THE INHIBITION OF THE VITAMIN K-DEPENDENT CARBOXYLATION OF GLUTAMYL RESIDUES IN PROTHROMBIN BY SOME COPPER COMPLEXES

M. P. ESNOUF, M. R. GREEN*, H. A. O. HILL* and S. J. WALTER

*Nuffield Department of Clinical Biochemistry, Radcliffe Infirmary, Oxford, OX2 6HE and *Inorganic Chemistry Laboratory, South Parks Road, Oxford, OX1 3QR, England*

1. Introduction

The biosynthesis of prothrombin and the other plasma clotting factors VII, IX and X, involves a vitamin K-dependent post-translational modification in which specific glutamyl residues are carboxylated on the γ-carbon to give γ-carboxyglutamyl residues [1,2]. The reaction requires the vitamin in its reduced form (vitamin K hydroquinone), molecular oxygen and carbon dioxide [3,4] together with a carboxylase system from liver microsomal fractions. Vitamin K_1 quinone is also active if added with NADH, the reduction being accomplished by an NADH-dependent vitamin K_1 reductase present in the liver microsomes [5]. The substrate for the reaction is either the hepatic prothrombin precursor that accumulates during vitamin K_1 deficiency in rats [6,7] or a penta-peptide based on residues 5--9 of prothrombin [8]. The reaction does not require ATP [4,9] or biotin [10].

It has been shown [11] that vitamin K_1 semi-quinone will reduce dioxygen to superoxide and that liver microsomes produce [12] superoxide by an NADPH-dependent process. We have shown [13] that the NADH-dependent generation of superoxide from normal rat liver microsomes is stimulated by the addition of vitamin K_1 and that superoxide dismutase (SOD) (10 μg/ml) scavenges the superoxide which is produced. We have also shown that carboxylation of the prothrombin precursor and pentapeptide is depressed by SOD, but only at >1 mg SOD/ml. This inefficiency of the superoxide dismutase in preventing carboxylation may be explained by the inability of the enzyme to reach the superoxide generating site. It was therefore decided to test several copper(II)-complexes, which have superoxide dismutase activity, as they may have easier access to the site of super-oxide production.

Richter et al. have reported [14] that the liver microsomal P-450 hydroxylation reaction is inhibited by the copper(II)—tyrosine complex. Although this reaction involves superoxide [15,16], no inhibition is seen with the dismutase in the intact system. The copper(II) amino acid chelates of lysine, histidine and tyrosine have been shown to catalyse superoxide dis-mutation efficiently using either the xanthine/xanthine oxidase system [17] or pulse radiolysis as a source of the superoxide ion [18,19]. By the latter assay, both SOD and the copper chelates have similar dismutase activity ($k \sim 10^9$ $M^{-1}.s^{-1}$), but using the xanthine/xanthine oxidase couple as the O_2^- source the copper chelates are less effective ($k \sim 10^6 - 10^7$ $M^{-1}.s^{-1}$). This may reflect the sensitivity of the superoxide dismutase activity of the copper chelates to weak ligand binding. The copper complexes of penicillamine [20], acetylsalicylic acid and diisopropyl salicyclic acid [21] are also effective O_2^- scavengers when assayed by the pulse radiolysis technique ($k \sim 10^9$ $M^{-1}.s^{-1}$).

The inhibitory effects of a number of metal com-plexes on the carboxylase activity have been investigated and compared with their superoxide dis-mutase activity.

2. Materials and methods

Male Sprague-Dawley rats (200—250 g) were housed individually in cages with a wide mesh floor to reduce

coprophagy. Rats were fed on a vitamin K-deficient diet adapted from that of Martin [22,23]. The drinking water contained 0.1% neomycin sulphate. After 10 days, when the plasma prothrombin had dropped to ~15% of normal, the rats were starved for 18 h, anaesthetized with diethylether and the livers excised. The microsomal fraction was prepared in 0.25 M sucrose, 80 mM KCl in 25 mM imidazole/HCl buffer (pH 7.5) according to [24] and the microsomal pellet resuspended in (2 ml/g liver) 0.25 M sucrose, 80 mM KCl in 25 mM imidazole/HCl buffer (pH 7.5) containing 2% Triton X-100.

