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Detection and investigation of the molecular nature of low-molecular-mass copper ions in isolated rheumatoid knee-joint synovial fluid

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Abstract **Low-molecular-mass copper(II) species have been detected and quantified in ultrafiltrates ($n = 7$) of rheumatoid synovial fluid (SF) by a highly-sensitive HPLC-based assay system with the ability to determine Cu(II) concentrations of $< 10^{-7}$ mol·dm^{-3}. High field ^{1}H NMR spectroscopy demonstrated that addition of Cu(II)$_{(aq.)}$ to isolated samples of RA SF ultrafiltrates resulted in complexation by histidine > alanine > formate > threonine > lactate > tyrosine > phenylalanine, their effectiveness in this context being in the given order. CD spectra of Cu(II)-treated samples of intact SF exhibited absorption bands typical of copper(II)-albumin complexes, in addition to a band attributable to a low-molecular-mass histidinate complex (λ_{min} 610 nm). Since both albumin and histidine are potent radical scavengers, these results indicate that any $^{\bullet}$OH radical generated from bound copper ions will be 'site-specifically' scavenged. Hence, low-molecular-mass copper complexes with the ability to promote the generation of $^{\bullet}$OH radical which can then escape from the metal ion co-ordination sphere (and in turn, cause damage to critical biomolecules) appear to be absent from inflammatory SF.**

Key words: Low-molecular-mass copper; Inflammatory joint disease; HPLC; ^{1}H NMR; Speculation; Synovial fluid

1. Introduction

Much debate has taken place regarding the involvement of oxygen-derived free-radical species in the pathogenesis of inflammatory joint diseases [1,2]. It is now well established that inflammation increases plasma levels of caeruloplasmin, an 'acute-phase' cuproprotein with associated antioxidant activity [3,4]. The higher antioxidant activity of the blood serum of patients suffering from rheumatoid arthritis (RA) relative to that of control serum has been attributed to the elevated level of caeruloplasmin present in the former [5].

Copper(II) species are known to exert deleterious effects in physiological environments. Indeed, copper ions have been implicated in mediating the production of oxygen-derived free radical species in vivo [6]. Caschin [7] has reported that the labile Cu(II) complex of glycylglycine causes an erosive arthropathy in guinea pigs, and has speculated that this may arise from the Cu(II) chelate-catalysed generation of oxygen-derived free radical species within the joint. Moreover, the copper ion-catalysed generation of hydrogen peroxide (H_2O_2) from homocysteine has been shown to give rise to endothelial cell injury, and an equivalent detrimental effect is caused by caeruloplasmin in the presence of homocysteine [8].

Copper(II) complexes of many anti-inflammatory compounds have exhibited enhanced therapeutic activity, relative to the parent ligands, in selected model systems [9–12]. Furthermore, in two models of acute inflammation (carrageenan-induced foot pad oedema and pleurisy), a copper deficiency was found to exert a pro-inflammatory effect [13]. However, using the 'phenanthroline assay', attempts to detect copper ions in a form which promotes the generation of $^{\bullet}$OH or 'crypto-$^{\bullet}$OH' radical species in fresh serum or SF collected from patients with RA have been unsuccessful [14].

Some anti-rheumatic drugs, including glucocorticosteroids, oligomeric 1:1 gold(I)-thiolate complexes and D-penicillamine, have been associated with lowered serum copper levels [15,16]. In male Wistar rats, administration of disodium aurothiomalate resulted in a significant reduction in the serum levels of copper with a corresponding five-fold increase in kidney copper deposition [16]. Gold(I)-thiolate complexes and D-penicillamine have been hypothesised to reduce total biofluid copper levels via their interaction with Cu(II) ions bound to the N-terminal binding site located on albumin [15]. A mixture of histidine, cysteine and Cu(II) ions has been demonstrated to mimic disodium aurothiomalate in inhibiting the sulphydryl-disulfide interchange reaction-mediated denaturation of human gamma globulin, bovine serum albumin and diluted human serum [17].

In view of the controversy associated with the role of Cu(II) complexes in rheumatoid arthritis, we have investigated the chemical nature of a number of Cu(II) species present in isolated RA SF samples. Moreover, we report for the first time the detection and quantification of trace levels (ca. 10^{-7} mol·dm^{-3}) of copper ions in ultrafiltrates of this biofluid by a novel high performance liquid chromatographic (HPLC) method. These studies are of particular relevance to the pro- and/or anti-inflammatory properties of Cu(II) species in RA, and the mode of action of potential anti-inflammatory copper ion complexes.

