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DIFFERENCE CIRCULAR DICHROISM STUDIES OF COPPER AND NICKEL BINDING TO D-PENICILLAMINE IN THE PRESENCE OF HUMAN SERUM ALBUMIN

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Abstract—The binding of copper and nickel to D-penicillamine in the presence of human serum albumin (HSA) has been studied using difference circular dichroism (CD). The difference CD spectra due to penicillamine in HSA–Cu^{2+}–penicillamine systems at pH 6 are indicative of ternary complexes between HSA, copper and penicillamine. On the other hand, the difference spectra due to penicillamine in HSA–Ni^{2+}–penicillamine systems resemble that due to the Ni^{2+}–penicillamine complex, indicating that mostly binary complexes are formed. The formation of stable complexes between penicillamine and copper or nickel (whether they are of the binary or mixed ligand type) may be important in the therapeutic action of this drug.

Copper is an essential nutritional element for animals and is present at the active site of such enzymes as lysyl and monoamine oxidases and superoxide dismutase. The nutritional essentiality (but not the physiological role) of nickel has been demonstrated recently [1]. Although these elements are essential, their presence in more than the necessary amounts leads to some toxic and pathological conditions. Wilson's disease is a hereditary metabolic disorder characterized by abnormally elevated levels of copper in liver, brain, kidneys and blood. Serum (and synovial plasma) copper levels are elevated and glutathione (GSH) levels are lowered in several inflammatory diseases such as rheumatoid arthritis [2]. Excess amounts of copper can inhibit glutathione reductase which lowers GSH levels and leads to toxic effects such as membrane damage. Nickel is known to be a carcinogen in man and experimental animals [3].

The exchangeable (or loosely bound) copper in serum is associated with the transport protein albumin. Human serum albumin (HSA) is also known to be the principal nickel binding protein. Both these metals form a square-planar complex at least with the N-terminal tripeptide sequence of bovine, human, rabbit and rat serum albumins [4–10]. However, when the metal ion (Cu^{2+} or Ni^{2+}) concentration in serum reaches much higher than normal levels, both the primary and secondary metal binding sites may be saturated and free metal ions can be present in the serum. Chelation therapy is then useful to block the toxic effects due to the excess of free metal ions. D-Penicillamine (β,β-dimethyl cysteine) is effective in treating both Wilson's disease and rheumatoid arthritis [11, 12]. D-Penicillamine (Pen) forms stable complexes with a variety of metal ions including Cu^{2+} and Ni^{2+}.

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Complex formation between Cu^{2+} and Pen has been studied quite extensively [13–19]. The complex formed between copper and Pen depends very much upon the conditions. At neutral pH and in the presence of chloride ions, the major species formed is violet in color and has mixed valence stoichiometry [13, 14, 16, 19]. From X-ray crystal structure studies, Birker and Freeman [20] determined that the mixed valence complex has the stoichiometry $[Cu_8^{(I)}Cu_6^{(II)}-(Pen)_{12}Cl]^{5-}$. The mixed valence copper chelate of Pen has exceptional stability compared to various thiol chelates of copper and has an absorption maximum in the 520–530 nm region.

To understand the role of Pen as a detoxifier of metal ions, it is necessary to know the type and nature of the complexes that are formed under physiological conditions. In the serum, Pen has to compete with natural ligands such as albumin and amino acids such as histidine. On the basis of spectroscopic studies, it has been concluded that ternary complexes of Cu^{2+}, Pen and amino acids are formed [21]. However, it has been reported that Pen is unable to remove the albumin-bound copper [19]. In this report, we present a circular dichroism study of the complexes formed between Pen and Cu^{2+} or Ni^{2+} in the presence of HSA.

MATERIALS AND METHODS

Reagent grade chemicals were used. D-Penicillamine (Sigma) and crystalline human serum albumin (Miles Laboratories) were used without further purification. The typical protein concentrations (estimated from the absorbance at 279 nm) of HSA solutions were of the order of 100 μM. The pH of the HSA solution (in 0.15 M NaCl) for binding experiments with copper was kept at 6.0 to avoid the formation of any oxo or hydroxo complexes. On the other hand, for experiments with Ni^{2+}, the pH of the albumin solution [0.1 M NaCl, 50 mM 4-(2-hydroxy-

ethyl)-1-piperazine-ethanesulfonic acid (HEPES)] was 7.4. It should, however, be noted that peptide deprotonation by Ni^{2+} is not complete until above pH 10.2. The albumin solutions were kept under nitrogen. The metal ion concentrations (about 100 mM) in stock $CuSO_4$ and $NiCl_2$ were estimated using atomic absorption spectroscopy.