The incubation mixture contained 1 ml microsomal suspension and 60 μM dithiothreitol, 1 mM NADH, 10 μCi $NaH^{14}CO_3$ in 1.25 ml total vol. The reaction was initiated with the addition of 100 μg vitamin K_1 (2-methyl-3-phytyl-1,4-napthoquinone) as a suspension in detergent. The concentration of the pentapeptide, Phe–Leu–Glu–Glu–Val, when included, was 2 mM.

All incubations were carried out in sealed tubes at 27°C for 30 min, after which the reaction was stopped by adding 5 ml 10% trichloroacetic acid and 0.5 ml 100 mM $NaHCO_3$ containing 0.9% NaCl and (10 mg/ml) bovine serum albumin. After leaving for 30 min at 4°C the precipitate was spun down and CO_2 was passed through the supernatant for 30 min. The trichloroacetic acid-insoluble material was redissolved and reprecipitated twice in 2 ml 2% Na_2CO_3 and 2 ml 10% trichloroacetic acid, respectively, and finally redissolved in 1 ml NCS tissue solubiliser (Amersham/Searle). Aquasol (New England Nuclear Co.) was added to samples of protein in NCS tissue solubiliser and gassed supernatant containing pentapeptide and samples counted were for ^{14}C activity in an LKB Wallac scintillation counter, model no. 81000.

Diaquo bis-(glycinato)–nickel(II) (nickel glycinate) [25], bis-(3,5-diisopropylsalicylato) (0,0)–copper(II) (copper diisopropylsalicyclic acid), tetrakis-μ-acetyl-salicylato–dicopper(II) (copper aspirinate), (D-penicillaminato)$_{2n}$(aqua)$_{2n}$(copper(II))$_n$ (copper penicillamine) [26] and bis-(L-tyrosinato)–copper(II) (copper tyrosine) [19] were prepared according to literature methods. Stock solutions of the copper and nickel complexes (1–8 mM) were made up in water and the metal content determined by atomic absorption spectroscopy.

Superoxide dismutase, isolated from bovine erythrocytes, was a gift from Dr J. V. Bannister (Department of Physiology and Biochemistry, University of Malta, Msida, Malta). The concentrations of stock solutions of superoxide dismutase were determined at 258 nm; (ϵ_{258} = 9840 M^{-1}.cm^{-1}) [27].

Solutions of the copper and nickel complexes and SOD were assayed for superoxide dismutase activity by a method similar to that in [28]. The 1 ml cuvette contained 4 μM riboflavin, 10 mM L-methionine, 50 μM NBT together with the sample to be assayed, all in 10 mM potassium phosphate buffer (pH 7.8). The sample was made up to 0.8 ml with buffer. After illuminating for 2 min, 5 cm from a twin set of 6 W fluorescence tubes, the increase in A_{550} due to the production of formazan was measured, using a Cary 17 spectrometer. The concentration of sample required to cause a 50% reduction in A_{550} increase was taken as a standard measure of its 'superoxide dismutase' activity.

Vitamin K_1 (Aquamephyton) was obtained from Merck, Sharpe and Dohme, Hoddesdon, $NaH^{14}CO_3$ from the Radiochemical Centre, Amersham and neomycin sulphate from Burroughs Wellcome, Berkhamstead.

L-Phenylalanyl–L-leucyl–L-glutamyl–L-glutamyl–L-valine was prepared as follows: N-t-butoxycarbonyl-γ-benzyl-L-glutamic acid N-hydroxysuccinimide ester was coupled with L-valine benzyl ester in the presence of hydroxybenztriazole and N,N' dicyclohexyl carbodiimide. After removal of the N-t-butoxycarbonyl group, the product was reacted with N-t-butoxy-carbonyl-γ-benzyl-L-glutamic N-hydroxysuccinimide ester. The N-t-butoxycarbonyl group was then removed from the tripeptide with trifluoroacetic acid. The tripeptide was coupled in the presence of hydroxybenztriazole and N,N' dicyclohexyl carbodiimide with N-t-butoxycarbonyl-L-leucine which, after removal of the N-t-butoxycarbonyl protecting group, was coupled with N-benzyloxy-carbonyl-L-phenylalanyl nitrophenyl ester. The protecting groups were then removed by hydrogenation to yield the pentapeptide. The product gave the correct amino acid analysis and was homogenous on thin-layer chromatography in three solvents.