2. Materials and methods

2.1. Reagents

Acetic acid, methanol, and sodium acetate were obtained from BDH Chemicals Ltd (Poole, Dorset, UK). Ammonium acetate and tetrabutylammonium bromide were purchased from Aldrich Chemical Co (Poole, Dorset, UK). Analytical grade $CuCl_2 \cdot 2H_2O$ was obtained from Hopkin and Williams Ltd. (Chadwell Heath, Essex, UK), and 2-(5-Bromo-2-pyridylazo)-5-(*N*-propyl-*N*-sulphopropyl-amino)-phenol (5-Br-PAPS) was purchased from Dojindo Chemical Co. (Japan). Distilled and de-ionised water (ELGA maxima water purifier) was utilised throughout these investigations.

2.2. Biofluid samples

Synovial fluid was aspirated for therapeutic reasons from the knee-

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joints of patients (n = 7) attending a rheumatology clinic who had been previously diagnosed as having RA as assessed according to the criteria of the American Rheumatism Association (ARA). RA sera (n = 7) were obtained in a similar manner. Control serum samples (n = 7) were obtained from normal healthy human volunteers. Biofluid samples were centrifuged at 7,000 × g for 15 min in order to remove cells and cell debris, and where required, ultrafiltered at 5,000 × g for 30 min using Millipore ultrafiltration devices with a membrane molecular weight cut-off of 5 kDa. All centrifugations were performed at 5°C. For the quantification of copper ion in inflammatory synovial fluid, serum and control normal serum ultrafiltrates by HPLC, samples were centrifuged and ultrafiltered within 1 h of aspiration.

2.3. NMR measurements

NMR spectra were recorded at a probe temperature of 25°C on a JEOL JNM-GSX 500 spectrometer operating in quadrature detection mode at 500.16 MHz for ^1H. Typically, 0.60 ml of each SF ultrafiltrate sample was placed in a 5-mm diameter NMR tube, and 0.07 ml of ^2H_2O was added to provide a field frequency lock. For intact biofluid samples, the broad protein resonances were suppressed by the Hahn spin-echo pulse sequence D[90°x–t–180°y–t–collect], where t = 60 ms. Pulsing conditions for single-pulse spectra were: 45° pulse angle, 2.730 s acquisition time, 2.270 s pulse delay, 32,768 computer points and 128 transients. Chemical shifts were referenced to external sodium 3-(trimethylsilyl)-[1,1,2,2-d_4]-propionate (d = 0.00 ppm). The intense H_2O signal was suppressed by the application of secondary irradiation at the water frequency. Microlitre aliquot additions of a 1.0×10^{-2} mol·dm^{-3} aqueous solution of copper(II) chloride were made using a pre-calibrated micropipette.

2.4. Ion-pair reversed-phase high performance liquid chromatographic analysis of copper(II) in biofluid ultrafiltrate samples

Synovial fluid ultrafiltrates (0.38 ml) were treated with 10 μl of ammonium acetate buffer (1.00 mol·dm^{-3}, pH 3.80). The Cu(II) ion-chelating pre-column derivatising agent 5-Br-PAPS (10 μl of a 5.00×10^{-3} mol·dm^{-3} aqueous solution) was added and the resulting solution thoroughly mixed. The sample was then heated in stoppered tubes at 95°C on a water bath for a period of 20 min to allow equilibration and, on cooling, 20 μl aliquots were used for HPLC analysis.

HPLC analysis was performed with an Applied Chromatography Systems 351 isocratic pump coupled to a Rheodyne injector fitted with a 20 μl loop. Detection was made using an ACS 750/16 variable wavelength detector coupled to an SE 120 chart recorder. Detection was monitored at a wavelength of 570 nm, using a flow-rate of 0.6 ml/min with a methanol/water [70:30 (v/v)] eluant containing 1.00×10^{-4} mol·dm^{-3} 5-Br-PAPS, 1.00×10^{-2} mol·dm^{-3} sodium acetate and 5.00×10^{-2} mol·dm^{-3} tetrabutylammonium bromide (ion-pair reagent). The eluant was buffered to pH 3.90 prior to the addition of methanol. A Spherisorb S5-ODS 1 column (250 mm × 4.5 mm) was employed in conjunction with a Spherisorb S5-ODS 1 guard column.

2.5. Circular dichroism spectroscopy

CD measurements were performed using quartz cells (1 cm) on a JASCO J600 spectrophotometer operating at ambient temperature.

