Copper Complexing by Growth Stimulating Tripeptide, Glycylhistidyllysine

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Glycylhistidyllysine (GHL) is a tripeptide, present in human blood plasma at a concentration of approximately 10^{-6} mol dm^{-3} [1], which alters the growth rate and survival of cultured hepatoma cells and hepatocytes [2]. The activity is synergistically enhanced by transition metal ions, optimal growth being reported when GHL (10^{-6} mol dm^{-3}) is introduced with copper or iron (5×10^{-7} mol dm^{-3}) [3]. This has led to the suggestion that the tripeptide's biological effects may arise from its ability to facilitate copper uptake into the cells [4].

The interaction between Cu(II) and GHL has been investigated by Lau and Sarkar using potentiometric, spectrophotometric and equilibrium dialysis methods [5]. They report formation constants for the binary complexes of GHL and Cu(II) as well as for the ternary species formed with histidine. In addition, they compared the relative affinities of the tripeptide and serum albumin for the metal ion. It was concluded from their results that GHL can effectively remove Cu(II) from the complex it forms with albumin and which acts as the major transport form of the exchangeable metal ion in plasma.

In order to examine the distribution of Cu(II) complexes formed by GHL in blood plasma, we included the tripeptide as a component in our established computer simulation model of the low-molecular-weight equilibria in the biofluid [6, 7]. The calculations were based on Lau and Sarkar's constants. They indicated that complexes involving GHL were unlikely to form in particularly significant concentration. This was surprising since there is considerable evidence to suggest that in cell culture experiments, at least, the formation of a 1:1 complex is physiologically important [8]. Accordingly, we have reinvestigated the binding between Cu(II) and GHL in an attempt to resolve this dichotomy.

Experimental

Formation constants were determined following our usual procedure [9, 10]. A microtitration vessel holding initial volumes of 2–4 cm^3 was used with a microcombination electrode (Russell pH Ltd., CMAW 757). Analytical grade reagents were used throughout. Potentiometric titrations were performed at 37 °C and an ionic strength of 150 mmol dm^{-3} using sodium chloride as the background electrolyte. Total ligand and metal concentrations ranged between 2 and 10 mmol dm^{-3}.

Glycyl-L-histidyl-L-lysine acetate (Sigma chemicals) — found C, 47.8%, N, 20.7%, H, 7.01%; calculated for $C_{16}H_{28}N_6O_6$ was C, 47.99%; N, 20.98%; H, 7.05%. L-Histidine (BDH) — found C, 46.1%; N, 27.1%; H, 5.80%; calculated for $C_6H_9N_3O_2$ was C, 46.6%; N, 27.1%; H, 5.85%.

Results and Discussion

The formation constants for the binding of Cu(II) by GHL alone and in the presence of histidine are shown in Table I. These values differ significantly in their composition and magnitude from those of Lau and Sarkar. Closer examination of their procedure [5, 11] suggests that this may be due to their use of very dilute experimental solutions. Error propagation analysis suggests that potentiometric determinations using concentrations less than about 1 mmol dm^{-3} are prone to be unreliable. Another difference between the respective methods concerns the form of GHL used: whereas in the present work the tripeptide was titrated as an acetate salt (and the data analysed as a ternary system), Lau and Sarkar converted it to the corresponding hydrochloride. However, it seems unlikely that this could be responsible for the discrepancies observed.

Using the ECCLES program [6], the distribution of Cu(II)–GHL complexes in human blood plasma was recalculated on the basis of the formation constants shown in Table I. The Plasma Mobilizing Index (P.M.I.) for the ligand was determined over a range of concentrations. This provides a measure of the tripeptides ability to mobilize protein-bound metal ions and to form low-molecular-weight complexes [7]. The results are shown in Fig. 1. Plasma concentrations of the Cu(II) complexes are clearly unaffected at physiological levels of the tripeptide.

The simulations show that the reason for this lack of copper complexation by GHL is the overwhelming presence of naturally-occurring amino acids, particularly histidine. Cu(histidinate)$_2$ is the predominant Cu(II) species in the biofluid but ternary complexes with histidine and other amino acids account for more than 80% of the metal ion in the low-molecular-weight fraction when [GHL] = 5.8×10^{-7} mol dm^{-3}.