3. Results

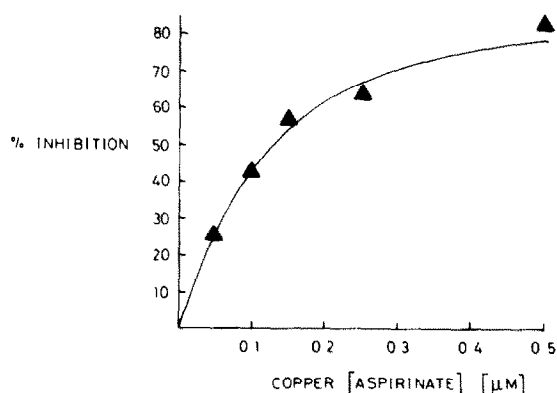The inhibition of formazan production from NBT provides a simple assay for superoxide dismutase

Fig.1. The percentage inhibition of formazan production from nitroblue tetrazolium caused by copper aspirinate. The copper aspirinate was assayed for 'superoxide dismutase' activity as in section 2.

activity. As shown in fig.1 copper aspirinate is a reasonable inhibitor, 50% inhibition occuring at 125 nM. Curves for the other copper complexes are substantially similar, 50% inhibition lying between 140 and 145 nM (table 1). No dismutase activity was observed using nickel glycinate at 8.3 μM. As expected the enzyme, bovine superoxide dismutase is a much better inhibitor with 50% inhibition occuring at ~1 nM.

Copper penicillamine, copper tyrosine and copper aspirinate inhibit the vitamin K-dependent incorporation of ^{14}C into trichloroacetic acid-insoluble material (fig.2a–c) at 10–400 μM. The incorporation was inhibited by 50% with copper aspirinate (65 μM), copper tyrosine (100 μM) and copper penicillamine (120 μM). Copper aspirinate is therefore more effective at lower concentrations than the penicillamine and tyrosine complexes. The copper diisopropyl-

Table 1
Concentration of the copper complexes and superoxide dismutase required to give 50% inhibition of formazan production from nitroblue tetrazolium

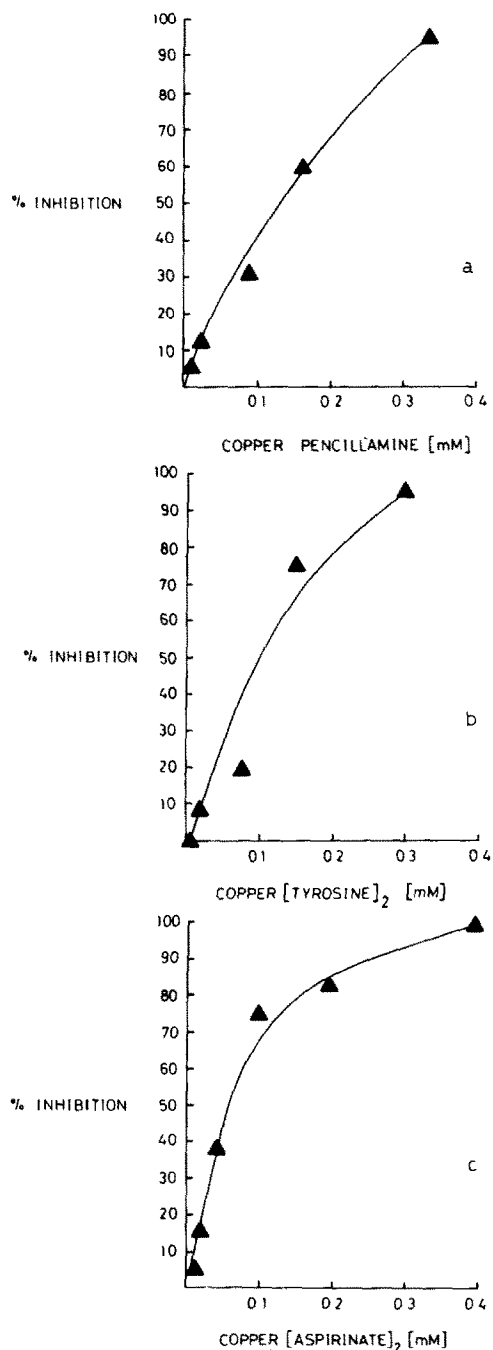|	Concentration causing 50% inhibition (nM)
Copper diisopropyl-salicyclic acid	145
Copper tyrosine	145
Copper penicillamine	140
Copper aspirinate	125
Superoxide dismutase	1

Fig.2. The percentage inhibition of ^{14}C incorporation into the trichloroacetic acid insoluble (protein) fraction of vitamin K-deficient rat liver microsomes by various copper complexes. Reactions were initiated by the addition of vitamin K_1. Samples were prepared and assayed as in section 2. (2a) + copper–penicillamine; (2b) + copper–tyrosine; (2c) + copper–aspirinate.

