Vol. 92, No. 2, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 29, 1980 Pages 470-477

EVIDENCE THAT CARNOSINE AND ANSERINE MAY PARTICIPATE IN WILSON'S DISEASE

Charles Eric Brown* and William E. Antholine

Departments of Biochemistry* and Radiology
The Medical College of Wisconsin
P.O. Box 26509, Milwaukee, Wisconsin 53226

Received December 3,1979

Summary: Carnosine, anserine and copper(II) ion all bind to specific sites on bovine serum albumin, and, in addition, both dipeptides chelate copper(II) ion in the absence of serum albumin. Thus a solution of dipeptide, copper(II) ion and serum albumin exhibits several complexes that arise from the competing binding reactions. Since a change in this complex equilibrium might occur in Wilson's disease, we have investigated the reactions between the various complexes with NMR and ESR spectroscopy. Serum albumin simultaneously binds the copper(II) ion and carnosine to separate sites rather than forming a mixed chelate, but carnosine still is capable of competing with serum albumin for subsaturating amounts of copper.

The primary lesion in Wilson's disease (hepatolenticular degeneration) is an inability of the liver to synthesize ceruloplasmin. This results in an increase in the lifetime of the copper-serum albumin complex that transports ingested copper from the gut to the liver (1,2). The build-up of this weak complex in the circulation appears to precede the deposition of copper in the kidneys, the edge of the cornea and specific regions of the brain, but the mechanism by which the copper is transported to these organs is not known.

We proposed that the copper may be transported as a chelate with carnosine (β-alanyl-L-histidine) and/or anserine (β-alanyl-1-methylhistidine) (3,4). This is supported by several lines of evidence. First, carnosine and anserine are good chelators of copper in the physiological range of pH and temperature (3-6). When copper(II) ion plus either carnosine or anserine are mixed in approximately equal concentrations in aqueous solution at pH 7.2 and ca 25°C, an equilibrium mixture of the type in the top half of Fig. 1 is produced. The equilibrium favors the dimeric copper complex, which may exhibit reduced dipolar coupling of the copper spins under these conditions compared to that in the solid state because of the vibrational motion represented in the upper right brackets of Fig. 1 (3,4). However, addition of a second ligand such as

0006-291X/80/020470-08$01.00/0

histidine, cysteine or glutathione, which would be present in vivo, stabilizes the monomeric structure by formation of mixed complexes and pulls the equilibrium in the top half of Fig. 1 to the left (4). Second, the highest activity in the body of carnosinase, which hydrolyzes carnosine and anserine to their component amino acids, is located in the kidney so that deposition of copper in this organ could be explained by hydrolysis of the transporting ligand (7). Third, there is evidence that these two dipeptides might enter the ganglia at the base of the brain. Carnosine and anserine are maintained at low concentrations in the circulation of healthy individuals, and mental retardation is associated with elevations of their concentrations in the circulation and/or urine that result from a number of apparently different metabolic disturbances (8-12). Cerebromacular degeneration, including damage to the optic nerve, has been demonstrated to accompany elevated urinary levels of carnosine and anserine in at least one family (13,14). Fourth, the deposition of copper and progressive neurological damage that results in Wilson's disease can be averted by administration of a chelator such as penicillamine (1).

For our proposed transport of copper from serum albumin to these organs to occur, however, carnosine and anserine must be capable of removing copper from its binding site on serum albumin. The intent of this investigation is to demonstrate that these dipeptides do indeed chelate copper in the presence of serum albumin and to delineate the conditions under which this can be expected to occur.

Experimental Procedures

Materials: L-Carnosine, bovine serum albumin, fraction V, and glycyl-L-tryptophan were purchased from Sigma Chemical Company. Sucrose was obtained from Schwarz/Mann. Copper(II) sulfate, 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 $CuSO_4$, BSA, dipeptide and sucrose. All stock solutions were freshly prepared in deionized water or D_2O, and spectra of the mixed samples were obtained within 24 hours. Samples were stored at 4°C. Solutions were adjusted to pH 7.2 with dilute HCl, NaOH or their deuterated analogues. No corrections were made for isotope effects.

