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MULTIPLE FORMS OF THE COBALT(II)-CARNOSINE COMPLEX

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Summary: Cobalt(II) ion and L-carnosine produce two different complexes when mixed in aqueous solution at pH 7.2. One complex has coordination of N-3 of the imidazole ring to the cobalt(II) and is produced when the concentration of peptide exceeds that of cobalt(II). The second complex has chelation of three nitrogen atoms of a single carnosine. This second complex produces a reversible oxygen carrier by making stable mixed chelates with additional carnosine, histidine or cysteine. These results indicate that cobalt complexes with mixed ligands should be of more importance in vivo than those with carnosine as the only ligand. They provide an explanation for the high activity and substrate specificity of carnosinase in kidney.

One of the highest in vivo concentrations of cobalt is in the kidney (1). The kidney is the primary route of excretion of cobalt (2) and also monitors the oxygen tension of blood (3,4). When the level of oxygen in the kidney is low, erythropoietin is released and stimulates the bone marrow to make red cells. Proliferation of red cells can also be produced by injection of $CoCl_2$, which has been suggested to cause anoxia in the kidneys by binding -SH groups (1,5). An apparently unrelated observation is that carnosinase is present at the highest in vivo activity in the kidney (6). The substrate of this enzyme, carnosine (β-alanyl-L-histidine), has been shown to form a complex with cobalt(II) that is a reversible oxygen carrier (7).

Together these findings suggest that the high activity of carnosinase in the kidney may be involved with the ability of this organ to monitor oxygen tension. Since little is known about the structure and stability of the cobalt-carnosine complex, we have investigated the ability of carnosine and its analogs to bind cobalt(II), the ability of these complexes to bind molecular oxygen and the effect of other ligands on the interaction of carnosine with cobalt(II).

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Experimental Procedures

Materials: L-Carnosine, L-anserine nitrate, and L-homocarnosine sulfate were purchased from Sigma Chemical Company. Sucrose was obtained from Schwarz/Mann. Cobalt(II) chloride, sodium hydroxide and hydrochloric acid were purchased from Fisher. Deuterium oxide (99.8% isotopic purity), deuterium chloride and sodium deuteroxide were from Merck, Sharp and Dohme.

Methods: All samples were prepared by mixing appropriately diluted stock solutions of $CoCl_2$, peptide and sucrose. All stock solutions were freshly prepared in distilled water, and spectra of the mixed samples were obtained within 24 hours. Samples were stored at 4°C. Solutions were adjusted to the appropriate pH with dilute HCl, NaOH or their deuterated analogs. No corrections were made for isotope effects.

All ESR spectra were obtained with spectrometers at the Biomedical ESR Center. The x-band spectra (9 GHz) were obtained with a Varian E-9 EPR spectrometer equipped with a dual sample cavity. Spectra of frozen samples were obtained by supporting the samples in the cavity with a finger dewar filled with liquid nitrogen. The ^{1}HMR spectra were obtained at ambient temperature (ca 30°C) with a Bruker WP-200 spectrometer with a 5 mm proton probe. The UV-visible absorption spectra were obtained with an Aminco DW-2 UV/Vis Spectrophotometer.

Results

Freshly made solutions of 910 μM $CoCl_2$ plus carnosine at concentrations 1, 5, 10, 100 and 1000 times that of the cobalt(II) ion yield ESR spectra, like the one in Fig. 1, that are characteristic of complexes with a molecule of oxygen bound to the axial coordination position of a chelated cobalt(II) ion with a Co-O-O angle of ca 120° (8,9). However, a combination of ^{1}HMR, ESR and UV-visible absorption spectroscopies provides data that the carnosine binds to the cobalt of these complexes in two different orientations. At ratios of carnosine to cobalt(II) of ca 1000:1, the ^{1}HMR signals of H-2 and H-4 of the imidazole ring are broadened equally with the amount of broadening being progressively less for protons farther removed from the imidazole ring (10). This indicates that N-3 of the imidazole ring of carnosine coordinates to the cobalt(II) ion (Table I). The stoichiometry of this complex in solution is not known but most likely involves coordination of carnosine to the four equatorial positions of the cobalt(II) ion. When the ratio of carnosine to cobalt(II) ion in solution approaches 1:1 a complex with different chemical and physical properties is produced. This complex can not be studied directly with ^{1}HMR spectroscopy because the resonances are too broad to detect, but model studies with anserine (β-alanyl-1-methylhistidine) and homocarnosine