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TABLE I. Formation Constant Data for GHL at 37 °C. I = 150 mmol dm^{-3} [NaCl]. Comparison of results with those constants obtained by Lau and Sarkar are given. $\beta_{pp'qr} = [M_qL_pL'_{p'}H_r]/[M]^q[L]^p[H]^r$ (L = GHL; L = K-histidine)

p	p'	q	r	Log $\beta_{pp'qr}$ values + Standard		Literature [5] log β	Sum of Squares in Residuals	MINIQUAD R Factor	Data Points
1	0	0	1	10.09	(0.007)	10.44			
1	0	0	2	17.47	(0.01)	18.37			
1	0	0	3	23.25	(0.01)	24.90	2.89×10^{-7}	0.004	168
1	0	0	4	27.05	(0.03)	27.81			
1	0	0	5	29.02	(0.03)				
Copper(II)									
1	0	1	0	14.83	(0.01)	16.44			
1	0	1	−1	5.87	(0.02)	7.48			
1	0	1	−2	−4.50	(0.03)	−3.74			
1	0	2	−1	13.95	(0.02)	–	5.39×10^{-6}	0.005	383
2	0	1	2	34.76	(0.07)	38.18			
2	0	1	1	27.38	(0.07)	30.83			
2	0	1	0	–		21.43			
2	0	1	−1	–		10.76			
2	0	1	−2	–		1.08			
1	1	1	3	36.49	(0.02)	–			
1	1	1	1	26.65	(0.05)	29.02			
1	1	1	0	18.59	(0.05)	21.09	5.65×10^{-6}	0.005	386
1	1	1	−1	9.25	(0.04)	11.45			
1	1	1	2	–		34.45			
Zinc(II)									
1	0	1	0	7.23	(0.007)				
1	0	1	−1	−1.99	(0.02)		5.75×10^{-6}	0.006	419
1	0	1	−2	−11.96	(0.02)				

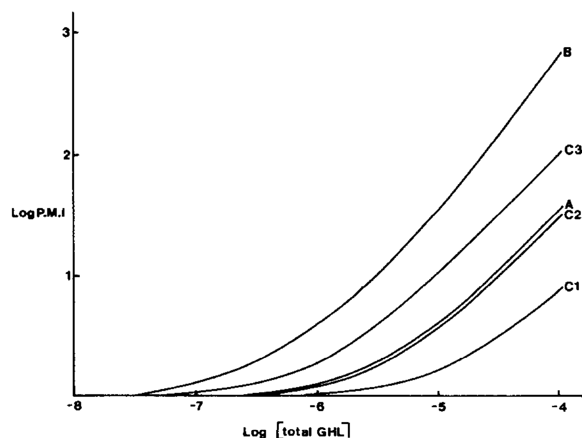

Fig. 1. Log PMI curves for GHL in human blood plasma and cell culture medium with Cu(II) ion. A: log PMI curve of this work: GHL physiological concentration 5.8×10^{-7} mol dm^{-3}. B: log PMI curve, using Lau and Sarkar's formation constants: GHL 5.8×10^{-7} mol dm^{-3}. C1: log PMI curve of cell culture medium at initial medium concentration: GHL cell culture concentration 10^{-6} mol dm^{-3}. C2: log PMI curve of cell culture medium at 50% reduction in concentration: GHL 10^{-6} mol dm^{-3}. C3: log PMI curve of cell culture medium at 75% reduction in concentration: GHL 10^{-6} mol dm^{-3}.

However, in cell culture experiments such as those reported in reference [3], the amino acid pool is not the same as in blood plasma. Further simulations were thus employed to take more realistic cell culture conditions into account. Concentrations of the amino acids in the incubation medium for neoplastic cells were introduced into the simulation model (90% Eagles basal medium, 10% Swims S-77 medium). Our formation constants were again used as input data. The tripeptide concentration was assumed to be 10^{-6} mol dm^{-3} and the total metal ion concentration was 5×10^{-7} mol dm^{-3} (approximating to the culture conditions). Results of the model reveal that at these initial concentrations of amino acids, tripeptide and copper, the ability of GHL to form a predominant species within the culture medium is very limited. This parallels the conclusions drawn from the blood plasma model.