3. Results

The HPLC-based method for the determination of low-molecular-mass (ultrafilterable) copper ions in biofluids described herein was developed and utilised since previously available assay systems do not appear to provide the sensitivity required for such investigations (ca. ≤1×10^{-7} mol·dm^{-3}). Analysis of Cu(II) in fresh RA SF and serum ultrafiltrate samples by this method revealed the presence of low-molecular-mass copper ion species in 6 out of a total of 7 samples examined (Fig. 1 and Table 1). The mean copper ion concentration for SF was $1.25 \pm 0.95 \times 10^{-7}$ mol·dm^{-3} (mean ± standard deviation) and for RA sera was $2.59 \pm 1.28 \times 10^{-7}$ mol·dm^{-3}. The mean cop-

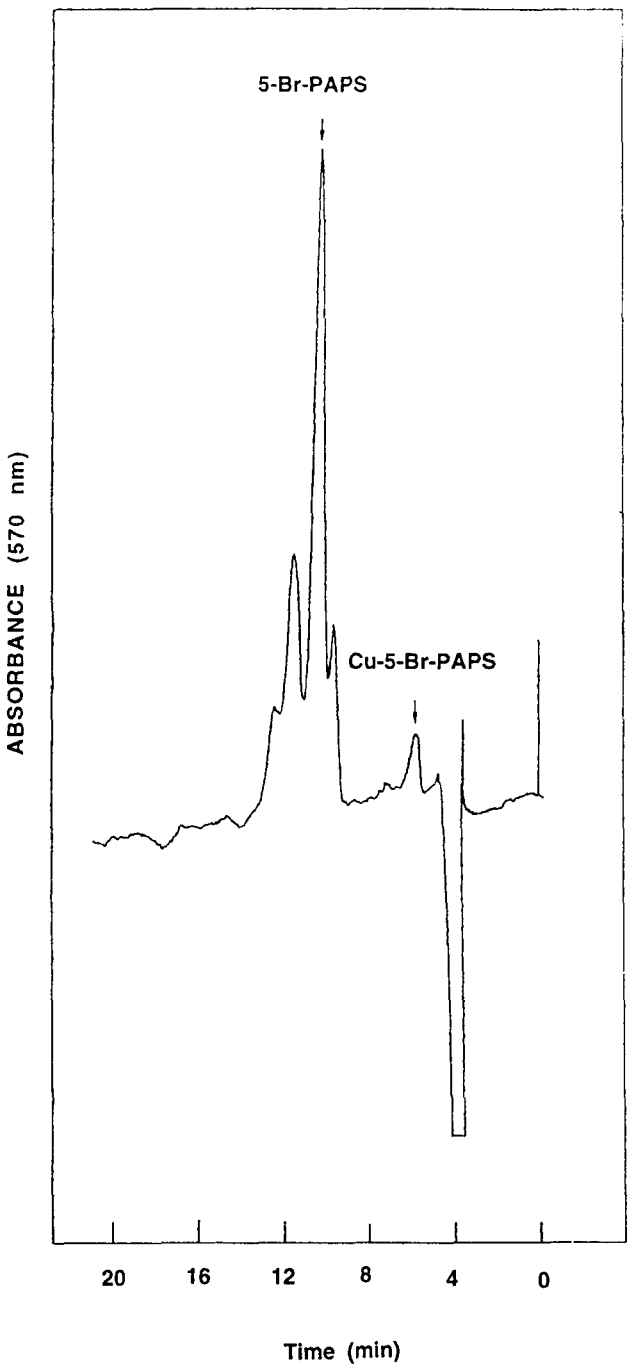

Fig. 1. HPLC analysis of copper(II) ions in isolated rheumatoid synovial fluid using 5-Br-PAPS as chromophore. A methanol/water (70:30, v/v) mobile phase was employed with a flow-rate of 0.60 ml/min. Detection was at 570 nm.

Table 1
Levels of copper ions in normal healthy human serum and rheumatoid arthritic knee-joint synovial fluid and sera

	Control sera	Rheumatoid sera	Rheumatoid synovial fluid
n	7	7	7
Mean age years	36 (28–46)	60 (47–67)	50 (22–76)
Ultrafilterable copper* ($\times 10^{-7}$ mol·dm^{-3})	3.45 ± 1.62 (1.5–5.6)	2.59 ± 1.28 (0.00–5.1)	1.25 ± 0.95 (0.00–3.0)

*Mean ± S.D.