The circular dichroism (CD) spectra were recorded on a JASCO 40 circular dichroism instrument using 50 mm cells at room temperature (25°). The JASCO 40 spectrometer was calibrated using camphor-10-sulfonic acid. The spectrometer was interfaced to a Nicolet 1180 computer, and each spectrum was the result of six or more accumulations. The concentration of Pen solutions was 100 mM. The binding experiments were performed by the addition of Cu^{2+} (or Ni^{2+}) and/or Pen stock solutions in microliter amounts using a Hamilton microsyringe with a constant delivery adaptor to 12 ml of HSA solution in the cell to obtain the desired molar ratios (relative to HSA). After each addition the solutions were mixed well with a magnetic stirrer while maintaining the solution under nitrogen. The difference CD spectra due to n molar equivalents of Pen were obtained by recording the CD spectrum due to HSA and n molar equivalents of Cu^{2+}/Ni^{2+} and storing the spectrum (spectrum a), recording the CD spectrum due to HSA and n molar equivalents each of Cu^{2+}/Ni^{2+} and Pen and storing the spectrum (spectrum b), and then subtracting spectrum a from spectrum b on the computer.

RESULTS AND DISCUSSION

Figure 1 shows the difference CD spectra due to one, two and three molar equivalents of Pen. In the 300–700 nm region studied, there are two extrema, a very small negative band around 432.5 nm and a relatively large positive band at about 620 nm. The amplitude of the extremum at 620 nm increased considerably upon the addition of increasing amounts of Pen. On the other hand there was only a very small increase in the amplitude of the negative extremum on increasing the molar equivalents of Pen. This may, however, represent an increase in the width and intensity of the neighboring positive extremum. In contrast to Cu^{2+}, the difference spectrum due to Pen for Ni^{2+} consisted of two negative bands and one positive band. The negative bands were centered at 508–518 nm and 325–330 nm while the positive band was located in the 435–445 nm region (Fig. 2). There was a gradual increase in the amplitudes of these extrema as the Pen (and Ni^{2+}) to HSA molar ratio increased.

Circular dichroism spectra due to induced d-d transitions are observed when copper and nickel bind to proteins [8]. It is our experience that, at equimolar amounts of Cu^{2+} and HSA, the difference CD spectrum due to Cu^{2+} has two negative bands at about 540–550 nm and 330–345 nm and one positive band in the 465–470 nm region, under the pH conditions of the present experiment.* There was a slight increase in the amplitudes of these extrema

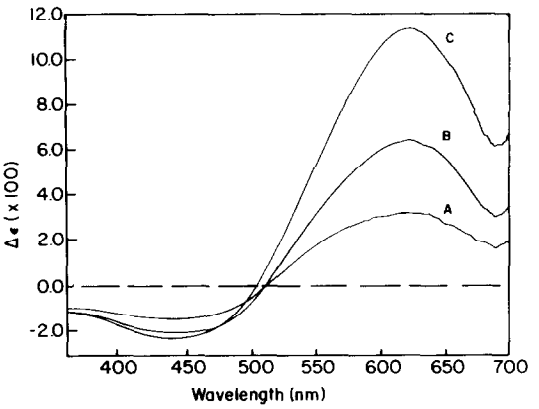

Fig. 1. Difference circular dichroism spectra due to penicillamine for the binding of copper to Pen in the presence of albumin. HSA concentration was 130.7 μM (0.1 M NaCl, pH 6.0). Each spectrum is the result of six accumulations. The Pen/HSA molar ratios are 1 for spectrum A, 2 for spectrum B, and 3 for spectrum C.

when the molar ratio was increased to 2. A further increase in the minimum at 540–550 nm may be due to the appearance of a maximum around 638–640 nm. There was a further gradual increase in the amplitude of the maximum for further increase in the $[Cu^{2+}]$/HSA molar ratio (not shown).

Complex formation between Pen and Cu^{2+} has been discussed in earlier work [13, 22]. The presence of a positive extremum at 620 nm and a very small negative extremum at about 432.5 nm means that Pen forms mixed complexes with Cu^{2+} and HSA since, in the difference spectra shown in Fig. 1, the d-d absorptions due to HSA and Cu^{2+} (and no Pen) have been subtracted. The steady increase at 620 nm in Fig. 1 on going from [HSA-1 Cu^{2+}-1 Pen] to [HSA-3 Cu^{2+}-3 Pen] probably indicates the same type of chelation by Pen with HSA–nCu^{2+} complexes, n

