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The Influence of L-cysteine on the Exchangeable Copper in Blood Plasma

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Approximately 5% of the copper in blood plasma is bound to albumin protein and was earlier shown to be in rapid equilibrium with copper in tissues [1]. Later studies [2, 3] established the presence of an equilibrium between this fraction and copper bound to amino acids in blood plasma as shown by the set of equilibria:

$$Cu^{II} + Albumin \rightleftharpoons [Cu^{II}(Albumin)] \quad (1)$$

$$Cu^{II}(Albumin) + AA^{*} \rightleftharpoons [Cu^{II}(AA)] + Albumin \quad (2)$$

$$Cu^{II} + AA \rightleftharpoons [Cu^{II}(AA)] \quad (3)$$

The amino acid complexes are thought to mediate the transport of copper through biological membranes by virtue of their small molecular size.

Although earlier studies [3] established histidine as the major ligand for Cu in the amino acid fraction, the low concentration of the metal ion and the complex equilibria have defied detailed experimental study to date. Instead, computer simulation models have evolved [4, 5] to delineate the speciation in this LMW fraction and have been put to uses such as determining the influence of chelating drugs on the LMW equilibria [6].

One caveat expressed by the developers [5–7] of the computer models in the neglect of the effect of the amino acid cysteine. This arises because of the lack of quantitative thermodynamic data on the cysteine-copper system, and so cysteine has been omitted from the Cu model calculations and the metal assumed to be in the Cu(II) form. It is noteworthy that cysteine is present in blood plasma (*ca.* 23×10^{-6} mol dm^{-3} [8]) and, from the model computations, ia a major ligand for Zn(II).

Excess cysteine is known to rapidly reduce Cu(II) according to the stoichiometry:

$$4CysH + 2Cu^{II} \rightarrow 1/n[Cu^{I}Cys]_n + Cys{-}Cys + 4H^{+} \quad (4)$$

However, there is an even larger excess of other amino acids in LMW which, by chelation, could presumably sufficiently stabilise the Cu(II) state by lowering the Cu(II)/Cu(I) redox potential.

Recently we have made use of simulated LMW solutions to investigate the interaction between penicillamine ($\beta\beta$-dimethylcysteine) and Cu(II) under physiological conditions [9]. We have now, with the aid of more sensitive instrumentation, extended this simulation to investigate the effect of cysteine, the results are reported here. Our strategy was to use aqueous solutions which had the same ratio of amino acids to exchangeable copper** (Cu_E) as those found in blood plasma. The concentrations in blood plasma were taken from the tabulations used in the computer simulation models [5] (see Table I). Apart from His and Cys the other amino acids, which form similar complexes with similar stability constants, were represented by L-alanine. This seems a reasonable assumption. The ratio of Ala/Cu used was thus *ca.* 3000/1. Substitution of Ala by Gly was found to have no effect on the results. Other individual amino acids that may substantially complex Cu(II) were investigated as a check on their possible influence. The ratio of His/Cu_E used was that in blood plasma, *i.e.* 85/1. Cystine, because of its limited solubility, could only be incorporated at the lowest concentrations. Finally, zinc chloride was added to give a Zn/Cu ratio of 16/1; the computer model predicts that some 80% of the Zn(II) is complexed to cysteine, which in turn would account for *ca.* 70% of the total cysteine [5].

Experimental

L-cysteine (Fluka 'puriss' grade) was used throughout without further purification. All other reagents were of the same or similar grade and used without further purification except for N-ethylmorpholine which was distilled before use [9]. The buffer solutions contained 0.1 mol dm^{-3} N-ethylmorpholine, 'AnalaR' HCl having been added to bring the pH to 7.4. All water used was distilled and de-ionized to a resistivity greater than 3 *M* ohm cm^{-1}. For the anaerobic studies the water was boiled for 15 minutes and cooled under a stream of deoxygenated N_2 and

*Abbreviations used throughout: AA, amino acids; LMW, low molecular weight plasma fraction; CysH, cysteine; Cys, cysteinate; Cys–Cys, cystine; His, histidinate; Ala, alaninate; Gly, Glycinate; DPPH, 2,2-diphenyl-1-picrylhydrazyl; Thr, threoninate; Asc, ascorbate; Cu_E, total exchangeable copper in blood plasma.

**Making the reasonable assumption for Cu(II) complexes that the equilibria (1)–(3) are rapidly established we have based our models on the total exchangeable Cu (*ca.* 1×10^{-6} mol dm^{-3}) even though most of this is initially bound to the albumin (*vide infra*).

TABLE I. Concentrations (mol dm^{-3}) used in the Blood Plasma Models and in Blood Plasma Itself.