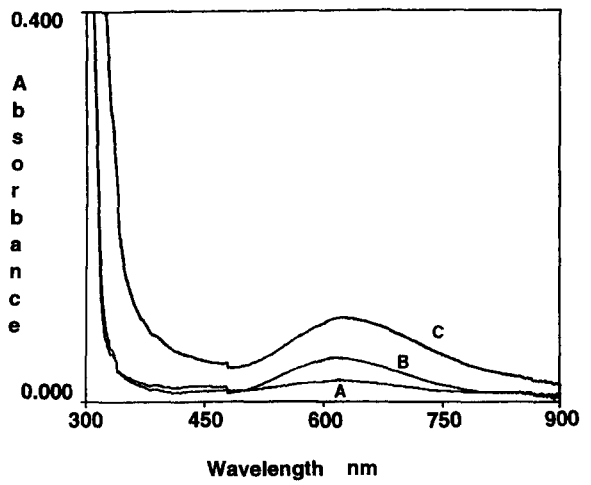

Fig. 2. Visible absorption spectra of RA SF ultrafiltrate before (A), and after addition of $Cu(II)_{(aq.)}$ at a concentration of 2.50×10^{-4} mol·dm^{-3} (B), and 5.00×10^{-4} mol·dm^{-3} (C).

per ion concentration for normal control sera was $3.45 \pm 1.62 \times 10^{-7}$ mol·dm^{-3}, a value which is comparable to previously reported levels [18] and significantly higher than that of RA serum ($P < 0.05$) and SF ($P < 0.01$) (one-way analysis-of-variance (ANOVA) performed on untransformed data; further comparisons of sample group means were made using Tukey's test). Doubly-distilled water, together with relevant buffer and reagent solutions served as control samples for this method and no copper ions were detectable therein. Data acquired on rheumatoid knee-joint SF samples are therefore at the lower limit of sensitivity for assays utilised previously for the detection of oxygen radical-generating low-molecular-mass copper ion species in RA SF. Without any form of pre-concentration, this assay system has the ability to detect copper ions at a lower limit of 5×10^{-8} mol·dm^{-3}.

Visible electronic absorption spectrophotometry was employed to primarily establish the molecular nature of the low-molecular-weight copper species present in RA SF. Fig. 2 shows the visible absorption spectrum of RA SF ultrafiltrate to which has been added increasing concentrations of aqueous copper(II) chloride. As expected, the visible absorption spectrum of RA SF ultrafiltrate exhibited no distinct features. Addition of increasing levels of $Cu(II)_{(aq.)}$ to these ultrafiltrates gave rise to the development of a broad band with a λ_{max} value of ca. 610 nm which was the major feature of all samples investigated. Previous investigations have established that this absorption band is characteristic of copper(II)-histidinate chelates [19]. No other absorption bands were detectable throughout the entire wavelength range investigated.

Circular dichroism (CD) spectra of rheumatoid synovial fluid ultrafiltrate samples exhibited no major features. However, the addition of $Cu(II)_{(aq.)}$ (5.00×10^{-3} mol·dm^{-3}) to these ultrafiltrates resulted in the appearance of an absorption minimum with a λ_{min} value of ca. 610 nm (Fig. 3), again characteristic of low-molecular-mass copper(II)-histidinate complexes [19]. As expected, CD spectra of cell-free, intact rheumatoid synovial fluids exhibited no characteristic features. However, addition of $Cu(II)_{(aq.)}$ to these samples resulted in albumin-binding of this transition metal ion, a phenomenon revealed by the appearance of absorption minima and maxima located at wavelengths of ca. 320, 400, 410 and 575 nm. Furthermore, a broad shoulder located at ca. 610 nm in these spectra is indicative of the presence of low-molecular-mass Cu(II)-histidinate species [19].

Further useful information concerning the chemical nature of low-molecular-mass copper(II) complexes present in inflammatory SFs was provided by high field ^{1}H NMR spectroscopy. The 500 MHz ^{1}H NMR spectrum of a typical isolated RA SF ultrafiltrate sample is shown in Fig. 4a. Equilibration of these macromolecule-free samples with increasing sequential concentrations of $Cu(II)_{(aq.)}$ for a period of 1 h at ambient temperature