The ^{1}HMR spectra were obtained at 26°C with a Nicolet NT-150 spectrometer with a 5mm probe. The chemical shifts were measured relative to tetramethylsilane in a coaxial capillary. The orientation in which the ligand binds the copper(II) ion was determined by assuming that the observed line broadening arises primarily from dipolar contributions. The binding constant of carnosine with copper(II) ion was not calculated because contact contributions to the observed line broadening could not be properly accounted for (3,4) and because the value of this constant will depend on the identity of the second ligand in the mixed chelate. The difficulties associated with measuring the binding constant of the carnosine-BSA complex are presented elsewhere (15). The x-band (9GHz) ESR spectra were recorded with a Varian E109 Century Series spectrometer. Spectra were obtained by supporting the frozen samples in the cavity with a finger dewar filled with liquid nitrogen. Sucrose (0.3 M) was added to these solutions to facilitate freezing.

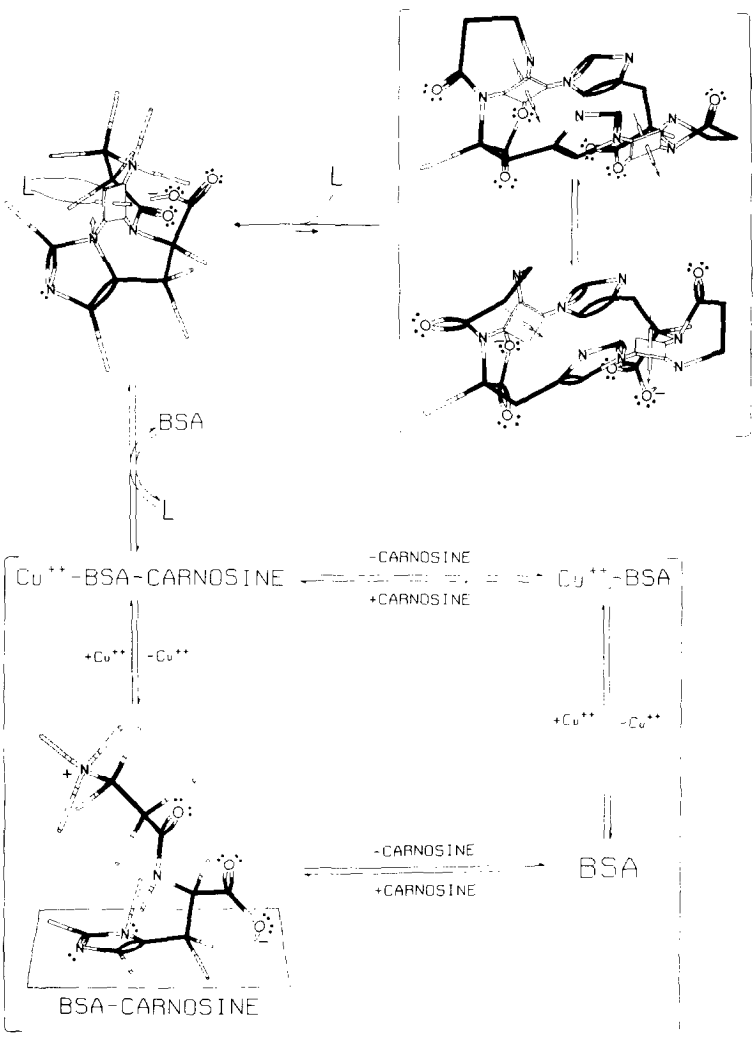

Fig. 1. The complex equilibria that occur in an aqueous solution of carnosine, bovine serum albumin and copper(II) ion at pH 7.2 and ambient temperature. L represents a second ligand such as histidine, cysteine, glutathione or a second molecule of carnosine. The plane in the structure of the BSA-carnosine complex represents the binding site on the albumin molecule.