Further simulations were then performed to determine the effect of amino acid depletion from the medium over a few days of growth. These might alter the relative complexing ability of GHL considerably. Reductions in amino acid concentrations of 50% and 75% were considered. The results are given in Table II. It can be seen that as the amino acid concentration

is reduced, GHL is indeed able to compete more effectively for Cu(II), ultimately forming the most predominant species. On the other hand, if a concomitant reduction in the concentration of the tripeptide occurs, either through hydrolysis or cellular uptake, the complexing is correspondingly reduced.

Conclusions

The following conclusions can thus be drawn from our results. GHL in human blood plasma is unable to compete effectively for Cu(II). In cell culture medium, when the amino acid concentration of the medium decreases with time, GHL may be able to compete for Cu(II) and hence may act to facilitate the transfer of the metal ion from the medium into the cells. The 1:1 complex of GHL and Cu(II) is the predominant species formed between this metal ion and ligand. Ternary complexes (with histidine) do not contribute to the biological effects of the tripeptide (see Table II). Thus, we conclude that whatever the growth-modulating properties of GHL *in vivo* may be, the formation of labile low-molecular-weight complexes with copper in blood plasma is not physiologically significant. The observed synergistic effects between Cu(II) and the tripeptide must, therefore, occur at the membrane or within the cell itself.

TABLE II. Computed Formation of GHL–Cu(II) Complexes in Human Blood Plasma and Cell Culture Medium.

Model[a]	Species	% Low molecular weight metal ions	log PMI
A	$Cu(GHL)^{+1}$	2.2	0.01
	$Cu(GHL)(HIS)(H)^{+1}$	0.2	
B	$Cu(GHL)^{+1}$	13.3	
	$Cu(GHL)(HIS)(H)^{+1}$	6.6	0.11
	$Cu(GHL)(HIS)^{0}$	1.9	
C1	$Cu(GHL)^{+1}$	5.3	0.03
	$Cu(GHL)(HIS)(H)^{+1}$	0.3	
C2	$Cu(GHL)^{+1}$	20.4	0.10
	$Cu(GHL)(HIS)(H)^{+1}$	0.6	
C3	$Cu(GHL)^{+1}$	45.4	0.29
	$Cu(GHL)(HIS)(H)^{+1}$	0.6	

[a]A: Blood Plasma Model using our constants: GHL physiological concentration 5.8×10^{-7} mol dm^{-3}. B: Blood Plasma Model using Lau and Sarkar's constants: GHL concentration 5.8×10^{-7} mol dm^{-3}. C1: Cell Culture Model at initial medium concentrations: GHL cell culture concentration 10^{-6} mol dm^{-3}. C2: Cell Culture Model at 50% reduction in medium concentration: GHL 10^{-6} mol dm^{-3}. C3: Cell Culture Model at 75% reduction in medium concentration: GHL 10^{-6} mol dm^{-3}. (GHL = glycylhistidyllysinate; HIS = histidinate).

References

1 L. Pickart and M. M. Thaler, *FEBS Letts., 104,* 1, 119 (1979).
2 L. Pickart and M. M. Thaler, *Nature New Biol., 243,* 65 (1973).
3 L. Pickart and M. M. Thaler, *J. Cell. Physiol., 102,* 129 (1980).
4 L. Pickart, J. H. Freedman, W. J. Loker, J. Peisach, C. M. Perkins, R. E. Stenkamp and B. Weinstein, *Nature, 288,* 715 (1980).
5 S. Lau and B. Sarker, *Biochem. J., 199,* 649 (1981).
6 P. M. May, P. W. Linder and D. R. Williams, *J. Chem. Soc. Dalton Trans.,* 588 (1977).
7 P. M. May and D. R. Williams, *FEBS Letts., 78,* 134 (1977).
8 J. H. Freedman, L. Pickart, B. Weinstein, W. B. Mims and J. Peisach, *Biochemistry, 21,* 4540 (1982).
9 A. Cole, P. M. May and D. R. Williams, *Agents and Actions, 11,* 296 (1981).
10 Z.-X. Huang, P. M. May, K. M. Quinlan, D. R. Williams and A. M. Creighton, *Agents and Actions, 12,* 536 (1982).
11 S. Lau and B. Sarker, *J. Chem. Soc. Dalton Trans.,* 491 (1981).