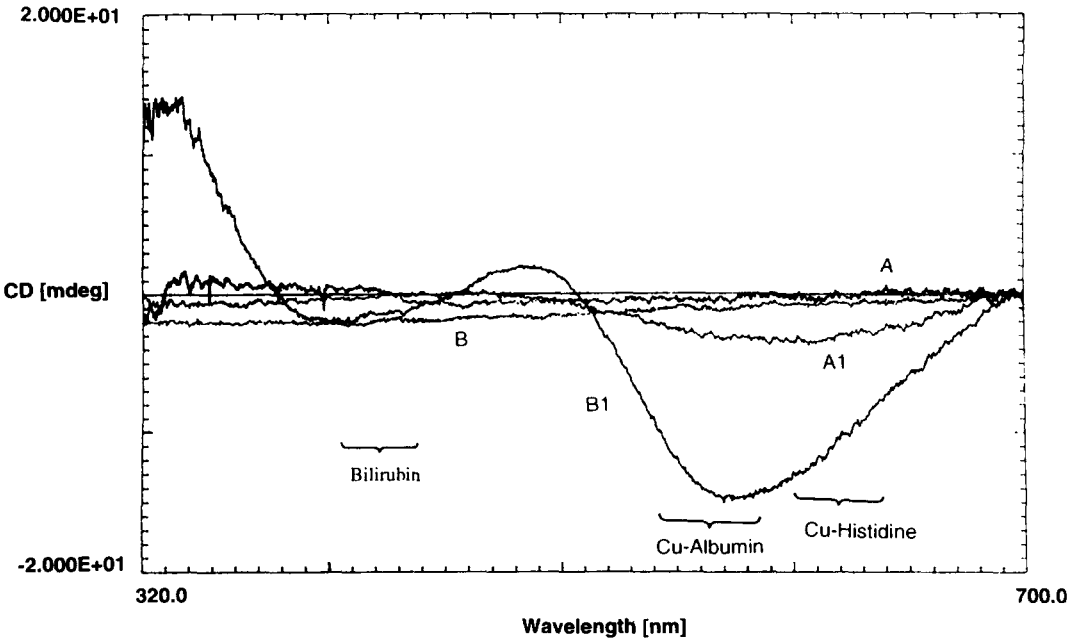

Fig. 3. Circular dichroism spectra of (A) RA SF ultrafiltrate; (A1) as (A), but after the addition of 5.00×10^{-3} mol·dm^{-3} $Cu(II)Cl_2$; (B) intact RA SF; (B1) as (B) but after the addition of 5.00×10^{-3} mol·dm^{-3} $Cu(II)Cl_2$. Spectrum B1 contains absorption bands characteristic of the Cu(II)-albumin complex.