Results

When BSA is added to an aqueous solution of carnosine, there is a small amount of line broadening of the ^{1}HMR spectral peaks arising from the two non-exchangeable protons on the imidazole ring of the carnosine (6.3 and 7.1 ppm, Fig. 2A). At lower magnetic fields this line broadening is more pronounced than that in Fig. 2A and has been used to demonstrate that carnosine binds to BSA as shown in Fig. 1 (15). The addition of various concentrations of copper(II) ion to an aqueous solution of carnosine (Fig. 2B-D left) causes line broadening of the peaks in the ^{1}HMR spectrum of the carnosine with the effect on the protons of the imidazole ring and methylene group (2.4 ppm) of the histidyl residue being detected at much lower concentrations of copper(II) ion than on those of the methylene groups (1.9 and 2.5 ppm) of the β-alanyl

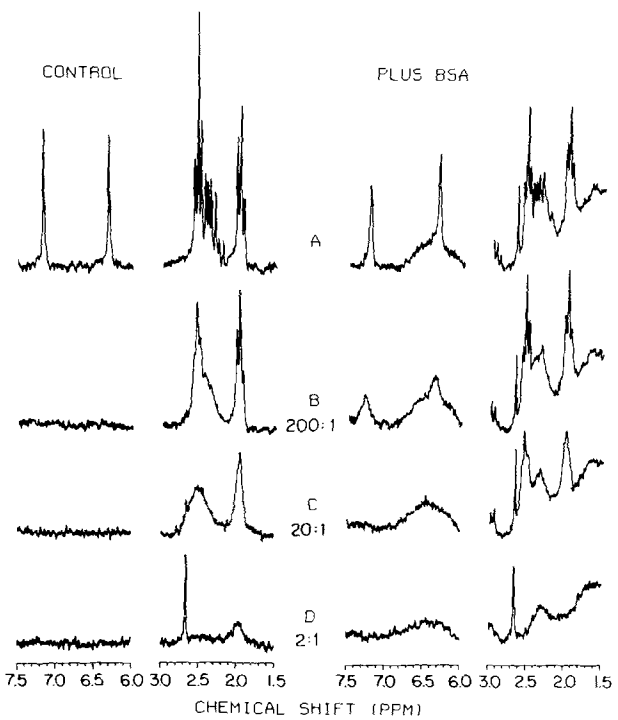

Fig. 2. The effect of 1 mM BSA on the ^{1}HMR spectrum of 20 mM carnosine in the absence (A) and presence (B-D) of copper(II) ion. The concentrations of added copper(II) ion range from 0.1 to 10 mM to produce the ratios of molar concentrations of carnosine-to-copper given between each set of spectra. The sharp peak at 2.7 ppm arises from an impurity in the NaOD.

residue. These changes in the recorded spectra arise from formation of the copper(II)-carnosine complexes presented at the top of Fig. 1 (3,4). The addition of BSA to solutions containing carnosine and copper(II) ion (Fig. 2B and C right) reduces the effect of the copper(II) on the line widths of the resonances of all the nonexchangeable protons on the carnosine molecule. Only when the concentration of added copper(II) ion greatly exceeds the concentration of BSA is the effect of the BSA on the linewidths of the proton resonances of carnosine not observed (Fig. 2D). There are two possible explanations of these results. Either the BSA is binding a substantial fraction of the copper(II) ion and thus is making less copper(II) ion available for chelating with the unbound carnosine in solution, or the BSA is binding virtually all of the added copper in Fig. 2B and C and the carnosine is experiencing relaxation enhancement by forming a mixed chelate with the copper bound to BSA. The second possibility is unlikely because binding of BSA to the copper(II)-carnosine chelate would be expected to increase rather than decrease the observed line widths of the proton resonances of carnosine, but a decrease could result if the residence time and distance of approach of the carnosine were sufficiently different in the mixed chelate to offset the effect of slow rotational motion of the BSA.

