 = 0$ and solid lines at $t = 16$ hours. For comparison, in part B, Ni(II)/glycyl-glycyl-L-histidine complex was substituted for Cu(II) ($t = 1$ hour).

Table III summarizes the increase in fluorescence emission intensities observed when some proteins were exposed to oxygen radicals generated by hydrogen peroxide/Ni(II)/glycyl-glycyl-L-histidine system.

Since dityrosine can be formed by oxidation with hydrogen peroxide alone,[17] relative fluorescence values shown in Table III represent the difference in fluorescence intensities observed at 400 nm for protein solutions with and without the glycyl-glycyl-L-histidine after 4 hours of incubation.

DISCUSSION

Many reports have shown that free Cu(II) ions only are good Fenton-catalysts and that consequently peptides which chelate Cu(II) ions inhibit the production of oxygen radicals. Inhibitory effect of some of them on the hydrogen peroxide reduction are reported in Table I.

In contrast, Ni(II) ions alone did not catalyse the hydroxyl-radical production from hydrogen peroxide but, when complexed with glycyl-glycyl-L-histidine, they became good Fenton-catalysts. Moreover, when an amino acid residue was added at the N-terminal and/or at the C-terminal of glycyl-glycyl-L-histidine, the catalytic effect

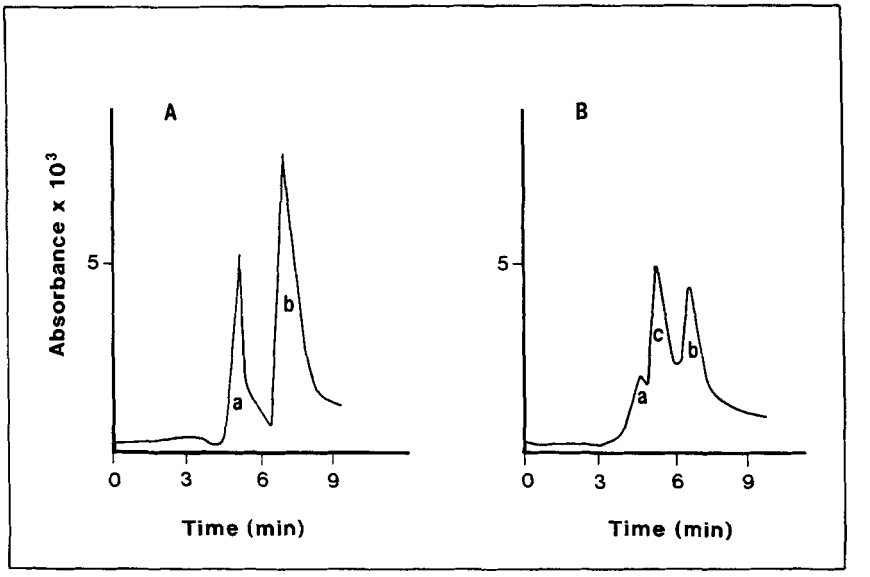

FIGURE 3 HPLC chromatograms of disulfide interchange promoted by the H_2O_2/Ni(II)/glycyl-glycyl-L-histidine system. A solution of 0.5 mM L-cystinyl-bis-L-phenylolanine (a) and 0.5 mM L-cystinyl-bis-L-tyrosine (b) was incubated with H_2O_2/Ni(II)/glycyl-glycyl-L-histidine. After one hour of incubation a new compound appeared (c) corresponding to L-cystinyl-bis-L-phenylolaline-L-tyrosine. The disulfide mixture was analyzed by HPLC on DEAE = Si 100 Serva column (4.6 × 250 mm). The column was eluted with potassium phosphate 30 mM, pH 7.2(2 mL/min). Detection was by UV absorption at 254 nm.

was reduced but remained significant, hence the glycyl-glycyl-L-histidyl sequence was critical to the effect.

As reported by Freeman,[18] Cu(II) and Ni(II) are chelated by peptides in the same way, but differences between the Cu(II) and Ni(II)-peptides systems were observed.

During the titration with alcali of glycyl-glycyl-glycyl-glycine performed in the presence of Cu(II) of Ni(II), the deprotonation of the three N(peptide) atoms was cooperative with Ni(II) ions, but not with Cu(II) ions.

A differing behaviour of the copper and nickel complexes to an increasing number of metal-N (peptide) bonds was also pointed out by Margerum and Dukes.[19] The stability of the Ni(II) systems increased relative to the stability of Cu(II) systems as the number of strong ligand field donors was increased. Differences in the ligand field stabilization effect could account for the formation and the behaviour of the mixed ligand complexes involved in the electron transfer mechanism from the metal-peptide complexes to oxygen.

The decrease in the rate of murexide bleaching in the presence of SOD of hydroxyl radical scavengers showed that superoxide anions and hydroxyl radicals were generated by the system: hydrogen peroxide/Ni(II)/peptide containing glycyl-glycyl-L-histidyl sequence.