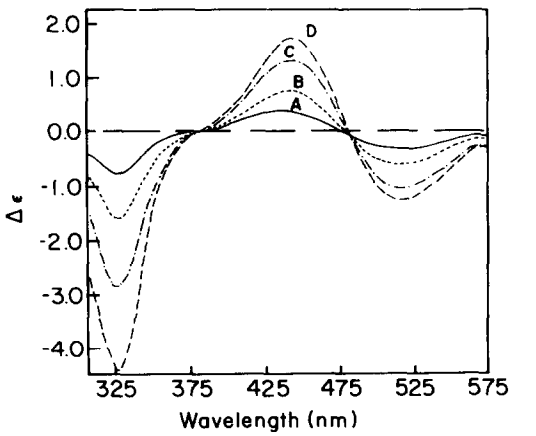

Fig. 2. Difference circular dichroism spectra due to penicillamine for the binding of nickel to Pen in the presence of HSA. HSA concentration was 130.4 μM (0.1 M NaCl, 0.05 M HEPES, pH = 7.4). Each spectrum was the result of six accumulations. The Pen/HSA molar ratios were 1 for spectrum A, 3 for spectrum B, 4 for spectrum C, and 6 for spectrum D.

* P. Mohanakrishnan and C. F. Chignell, unpublished results.

Table 1. Difference circular dichroism due to penicillamine*

Metal	No. of molar equivalents of Pen	λ_1 (nm)	$\Delta\varepsilon_{\lambda_1}$ †	λ_2 (nm)	$\Delta\varepsilon_{\lambda_2}$ †	λ_3 (nm)	$\Delta\varepsilon_{\lambda_3}$ †
Copper	1	620	3.153×10^{-2}	432.5	-1.483×10^{-2}		
	2	620	6.365×10^{-2}	432.5	-2.08×10^{-2}		
	3	620	11.286×10^{-2}	432.5	-2.334×10^{-2}		
Nickel	1	508	−0.305	435.0	0.361	327.5	−0.774
	2	513.5	−0.600	441.0	0.723	331.5	−1.603
	4	516.5	−1.055	445.0	1.302	326.5	−2.877
	6	516.5	−1.547	444.0	1.715	326.5	−4.426

* Difference circular dichroism due to n molar equivalents of Pen = CD of (HSA-n metal-nPen) − CD of (HSA-n metal).

† $\Delta\varepsilon$ values were calculated using a molecular weight of 67,000 daltons for HSA.

being 1 to 3. Since only one molar equivalent of Cu^{2+} can bind at the N-terminal site, this also means that Pen forms HSA–nCu^{2+}–nPen mixed complexes despite the nature of the copper binding site of albumin. The decrease of the absorbance at 420 nm for copper–albumin (1:1) complex on addition of equimolar amounts of Pen has earlier been explained by Sugiura and Tanaka [16] as due to the formation of the mixed albumin–copper–Pen complexes. These authors have also demonstrated the formation of mixed BSA–copper–Pen complexes by performing gel filtration on a Sephadex G-25 column of BSA–Cu^{2+}–Pen systems using [^{3}H]Pen and $^{64}Cu^{2+}$.

According to McCall *et al.* [23] the actual mechanism of action of Pen during therapy is probably more complex than a simple chelation of copper by Pen in order to account for the observed increase in copper excretion. Laurie and Prime [19] state that Pen is unable to mobilize Cu^{2+} that is bound to albumin. The results of the present study, as well as those of Sugiura and Tanaka [16], clearly demonstrate the formation of mixed complexes involving Pen, Cu^{2+} and HSA. Since albumin is a carrier protein, it is quite probable that the mixed complexes might be intermediates in the mobilization of copper. The difference between the results by different groups may be due to the different experimental conditions employed.

The difference CD spectra due to Ni^{2+} alone in Ni^{2+}–albumin complexes at pH 7.4 exhibit a negative extremum at 473 nm and a positive extremum in the 407–410 nm region.* The amplitudes of these bands increase steadily when the [Ni^{2+}]/HSA molar ratio increased from 1 to 3. The difference spectrum due to Ni^{2+} alone in the Pen–Ni^{2+} complex (1:1) at pH 7.4 has a negative band at about 517 nm ($\Delta\varepsilon = -0.336$), a strong positive band at about 445 nm ($\Delta\varepsilon = 0.444$), and second negative band at 337.5 nm ($\Delta\varepsilon = -0.871$). The results from the difference CD due to Pen for HSA–Cu^{2+}–Pen and HSA–Ni^{2+}–Pen systems are listed in Table 1. From the data listed for HSA–Ni^{2+}–Pen, it is obvious that, at least at physiological pH, the complexes formed are just binary and not mixed (ternary) complexes involving albumin.

The present study clearly demonstrates the *in vitro* formation of ternary complexes involving Pen, Cu^{2+} and HSA. However, the role of ternary complexes in mobilizing albumin-bound copper *in vivo* has to be determined experimentally. Until this has been done, it is not possible to ascertain the therapeutic importance of these mixed complexes.

* P. Mohanakrishnan and C. F. Chignell, unpublished results.

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