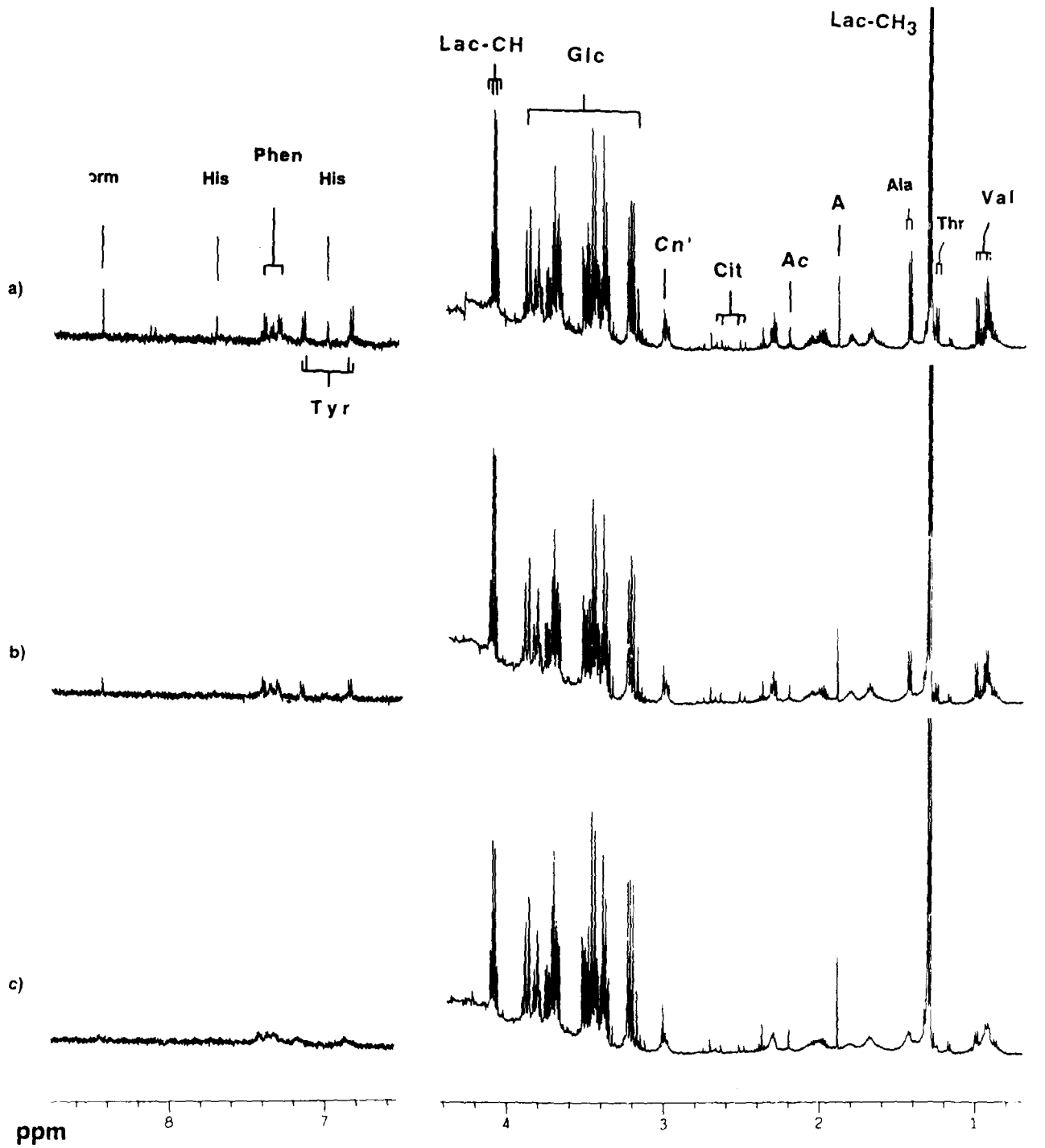

Fig. 4. 500 MHz ^{1}H NMR spectra of (a) RA SF ultrafiltrate, and the same sample following equilibration with Cu(II) at concentrations of (b) 1.40×10^{-5} and (c) 7.20×10^{-5} mol·dm^{-3}. Abbreviations: A, acetate-CH_3; Ac, acetone-CH_3; Ala, alanine-CH_3; Cit, citrate-CH_2; Cn′, creatine N-CH_3; Glc, glucose ring protons; Form, formate-H; His, histidine imidazole ring protons; Lac-CH_3 and CH, lactate-CH_3 and CH groups; Phen, phenylalanine aromatic ring protons; Thr, threonine-CH_3; Tyr, tyrosine aromatic ring protons; Val, valine-CH_3 group.

gave rise to the line-broadening of selected resonances present in their spectra. At an added Cu(II) concentration of 1.43×10^{-5} mol·dm^{-3}, complexation by histidine occurs resulting in the complete disappearance of its imidazole ring proton resonances from the spectrum. This selective removal of the histidine C_2H and C_4H imidazole ring proton resonances reflects the powerful Cu(II) chelating ability of this amino acid [19]. At this concentration of added Cu(II), the intensity of the formate resonance (δ = 8.46 ppm) also diminished and an unassigned resonance located at 7.98 ppm disappeared from the spectrum. In the high field spectral region, corresponding reductions in the intensities of the alanine and threonine resonances were readily detectable. Further addition of Cu(II) (to a total concentration 7.15×10^{-5} mol·dm^{-3}) gave rise to a total broadening of the alanine and threonine methyl group doublet signals, indicative of their involvement in complexation of this metal ion. Indeed, at this level of added Cu(II), resonances in the aromatic region were completely removed from the spec-

trum (an observation revealing further Cu(II) ion complexation by tyrosine and phenylalanine), whereas the line-widths of resonances attributable to glucose, citrate and 3-D-hydroxybutyrate were not significantly influenced. At an added Cu(II) concentration of 2.86×10^{-4} mol·dm^{-3}, much broadening of resonances occurred throughout the entire spectrum.