We attempted to explain the well documented toxic effect of nickel *in vivo*, by exposing some peptides and proteins to radicals generated by the system hydrogen peroxide/Ni(II)/glycyl-glycyl-L-histidine. The observed effects were similar to those produced with oxygen radicals generated by Fe(II), Cu(II) or water radiolysis: pheny-

TABLE I

Oxidation rates of murexide (22×10^{-6} M) and nitrosodimethylaniline (22×10^{-6} M) and reduction rates of nitroblue tetrazolium (22×10^{-5} M) in the presence of hydrogen peroxide (10^{-2} M), metal ions: Cu(II) (10^{-6} M) or Ni(II) (10^{-4} M) and various peptides (10^{-4} M). The solution absorbances were measured at 465 nm for murexide ($\varepsilon = 14250\,M^{-1}cm^{-1}$), 440 nm for nitrosodimethylaniline (NDMA, $\varepsilon = 34400\,M^{-1}cm^{-1}$), and 560 nm for nitroblue tetrazolium (NBT, $\varepsilon = 28600\,M^{-1}cm^{-1}$). The nickel concentrations used are 100-times higher than the copper concentrations to obtain suitable concentrations of the nickel-peptide complex in the solution. With nickel, the excess of peptide remains without effect on the radical formation since it is not a radical scavenger but only a metal chelator. Values are means of three independent determinations.

Metal Chelator	Metal ion	Transformation μM/min Murexide	NDMA	NBT
None	Cu	0.19	0.07	0.23
	Ni	0	0	0
Glycyl-glycyl-L-histidine	Cu	0	0	0
	Ni	0.75	0.12	0.74
Glycyl-glycyl-L-histidyl-glycine	Cu	0	0	0
	Ni	0.60	0.10	0.55
Glycyl-glycyl-glycyl-L-histidine	Cu	0	0	0
	Ni	0.10	0	0.02
L-alanyl-glycyl-glycyl-L-histidine	Cu	0	0	0
	Ni	0.10	0	0.02
Glycyl-glycyl-L-histidyl-L-alanine	Cu	0	0	0
	Ni	0.40	0.07	0.50
Glycyl-L-phenylalanine	Cu	0.08	0	0
	Ni	0	0	0
Glycyl-glycine	Cu	0.19	0.02	0.03
	Ni	0	0	0
Glycyl-glycyl-glycine	Cu	0.19	0.02	0.03
	Ni	0	0	0

Negative results were obtained with the following peptides:

Glycyl-L-histidine	L-histidyl-glycine	L-carnosine
L-anserine	L-tyrosyl-glycine	Glycyl-L-tyrosine
L-tryptophyl-glycine	Glycyl-L-tryptophyl-glycine	Glycyl-glycyl-L-tryptophyl-L-alanine
β alanyl-L-tyrosine	Glycyl-glycyl-L-tyrosine	Glycyl-L-histidyl-glycine
L-histidyl-glycyl-L-lysine	Glycyl-L-histidyl-L-lysine	Glycyl-L-alanyl-L-phenylalanine
L-alanyl-L-histidyl-L-lysine	L-phenylalanyl-glycine	Glycyl-glycyl-L-phenylalanine

TABLE II

Loss of tryptophan following exposure of enzymes (0.33 mg/mL) to oxygen radicals generated by the H_2O_2/Ni(II)/glycyl-glycyl-L-histidine system. Results represent percentage loss of fluorescence emission intensity measured at 345 nm (excitation at 290 nm), after 1 hour of incubation.

Protein	Tryptophan loss (%)
Glutamic dehydrogenase	30
Lactic dehydrogenase	35
Hexokinase	24
Bovine serum albumin	44
Chymotrypsin	8

TABLE III

Production of dityrosine following exposure of enzymes to oxygen radicals generated by the H_2O_2/Ni(II)/glycyl-glycyl-L-histidine system. Experimental conditions were the same as in Table I. Results represent dityrosine content measured by fluorescence emission intensity at 400 nm (excitation at 325 nm), after 4 hours of incubation and for 1 mg/ml protein solutions.

Protein	Bityrosine fluorescence
Glutamic dehydrogenase	1.0
Lactic dehydrogenase	0.9
Hexokinase	1.66
Bovine serum albumin	1.58
Chymotrypsin	2

lalanines were hydroxylated (Figure 3), disulfides were interchanged (Figure 4), tryptophanes were destroyed (Table II) an dityrosines were formed (Table III).

Since iron and nickel have similar valence electronic structures, we tested the effect of metal chelators such as EDTA and EGTA which are known to activate the Fenton catalytic effect of iron. These chelators inhibited the effect of Ni(II)/glycyl-glycyl-L-histidine complex on the reduction of hydrogen peroxide probably by extracting Ni from its complex.

Our results confirm the hypothesis that peptides containing glycyl-glycyl-L-histidyl sequences could stimulate the reduction of hydrogen peroxide by Ni(II), and promote the generation of oxygen free radicals. They suggest that the toxic effects of nickel *in vivo*, might be partly explained by the ability of some peptides to convert Ni(II), into a Fenton-catalyst.

Several experiments have shown that Ni(II) ions enter nuclei and strongly bind DNA.[20] That binding could induce conformational and/or structural changes of DNA,[21] modifying gene expression without direct damage DNA, and, in the presence of hydrogen peroxide, cleavages of DNA by oxygenated active intermediates.[22]

The disproportionation of hydrogen peroxide postulates the involvement of the Ni(III)/Ni(II) redox couple acting as a Fenton catalyst. Our results confirm such an assumption since the protein damaging oxidant is produced only when nickel ions are chelated by the peptide and has an "hydroxyl radical like" reactivity.

But the absolute necessity of a chelation of the nickel ions for the disproportionantion of hydrogen peroxide observed in all nickel studies and the inability of organic scavengers to inhibit DNA damage reported by Kawanishi *et al.*,[22] seem to be the consequences of the chemical nature of the Fenton-generated radical. That radical could be, by analogy with the postulated crypto-hydroxyl radical,[23] a hydroxyl radical complexed to the metal and its organic ligand (peptide or DNA). Its breakdown would complete the Fenton reaction to produce the hydroxyl radical.

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