	Model Solutions						Blood Plasma [a]
	PM5	PM9	PM10	PM11	PM12	PM13	
Cu[b]	$1 \cdot 10^{-4}$	$1 \cdot 10^{-5}$	$2 \cdot 10^{-5}$	$4 \cdot 10^{-5}$	$1 \cdot 10^{-6}$	$1 \cdot 10^{-6}$	$1 \cdot 10^{-6}$
Zn[b]	$16 \cdot 10^{-4}$	$16 \cdot 10^{-5}$	$32 \cdot 10^{-5}$	$64 \cdot 10^{-5}$	$16 \cdot 10^{-6}$	$16 \cdot 10^{-6}$	$16 \cdot 10^{-6}$
L-His	$85 \cdot 10^{-4}$	$85 \cdot 10^{-5}$	$170 \cdot 10^{-5}$	$340 \cdot 10^{-5}$	$85 \cdot 10^{-6}$	$85 \cdot 10^{-7}$	$85 \cdot 10^{-6}$
L-Ala	$30 \cdot 10^{-2}$	$30 \cdot 10^{-3}$	$60 \cdot 10^{-3}$	$120 \cdot 10^{-3}$	$30 \cdot 10^{-4}$	$30 \cdot 10^{-4}$	$\sim 28 \cdot 10^{-4}$ [c]
L-Cys	–	–	–	–	–	–	$23 \cdot 10^{-6}$
L-Cys	–	–	–	–	–	$40 \cdot 10^{-6}$	$40 \cdot 10^{-6}$
L-Thr	–	–	–	–	–	–	$15 \cdot 10^{-5}$
L-Asc	–	–	–	–	–	–	$43 \cdot 10^{-6}$
pH	7.4	7.4	7.4	7.4	7.4	7.4	7.4
NaCl	0.15	0.15	0.15	0.15	0.15	0.15	0.15

[A] Taken from ref. 5. [b] Added as their chloride salts. [c] Total amino acids.

all solutions were prepared under a blanket of N_2. All cysteine solutions were freshly prepared in de-oxygenated buffer solution and stoppered under a N_2 atmosphere. The cysteine content, after reaction with the Cu solutions, was determined either by Ellman's colorimetric method [10] or by conversion to the carboxymethyl derivative [8] and determination of this with an amino acid auto-analyser (Locarte Mk. IV model with a lower limit of detection of 10^{-9} mol dm^{-3}).

UV/VIS absorption spectra were measured at 37 °C using a Perkin Elmer 555 spectrophotometer. ESR spectra were recorded on solutions frozen in liquid N_2 using a Brüker ER 200D spectrometer operating at an approximate frequency of 9.47 GHz and using the DPPh signal as reference. Circular dichroism spectra were measured at room temperature on a C.N.R.S./Roussel–Jouan Dichrographe Mk. III Instrument.

Results

a) Anaerobic Conditions, 37 °C

Addition of cysteine to plasma model PM5 caused a reduction in UV/VIS, ESR and CD spectral absorptions attributable to Cu(II). Complete reduction of Cu(II) occurred at a Cys/Cu ratio of 5/1. Changes in Cu level had negligible influence on the results, neither did the additional presence of threonine (150×10^{-4} mol dm^{-3}). Interestingly the use of L-ascorbate, instead of Cys, to give the same Asc/Cu_E ratio as in plasma, resulted in no reduction of the Cu(II).

b) Aerobic Conditions, 37 °C

Exposure to air of the final cysteine-containing solutions above in all cases resulted in the full return of the original Cu(II) absorptions. Aqueous air-saturated solutions at 37 °C and 0.15 mol dm^{-3} ionic strength have a dissolved oxygen concentration of $1.8–2.1 \times 10^{-4}$ mol dm^{-3} [11]. This is of the same order as that (excluding the haemoglobin bound O_2) found in blood plasma, $0.55–1.34 \times 10^{-4}$ mol dm^{-3} [12, 13]. Cu(II) is well known to catalyse the oxidation of Cys by O_2, and depicted in the Scheme:

$$\begin{array}{lll} \text{Cysteine} & \text{Cu(II)} & H_2O_2 \\ & & \\ \text{Cystine} & \text{Cu(I)} & O_2 \end{array} \qquad (5)$$

The re-oxidation of Cu(I) to Cu(II) is fast [14–16].

The critical factors in the kinetics of the reaction are hence both the Cys/Cu and O_2/Cu ratios. We therefore set out to determine the extent of the catalysed oxidation using air-saturated solutions at lower Cu levels (the limit for Cu(II) measurement by UV/VIS spectroscopy was *ca.* 10^{-5} mol dm^{-3} using a 10 cm path length). After addition of Cys further air exposure was avoided in all cases.