TABLE 1

^{1}HMR Spectral Line Broadening of Carnosine, Histidine and Cysteine by Co^{++}

Line Broadening[b] ($\frac{\Delta \text{plus } Co^{++}}{\Delta \text{without } Co^{++}}$)	Composition of Solution[a]						
	Carnosine				Histidine		Cysteine
	$H\text{-}2^{c}_{im}$	$\beta\text{-}CH_2\text{-}his$	$\alpha\text{-}CH_2\text{-}ala$	$\beta\text{-}CH_2\text{-}ala$	$H\text{-}2^{c}_{im}$	$\beta\text{-}CH_2$	$\beta\text{-}CH_2$
Expt. 1							
2.3	1.8	1.0	1.0		---	---	---
Expt. 2							
3.4	ca5	ca7	ca3		3.2	ca4.7	---
Expt. 3							
1.7	ca2.9	3.3	1.8		---	---	1.8

[a] Three experiments were performed. The first sample consists of 20 mM carnosine plus 100 μM $CoCl_2$ in D_2O. The second and third samples also contain 20 mM histidine and 20 mM cysteine, respectively. The pH was maintained at 7.2±0.1, and all spectra were measured at 200 MHz.

[b] When a small molecule binds to a paramagnetic metal ion the observed spin-spin relaxation times represent an average of the relaxation times in the bound and free states if $T_{2\text{bound state}} >> \tau$ and $(\Pi\ \delta_{fb} T_{2\text{bound state}}) << 1$ where τ is the lifetime of the bound state, δ_{fb} is the chemical shift difference between the bound and free states, and $T_{2\text{bound state}}$ is the spin-spin relaxation time of the ligand proton when the ligand is on the paramagnetic metal ion. Since $1/T_2 = \pi(\Delta\nu)$ where $\Delta\nu$ is the line width at half height of the peak in the ^{1}HMR spectrum, the spin-spin relaxation rates of the protons on a ligand molecule, and thus the ability of that ligand to bind to a given paramagnetic metal ion, can be measured from the line widths of the peaks in the spectrum.

[c] The subscripts refer to the imidazole ring and methylene group of the histidinyl residue and to the methylene groups of the β-alanyl residue, respectively. The 1.8-fold spectral broadening of the methylene protons of the histidinyl residue in Expt. 1 indicates that some coordination of the peptide nitrogen atom may occur at this ratio of carnosine to cobalt(II) ion. A similar coordination of the primary amino group of histidine in Expt. 2 is suggested. The decreased line broadening in Expt. 3 as compared to Expt. 2 indicates that, although the orientation of carnosine binding appears to be the same, the residence time is less and/or the approach is more distant with added cysteine as compared to histidine.

(γ-aminobutyryl-L-histidine) provide indirect evidence for its structure in solution. Neither of these analogs yields ESR spectra of the type in Fig. 1 or UV-visible absorption spectra that are characteristic of cobalt(II) oxygen carriers (see below) as does carnosine when mixed with cobalt(II) under identical conditions. This implies that N-1 of the imidazole ring of carnosine binds to the cobalt(II) ion since the methyl group on this nitrogen atom inhibits the formation of the analogous complex by anserine. Since homocarnosine, which would form a seven membered ring upon coordination of the peptide and terminal amino nitrogen atoms, does not form a similar complex with cobalt(II), the six membered ring formed by chelation of carnosine appears to be necessary for the stability of the complex. The same is true for the

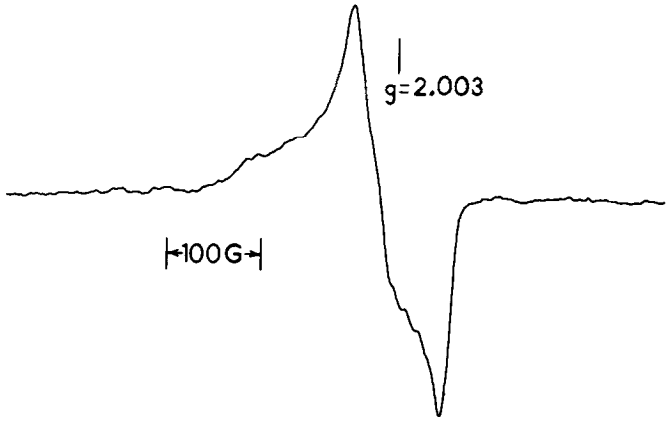

Fig. 1. The ESR spectrum of a frozen (-196°C) aqueous solution at pH 7.2 of 910 μM $CoCl_2$ plus 4.5 mM carnosine and 0.3 M sucrose. The sample was frozen in liquid nitrogen immediately after mixing the cobalt and carnosine. Spectrometer settings: modulation amplitude, 5 Gauss; modulation frequency, 100 KHz; incident power, 20 mW; gain, 10×10^2.