Table 2

The inhibition of ^{14}C incorporation into the trichloroacetic acid-soluble (containing pentapeptide)
and insoluble (protein) fractions of vitamin K-deficient rat liver microsomes

	Pentapeptide dpm/ml incubation mix	% Inhibition	Protein dpm/ml incubation mix	% Inhibition
No additions	3230		723	
Vitamin K hydroquinone + copper aspirinate	11 258		2289	
360 μM	2584	100	882	93
180 μM	7004	52	1640	41
90 μM	11 560	0	2170	6

salicyclic complex is not sufficiently soluble to be studied at >80 μM; at this concentration it caused no reduction of ^{14}C incorporation. Nickel glycinate, 208–830 μM, caused no inhibition either in the trichloroacetic acid-insoluble or -soluble material. Table 2 shows the results from an experiment in which copper aspirinate was included and the incorporation into both protein and pentapeptide was measured. In this case vitamin K hydroquinone (80 μg/ml) was added as a solution in ethanol having been reduced by shaking an ethereal solution of the vitamin over sodium dithionite in water [29].

4. Discussion

It has been suggested [30] that the superoxide anion, or hydrogen peroxide derived from it, is involved in the formation of the carboxylating species in the synthesis of prothrombin. The inhibition of vitamin K-dependent carboxylation by SOD (50% inhibition at ~600 μM; estimated from [13]) has been reported [13]. Since this represents rather ineffective inhibition it was concluded that access of the enzyme to the carboxylase complex may be impeded. Hence the inhibition of the microsomal carboxylating system by low molecular weight copper chelates is of interest. Small copper complexes have already been shown to be effective in scavenging for superoxide in a microsomal P-450 hydroxylating system [14]. No data are available concerning their solubility in lipid or membraneous environments, but their smaller size may afford them greater access to the site of super-oxide production.

The copper complexes are more effective inhibitors

of carboxylation than SOD (50% inhibition at ~100 μM compared to 600 μM). However if the poorer performance of the copper complexes as catalysts for the disproportionation of superoxide (table 1) is taken into account, they are many-times more effective inhibitors of carboxylation. This is shown by the high ratio of that concentration required to give 50% inhibition of formazan production to the concentration required to give 50% inhibition of carboxylation (table 3). The superoxide ion is the only known substrate for superoxide dismutase though this may not be so for the copper complexes because they may, for example, act as general radical scavengers. The observation that superoxide production from rat liver microsomes is stimulated by vitamin K [13] has also been confirmed by the technique of spin trapping [31].

Table 3

The ratios of the 'superoxide dismutase' activity of the copper complexes and SOD to the inhibition of ^{14}C incorporation into the protein fraction of vitamin K-deficient rat liver microsomes

	Ratio[a]
Cu–penicillamine	1.15
Cu–tyrosine	1.50
Cu–aspirinate	1.92
SOD[b]	0.0017[b]

[a] $\dfrac{50\% \text{ inhibition of formazan production}}{50\% \text{ inhibition of protein carboxylation}} \times 10^3$

[b] The concentration of superoxide dismutase required to inhibit protein carboxylation by 50% was estimated from the data in [13]

The suggestion [13] that the superoxide ion is involved in vitamin K_1-dependent carboxylation of glutamyl residues in preprothrombin, is reinforced.

Acknowledgements

References

[1] Stenflo, J. (1974) J. Biol. Chem. 249, 5527–5535.

[2] Esmon, C. T., Sadowski, J. A. and Suttie, J. W. (1975) J. Biol. Chem. 250, 4744–4748.

[3] Sadowski, J. A., Esmon, C. T. and Suttie, J. W. (1976) J. Biol. Chem. 251, 2770–2776.

[4] Jones, J. P., Gardner, E. J., Copper, T. G. and Olson, R. E. (1977) J. Biol. Chem. 252, 7738–7742.

[5] Martius, C., Ganser, R. and Viviani, A. (1975) FEBS Lett. 59, 13–14.

[6] Shah, D. V., Suttie, J. W. and Grant, G. A. (1973) Arch. Biochem. Biophys. 159, 483–491.