A typical proton Hahn spin-echo NMR spectrum of intact (macromolecule-containing) RA SF containing 1.00×10^{-4} mol·dm^{-3} added L-histidine is shown in Fig. 5. Incubation of this sample with $Cu(II)_{(aq.)}$ (final concentration 1.00×10^{-4} mol·dm^{-3}) for a period 1 h at ambient temperature resulted in a selective broadening of the imidazole ring proton resonances of pre-added histidine (δ = 7.05 and 7.77 ppm), indicating that this is a primary site for copper(II) ion chelation in inflammatory synovial fluids. Despite the addition of exogenous histidine to the intact RA SF sample, the intensity of the alanine-CH_3 group doublet resonance is also significantly diminished after equilibration with Cu(II). Moreover, the highly-coupled glutamine β- and γ-CH_2 group multiplets (δ = 2.04 and 2.40 ppm) are completely removed from the spectrum after treatment with this concentration of added Cu(II). Prolonged equilibration (12 h) of Cu(II)-loaded RA SF samples (2.40×10^{-4} mol·dm^{-3}Cu(II)) with an excess of the hexadentate chelator EDTA (4.00×10^{-4} mol·dm^{-3}) had no effect on the high frequency (low field) region of spectra, (data not shown), demonstrating that this powerful hexadentate chelator does not have the capacity to remove Cu(II) ions chelated to endogenous histidine under the experimental conditions employed here.

4. Discussion

This investigation was specifically aimed at elucidating the precise molecular nature of non-caeruloplasmin-bound Cu(II) species present in inflammatory SF samples, particularly low-molecular-mass complexes which reportedly promote the generation of $^{\bullet}OH$ radical in the presence of endogenous reductants [1]. Our HPLC-based Cu(II) detection assay, based on the development and subsequent detection of a chromophoric Cu(II)-5-Br-PAPS complex [20], selectively detects and measures low-molecular-mass Cu(II) in 'fresh' rheumatoid SF (analysis was performed within 1 h of collection). Rapid analysis of fresh biofluid samples was necessary to prevent copper ion liberation from proteins via metalloproteinase activity. Addition of metalloproteinase inhibitors such as EDTA or 1,10-phenanthroline precludes the accurate measurement of low-molecular-mass copper(II) since these reagents are powerful chelators of this metal ion. The differences observed between our results and those of previous investigations are explicable by the increase in sensitivity afforded by this technique.

The complexation of this redox-active transition metal ion by albumin in biofluids is a well established phenomenon, and exchange of Cu(II) ions from albumin to low-molecular-mass chelators has been speculated to give rise to the re-distribution of Cu(II) species amongst further biofluid components, and also cells. The studies conducted here have provided much useful information concerning the nature of this process and the molecular nature of the species involved. In a previous computational study, a theoretical distribution of Cu(II) species has been determined from the stability constants for complexes of Cu(II) with amino acids present in blood plasma [21]. A major limitation of this approach, however, is its inability to incorporate proteins and peptides into the experimental models employed. In the experiments conducted here, such limitations are clearly circumvented.

CD and visible absorption spectroscopic studies conducted on RA SF ultrafiltrates provided evidence for the complexation

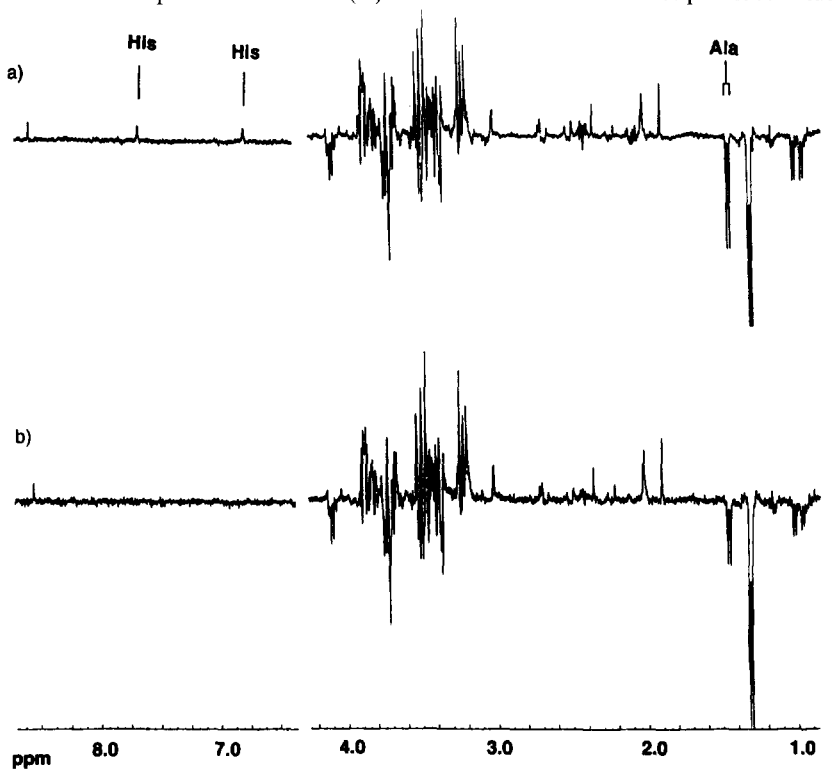

Fig. 5. 500 MHz ^{1}H Hahn spin-echo NMR spectra of RA SF containing added exogenous His (2.0×10^{-4} mol·dm^{-3}) (a), and the same sample following incubation with Cu(II) at a concentration of 1.0×10^{-4} mol·dm^{-3} (b). Abbreviations: as Fig. 4.