stability of the copper(II)-carnosine complex (11,12,13). These observations suggest that carnosine binds to cobalt(II) ion with N-1 of the imidazole ring and the peptide and terminal amino nitrogen atoms (Fig. 2(middle)) when the ratio of carnosine to cobalt approaches 1:1. This conclusion is supported by the observation that addition of cysteine or histidine to a solution of cobalt(II) ion containing a 200-fold excess of carnosine causes the orientation of the bound carnosine to change from that in Fig. 2(left) to that in Fig. 2 (middle) (Table 1). The ^{1}HMR spectral line broadening indicates that the orientation of the carnosine molecule with three point coordination is more stable (i.e. has a longer residence time on and/or a closer approach to the cobalt(II) ion) than the orientation with only one point coordination by N-3. The most likely structure of these mixed complexes in solution is that in Fig. 2(middle) (i.e. the tridentate carnosine complex) with the fourth equatorial coordination position of the cobalt(II) ion bound to the -SH group of cysteine or the imidazole ring of histidine (Table 1). Keep in mind that the axial ligand of these complexes is a molecule of oxygen (8,9).

It must be noted, however, that the ESR spectra of the type in Fig. 1 and the ^{1}HMR spectral line broadening as in Table 1 disappear with time. The

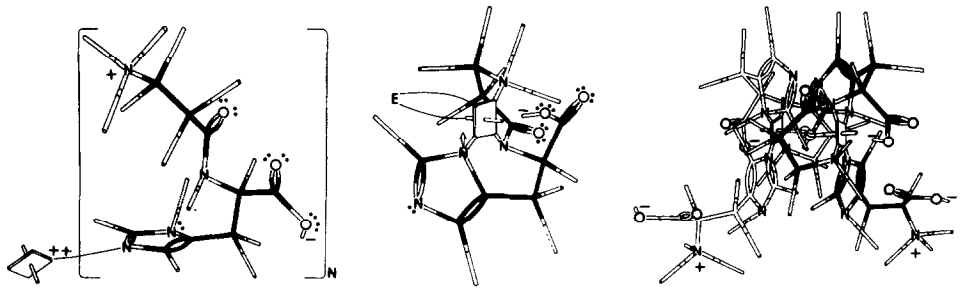

Fig. 2. Structures of the cobalt(II)-carnosine complexes. When carnosine is present in excess it binds to the cobalt(II) ion in the orientation at the left. The number of carnosine molecules, N, is expected to vary between 1 and 4 depending on how large an excess of carnosine is present in the solution. The middle structure is formed when cobalt(II) ion and carnosine are present at equimolar concentrations. This complex is unstable toward oxidation to cobalt(III). The E indicates the two positions of the cobalt that are free to coordinate H_2O and O_2 or to bind to the active site of carnosinase. The arrow on N-1 of the imidazole ring represents the location of the hydrogen atom in carnosine and of the methyl group in anserine. Addition of an equimolar concentration of histidine stabilizes the binding of carnosine in this orientation and results in formation of the binuclear cobalt(II) oxygen carrier at the right.

complex formed in the presence of a ca 1000-fold excess of carnosine (Fig. 2 (left)) slowly oxidizes overnight to the red cobalt(III) form. The complex formed by carnosine and cobalt at equal concentrations (Fig. 2(middle)) oxidizes very rapidly (in a matter of seconds at concentrations above 100 mM) to its cobalt(III) form. The laser Raman spectrum of this complex exhibits weak bands at 640cm^{-1} and 975cm^{-1} that would be expected for a cobalt(III) complex but does not exhibit the intense band at 805cm^{-1} that is characteristic of a peroxo-bridged binuclear cobalt(III) complex (14). In contrast, the complexes formed with intermediate ratios of carnosine to cobalt(II) do not oxidize to cobalt(III) even after prolonged storage at room temperature. These stored complexes do not yield an ESR signal or exhibit paramagnetic line broadening of the ^{1}HMR spectrum of the ligand, but they do exhibit the charge transfer band at 345 nm (Fig. 3) that is characteristic of the diamagnetic, binuclear cobalt(II) oxygen carriers (15). This yellow-brown diamagnetic complex also is produced within two hours by the mixed complexes with either histidine or cysteine. These results indicate that the paramagnetic cobalt(II)-oxygen