[7] Shah, D. V. and Suttie, J. W. (1974) Biochem. Biophys. Res. Commun. 60, 1397–1402.

[8] Suttie, J. W., Hageman, J. M., Lehrman, S. R. and Rich, D. H. (1976) J. Biol. Chem. 251, 5827–5830.

[9] Esmon, C. T. and Suttie, J. W. (1976) J. Biol. Chem. 251, 6238–6243.

[10] Friedman, P. A. and Shia, M. A. (1977) Biochem. J. 163, 39–43.

[11] Patel, K. B. and Willson, R. L. (1973) J. Chem. Soc. Faraday Trans. I, 69, 814–825.

[12] Bartoli, G. M., Galeotti, T., Palombini, G., Parisi, G. and Azzi, A. (1977) Arch. Biochem. Biophys. 184, 276–281.

[13] Esnouf, M. P., Green, M. R., Hill, H. A. O., Irvine, G. B. and Walter, S. J. (1978) Biochem. J. 174, 345–348.

[14] Richter, C., Azzi, A., Weser, U. and Wendel, A. (1977) in: Superoxide and Superoxide Dismutases (Michelson, A. M. et al. eds) pp. 375–385, Academic Press, London.

[15] Strobel, H. W. and Coon, M. J. (1971) J. Biol. Chem. 246, 7826–7829.

[16] Coon, M. J., Strobel, H. W., Autor, A. P., Heidema, J. and Duppel, W. (1972) in 'Biological Hydroxylation Mechanisms' (Boyd, G. S. and Smellie, R. M. S. eds) pp. 45–54, Academic Press, New York.

[17] Joester, K.-E., Jung, G., Weber, U. and Weser, U. (1972) FEBS Lett. 25, 25–28.

[18] Brigelius, R., Spöttl, R., Bors, W., Lengfelder, E., Saran, M. and Weser, U. (1974) FEBS Lett. 47, 72–75.

[19] Brigelius, R., Hartmann, H.-J., Bors, W., Saran, M., Lengfelder, E. and Weser, U. (1975) Hoppe-Seyler's Z. Physiol. Chem. 356, S. 739–745.

[20] Lengfelder, E., Saran, M. and Bors, W. (1977) Communicated at the International Conference on Singlet Oxygen and Related Species in Chemistry and Biology. Pinawa, Manitoba, Canada, August 21–26, 1977.

[21] Younes, M., Lengfelder, E., Zienau, S. and Weser, U. (1978) Biochem. Biophys. Res. Commun. 81, 576–580.

[22] Martin, A. D. (1979) Vertebrate Biochemistry Section, Pest Infestation Control Laboratory, Ministry of Agriculture, Fisheries and Food, Hook Rise South, Tolworth, Surbiton, Surrey KT6 7NF, England, personal communication.

[23] Mameesh, M. S. and Johnson, B. C. (1959) Proc. Soc. Exp. Biol. Med. 101, 467–469.

[24] Helgeland, L. and Laland, S. (1962) Biochim. Biophys. Acta 62, 200–202.

[25] McAuliffe, C. A. and Perry, W. D. (1969) J. Chem. Soc. A, 634–636.

[26] Sorenson, J. R. J. (1976) J. Med. Chem. 19, 135–148.

[27] Weser, U., Bunnenberg, E., Cammock, A., Djerassi, C. Flohé, L., Thomas, G. and Voelter, W. (1971) Biochim. Biophys. Acta 243, 203–213.

[28] Beauchamp, C. and Fridovich, I. (1971) Anal. Biochem. 44, 276–287.

[29] Fieser, L. F., Tishler, M. and Wendler, N. L. (1940) J. Am. Chem. Soc. 62, 2861–2866.

[30] Esnouf, M. P., Green, M. R., Hill, H. A. O., Irvine, G. A. and Walter, S. J. (1979) in: Oxygen Free Radicals and Tissue Damage, Ciba Found. Symp. 65 (new ser.) pp. 187–197, Elsevier/Excerpta Medica, Amsterdam, New York.

[31] Esnouf, M. P., Burgess, A. I., Walter, S. J., Green, M. R., Hill, H. A. O. and Okolow-Zubkowska, M. J. (1979) Proc. 8th Steenbock Symp. Vitamin K metabolism and vitamin K-dependent proteins; Wisconsin, Madison, June 10–13, 1979 (Suttie, J. W. ed) in press.