Addition of subsaturating concentrations of copper(II) ion to BSA produces an ESR spectrum like that in Fig. 3A, and addition of subsaturating concentrations of carnosine to this complex has no effect on the ESR spectrum (Fig. 3B). When BSA is added to a solution of the dimeric copper(II)-carnosine complex, the characteristic seven-line pattern in the g_{II} region of the spectrum that is characteristic of cupric dimers is lost and a spectrum like that in Fig. 3A is obtained. Thus, BSA, like histidine, cysteine and glutathione, inhibits the formation of the dimeric copper(II)-carnosine complex. However, since the ESR spectrum of copper(II)-BSA is identical in both the presence and absence of carnosine, BSA does not appear to do so by formation of a more stable mixed complex in which both carnosine and the second ligand

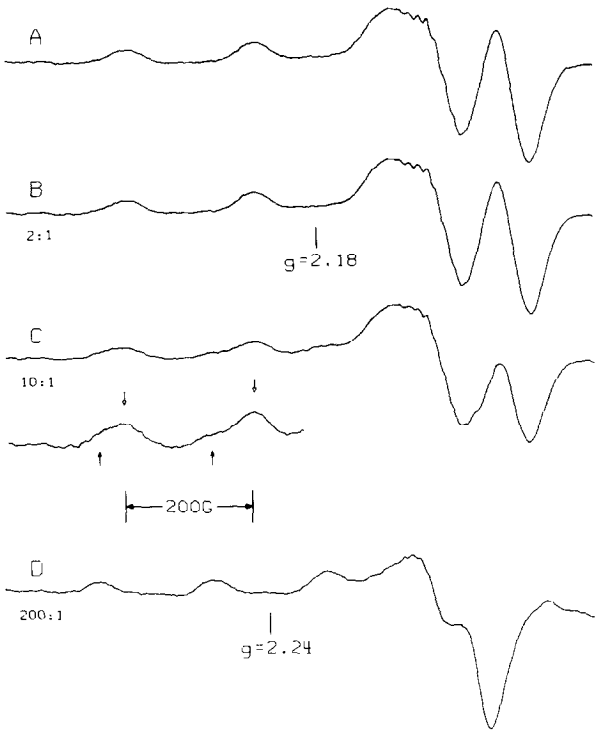

Fig. 3. The effect of a mixture of carnosine and histidine on the ESR spectrum of 0.1 mM copper(II) ion in the presence of 1 mM BSA. Spectrum A is the control spectrum of copper(II)-BSA, whereas spectra B-D were recorded in the presence of carnosine and histidine, both of which were added to a final concentration of 0.2, 1 and 20 mM, respectively. The ratios of molar concentrations of carnosine-to-copper are given below each spectrum. Spectrum D is as expected for the mixed chelate with both carnosine and histidine as ligands. The involvement of both ligands in this complex was substantiated by its ^{1}HMR spectrum. The inset below spectrum C demonstrates the spectral contributions from the copper(II)-BSA and copper(II)-carnosine/histidine complexes.

bind to the same copper(II) ion. Carnosine and copper(II) ion bind to separate sites on the BSA molecule.[+] Addition of sufficient carnosine to saturate its binding site on BSA leaves unbound carnosine in solution that effectively competes with BSA for available copper (Fig. 3C and D). Conversely, the concentration of carnosine required to form a detectable amount of copper-carno-

[+]We cannot distinguish between the possibilities of carnosine and copper binding to totally unrelated sites on BSA or of carnosine and copper competing for the same sites on BSA with one binding each site to the exclusion of the other. Since available evidence suggests that both the carnosine and copper binding sites contain a histidyl residue (2,15), the second possibility should not be overlooked.

sine in the presence of a fixed concentration of serum albumin decreases as the concentration of copper is increased (from ^1H relaxation measurements).

Other small peptides and analogues of carnosine that we have studied tend not to produce as strong chelates with copper(II) ion as do carnosine and anserine and also would not be preferentially concentrated near the copper in the circulation since they do not bind to serum albumin (3,15). Glycyl-L-tryptophan, which forms a complex with copper(II) ion (g_{11}=2.22, A_{11}=165G) and also binds to a specific site on bovine serum albumin that is different from that for carnosine (15), was found not to compete with serum albumin for copper under any of the conditions that permitted carnosine to do so.