of added Cu(II) by histidine. Moreover, ^{1}H NMR studies performed on protein-free (ultrafiltered) synovial fluid demonstrated that complexation of added Cu(II) ion by the amino acids histidine and alanine predominates. This observation is consistent with the calculated formation constant of 10^{17} for the ternary Cu(II)·HisO·AlaO· species. Indeed, in the computational speciation study of Furnival et al. [21], histidine was found to be incorporated in all copper(II)-amino acid complexes predicted to be present in the low-molecular-mass fraction of human blood plasma.

Decreased serum histidine levels have been reported in patients with rheumatoid arthritis [22]. However, with mean normal and rheumatoid levels of 1.192×10^{-4} and 1.005×10^{-4} mol·dm^{-3} respectively, little Cu(II) binding capacity is lost. Moreover, rheumatoid synovial fluid histidine levels have been reported to be higher than those of matched sera [23].

Previous studies have demonstrated that H_2O_2 attack on Cu(II)-histidine species generates four products: asparagine, aspartic acid, aspartylurea, and formyl asparagine [24], products which, to the best of our knowledge, do not display any pro-inflammatory properties. In addition, Cu(II)-histidinate complexes have been shown to exhibit superoxide dismutase (SOD)-mimetic activity [25]. The rate constant for the dismutation of $O_2^{\bullet-}$ by Cu(II)-His species is of a similar order of magnitude to that of SOD itself. Further additions of Cu(II) solutions to rheumatoid SFs resulted in selective broadenings of the threonine, tyrosine and phenylalanine signals, the latter two aromatic amino-acids being effective hydroxyl radical scavengers (second order rate constants, $k_2 = 3.70$ and 3.50×10^9 mol^{-1}·dm^3·s^{-1}, respectively).

CD and ^{1}H Hahn spin-echo NMR experiments indicate that the protein fraction of intact SF complexes a large proportion of added Cu(II) ions. Addition of 2.00×10^{-4} mol·dm^{-3} Cu(II) to intact synovial fluid resulted in only a marginal broadening of the ^{1}H NMR signals of low-molecular-mass components present in the spectrum. However, addition of these levels of added Cu(II) to protein-free RA SF ultrafiltrates gave rise to an extensive broadening of several resonances present in their spectra. The concentration of albumin in rheumatoid synovial fluid is ca. 3.1×10^{-4} mol·dm^{-3} [26], facilitating binding of up to 100% of added Cu(II) ions. However, under the experimental conditions employed in this study, we detected Cu(II) ion complexation by histidine, indicating that significant quantities of added Cu(II) are chelated by this endogenous ligand.

Of special relevance to this study is the ability of the Cu(II) complexes thus formed to exert either a therapeutic or deleterious role in the inflammatory process. From previous work, Cu(II) bound to albumin is redox-active and participates in mediating the generation of hydroxyl radical via interaction with hydrogen peroxide and superoxide. However, this process has been shown to result in oxidative damage only to albumin which effectively acts as a powerful $^{\bullet}$OH radical scavenger, i.e. a 'site-directed' scavenging of $^{\bullet}$OH radical by this protein.

5. Conclusions

Low levels of low-molecular-mass Cu(II) species were demonstrated to be present in 'fresh' rheumatoid synovial fluid. Using Hahn spin-echo ^{1}H NMR, visible absorption and CD spectroscopies, we have demonstrated that Cu(II) associates with both protein and low-molecular-mass species in cell-free macromolecule-containing SF. Under the conditions employed, we detected Cu(II) complexation by albumin and amino acids (specifically, histidine and alanine). In macromolecule-free SF, very small quanties of Cu(II) were required to selectively and totally broaden the histidine resonances, indicating its preferential complexation by this amino acid and reflecting the important role played by it in Cu(II) homeostasis in vivo.

These data are of significance in the design and screening of new Cu(II)-containing, and potential Cu(II)-chelating compounds as anti-inflammatory/anti-arthritic agents in vivo. Prior knowledge of the interactions of therapeutic agents with biofluid components is especially pertinent to the search for new drugs for the treatment of inflammatory diseases.

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