The addition of Cys to air-saturated solutions PM9, PM10 and PM11, giving a Cys/Cu ratio of 23/1, resulted in an initial substantial loss of the Cu(II) 640 nm absorption followed by a slower re-oxidation back to Cu(II). The times for full return of the 640 nm band being *ca.* 7, 14 and 30 minutes for PM9, PM10 and PM11 respectively. Concomitant with the return of the 640 nm band the Cys level (determined by Ellman's method [10]) decreased, reaching zero at the same time as the 640 nm band was fully restored.

c) Influence of Cu Concentration on the Oxidation of Cysteine at 37 °C

From the above results the catalysed Cys oxidation is obviously strongly dependent upon the Cu

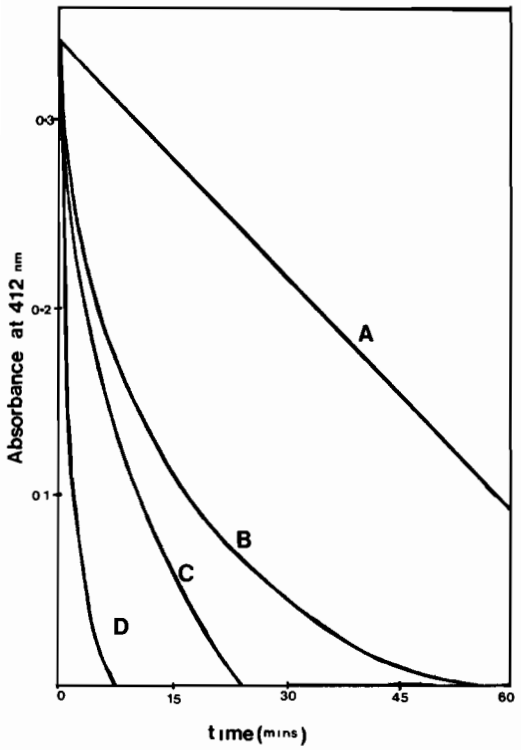

Fig 1 Influence of the Cu concentration upon the oxidation of cysteine in the plasma model solution at 37 °C Copper levels A, 0, B, 1×10^{-8}, C, 1×10^{-7}, D, 1×10^{-6} mol dm^{-3} All other concentrations as for solution PM12 Cys determined by Ellman's method

concentration The influence at lower Cu levels is shown in Fig 1 The rapid conversion to cystine in solution PM12 was confirmed by the lack of detection of any cysteine, after 5 minutes reaction time, on the amino acid auto-analyser The additional presence of cystine itself (PM13) prior to Cys addition had no influence on the oxidation rate Even when the cysteine added was elevated to represent the total cysteine and cystine in blood plasma (106×10^{-6} mol dm^{-3}) the oxidation to cystine was still complete within 5 minutes with solution PM12

Conclusions

The facile reaction observed between Cu(II) and Cys, despite the overwhelming amount of other amino acids present, can be attributed to three factors (*i*) The inevitable presence of minute amounts of free Cu(II) ion, estimated as *ca* 1×10^{-16} mol dm^{-3} in blood plasma [7] (*ii*) The labile nature of the chelates of Cu(II) and Zn(II) (*iii*) A very favourable thermodynamic interaction between Cu(II) and Cys prior to the redox process This latter fact is included because of the lack of reaction observed with Asc although this is known to rapidly reduce simple Cu(II) chelates [17]. The lack of reaction with ascorbate gives further credibility to our model solution approach as ascorbate is found as such in blood plasma

The use of the total 'exchangeable Cu' in our model approach is based on the claim of rapid equilibrium between the Cu bound to amino acids and that bound to albumin [2] Copper-64 distribution studies show a rapid Cu clearance from blood plasma [1, 18, 19] in keeping with rapidly established equilibria Thus, although some 99% of this Cu is bound to albumin, our results suggest that Cu(II) and cysteine cannot exist in the blood plasma LMW fraction Under anaerobic (or pseudo-anaerobic) conditions rapid reduction to Cu(I) would occur with the stoichiometric conversion of Cys to its oxidised form (Reaction 4) Under aerobic conditions the Cys would be quantitatively and rapidly converted to cystine Since cysteine is apparently found in blood plasma [8] then the Cu must be in the reduced Cu(I) state

The dichotomy here is the observed lack of reaction between cysteine and Cu(II) bound to albumin [2, 20], although these were strictly long-time *in vitro* studies However, we have [21] also observed that the reduction of Cu(II) chelates with the anion MoS_4^{2-} is considerably slower with albumin as the ligand In this case the equilibria involving Cu–albumin may not be so rapid and the appropriate Cu level is then *ca* 6×10^{-9} mol dm^{-3}, at which level (see Fig 1) the reaction with Cys is slower than the clearance rate of Cu from blood plasma The influence of albumin is currently being investigated

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