Discussion

Since the onset of the toxic effects of copper on the brain of Wilson's disease patients is observed only after several years of life, it would be expected that the fraction of total copper in the serum that is transported to the brain at any one time should be very small. The complex system of competing equilibria between carnosine, serum albumin and copper that we describe provides a mechanism by which this transport of small amounts of copper might occur. The observation that serum albumin binds carnosine and copper(II) ion to separate sites instead of forming a mixed copper(II) chelate has several physiological implications. First, carnosine is not likely to stabilize the copper(II)-BSA complex since a mixed chelate is not formed. Instead, carnosine and serum albumin compete for available copper. Second, because serum albumin binds both carnosine and copper to separate sites, the concentration of carnosine must be elevated high enough to saturate its binding site on serum albumin and thereby yield enough free carnosine in solution to compete for copper. Since our experiments indicate that serum albumin is effective in keeping the copper and carnosine separated until the concentration of carnosine is almost equimolar with that of serum albumin, it is unlikely that the variations in carnosine concentration that are found in the circulation would have a significant effect on the transport of copper

from the gut to the liver of an otherwise normal individual. Third, elevation of the concentration of copper can be expected to increase the ability of carnosine to form a chelate in the circulation. The presence of a second ligand in the serum such as histidine, which does not itself bind to serum albumin, stabilizes the copper-carnosine chelate by forming a mixed complex (3,4) and thus hinders the reverse reaction. In severe cases of Wilson's disease the concentration of copper in the serum is greatly elevated and the lifetime of the copper-serum albumin complex *in vivo* is extended (1). Under these conditions the equilibrium between bound and free copper makes more unbound or very weakly bound copper available to other ligands in the serum. It is quite possible that carnosine and anserine remove a small fraction of the bound copper from the serum albumin in the circulation and then take part in its transport to the kidneys and specific regions of the brain of individuals with Wilson's disease.

Acknowledgements

We thank the Biomedical ESR Center for the use of the ESR spectrometer. This investigation was supported by a Biomedical Research Support Grant from The Medical College of Wisconsin, NIH Grant 20737 and NIH Grant RR-01008.

References

1. Walshe, J.M. (1967) Brain, 90, 149-176.
2. Boas, J.F., Pilbrow, J.R. and Smith, T.D. (1978) Biological Magnetic Resonance, Vol. 1, Berliner, L.J. and Reuben, J., eds., pp. 277-342, Plenum Press, New York.
3. Brown, C.E. and Antholine, W.E., J. Chem. Soc. Dalton Trans., in press.
4. Brown, C.E. and Antholine, W.E., J. Phys. Chem., in press.
5. Viola, R.E., Hartzell, C.R. and Villafranca, J.J. (1979) J. Inorg. Biochem., 10, 281-292.
6. Viola, R.E., Hartzell, C.R. and Villafranca, J.J. (1979) J. Inorg. Biochem., 10, 293-307.
7. Margolis, F.L., Grillo, M., Brown, C.E., Williams, T.H., Pitcher, R.G. and Elgar, G.J. (1979) Biochim. Biophys. Acta, 570, 311-323.
8. Perry, T.L., Hansen, S., Tischler, B., Bunting, R. and Berry, K. (1967) New England J. Med., 277, 1219-1226.
9. Perry, T.L., Hansen, S., and Love, D.L. (1968) Lancet, June 8, 1229-1230.
10. Van Heeswijk, P.J., Trijbels, J.M.F., Schretlen, E.D.A.M., Van Munster, P.J.J. and Monnens, L.A.H. (1969) Acta Paediat. Scand., 58, 584-592.
11. Murphey, W.H., Patchen, L., Lindmark, D.G. (1972) Clin. Chim. Acta, 42, 309-314.
12. Murphey, W.H., Lindmark, D.G., Patchen, L.I., Housler, M.E., Harrod, E.K., and Mosovich, L. (1973) Pediat. Res., 7, 601-606.
13. Bessman, S.P. and Baldwin, R. (1962) Science, 135, 789-791.
14. Tocci, P.M. and Bessman, S.P. (1967) Amino Acid Metabolism and Genetic Variation, Nyhan, W.L., ed., pp. 161-168, McGraw-Hill, New York.
15. Brown, C.E., Margolis, F.L., Williams, T.H., Pitcher, R.G. and Elgar, G.J. (1979) Arch. Biochem. Biophys., 193, 529-542.