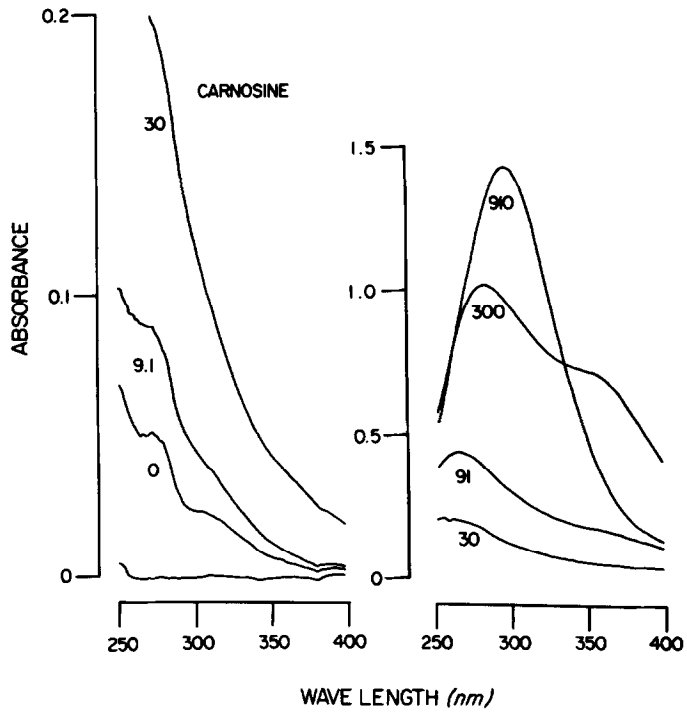

Fig. 3. UV-visible absorption spectra of the complexes formed by mixing carnosine and cobalt(II) ion in aqueous solution at pH 7.2±0.2. The concentration of carnosine was 9.1 mM in all cases, and the μM concentrations of $CoCl_2$ are given by the numbers in the figure. The samples were stored in the presence of air for 24 hours before recording the spectra to permit maximal oxygen uptake by the complex. It should be noted that the spectra from 400 to 700 nm of the reversible oxygen carrier and of the irreversibly oxidized complex with carnosine are qualitatively the same as those reported earlier with histidine (7).

complexes that do not oxidize to cobalt(III) bind a second chelated cobalt(II) ion to the oxygen molecule instead. When the uncoordinated equatorial position of the cobalt(II) ion in Fig. 2(middle), labeled E, binds a molecule of water, the oxidation to cobalt(III) is favored (15). However, coordination of this equatorial position by cysteine or by the imidazole ring of either histidine or a second molecule of carnosine stabilizes the cobalt(II) against oxidation and favors the diamagnetic, oxygen-bridged cobalt(II) dimer (Fig. 2(right)).

Discussion

The chemistry of cobalt(II) complexes with carnosine as the only ligand is not likely to be important *in vivo* where the presence of other ligands

with imidazole and -SH functional groups favor the formation of more stable mixed complexes. The fact that these mixed complexes are more stable toward oxidation to the cobalt(III) form indicates that they might form reversible complexes with molecular oxygen in organs that contain carnosine and high levels of cobalt. Thus, the high activity of carnosinase in kidney might be necessary to guarantee proper functioning of the oxygen sensor that regulates the release of erythropoietin. If carnosine were permitted to accumulate in the kidney, it would affect the binding of cobalt in this organ to -SH and imidazole groups. This is completely analogous to the proposed mechanism by which elevated cobalt in the kidney produces polycythemia. It should be noted that the high substrate specificity of carnosinase, which can be activated by cobalt, also can be explained by the ability of carnosine to form these mixed complexes with the activating metal ion of this enzyme. When carnosine is mixed with crude preparations of carnosinase, the ^{1}HMR spectral line broadening of the carnosine resonances from interaction of the substrate with the activating metal indicates that chelation of at least N-1 of the imidazole ring of both the substrate, carnosine, and the product, histidine, occurs on the enzyme surface (10). The proton resonances of anserine and homocarnosine, which do not produce stable complexes with cobalt and also are poor substrates of carnosinase (16), are not broadened, indicating that these dipeptides do not bind to the activating metal. The protein portion of the enzyme appears to stabilize the chelation of carnosine with the orientation in Fig. 2(middle) and then to catalyze the addition of water across the coordinated dipeptide bond.

Acknowledgements

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