Evidence for the Involvement of Superoxide in Vitamin K-Dependent Carboxylation of Glutamic Acid Residues of Prothrombin

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The formation of vitamin K epoxide and the vitamin K-dependent carboxylation of glutamic acid residues present in synthetic substrates and decarboxyprothrombin are both inhibited by superoxide dismutase. Catalase only inhibits the generation of vitamin K epoxide, suggesting that the carboxylation and epoxidation reactions are not interdependent.

Since the demonstration that the carboxylation of specific glutamic acid residues in the *N*-terminal region of prothrombin was a vitamin K-dependent process (Stenflo, 1974; Shah & Suttie, 1974), considerable attention has been focused on the mechanisms of this reaction. The vitamin K-dependent carboxylation of glutamic acid, unlike other biological carboxylation reactions, does not require biotin (Friedman & Shia, 1977) and is insensitive to antagonists of ATP (Sadowski *et al.*, 1976). The minimum requirements for vitamin K-dependent carboxylation are vitamin K, bicarbonate, molecular oxygen and NADH, in addition to a microsomal fraction prepared from the livers of vitamin K-deficient rats. The substrate for the reaction is either the prothrombin precursor that is bound to the microsomal fraction and accumulates in the livers of vitamin K-deficient rats (Shah *et al.*, 1973) or a synthetic peptide substrate based on residues 5–9 of bovine or rat prothrombin (Suttie *et al.*, 1976; Houser *et al.*, 1977). The carboxylation of either the protein or the peptide substrate can be achieved by adding vitamin K as the quinol and omitting the NADH (Sadowski *et al.*, 1976). Since rat liver microsomal preparations contain an NADH-dependent vitamin K reductase (Martius *et al.*, 1975), it is likely that the active form of the vitamin is either the semiquinone or quinol. Jones *et al.* (1977) have concluded that CO₂ rather than bicarbonate is the source of the carbon for the carboxylation, and proposed that the vitamin K reacts with CO₂ to produce a carboxylated intermediate. However, Patel & Willson (1973) have shown that reduced vitamin K reacts with molecular oxygen to form superoxide, and it is possible that the superoxide generated by this reaction reacts with CO₂ to form an active carbon intermediate. The experiments described here investigate the possibility that the formation of superoxide by reduced vitamin K might be linked with the carboxylation reaction.

Experimental

Male Sprague–Dawley rats (200–280 g) housed in individual cages were fed on a vitamin K-deficient diet (Mameesh & Johnson, 1959), and their drinking water contained 0.1 % neomycin sulphate (Burroughs Wellcome, Berkhamstead, Herts., U.K.). After 10 days, when the prothrombin concentrations were less than 20 % of normal, the livers were removed and the microsomal fraction (hereafter referred to as microsomes) prepared as described by Suttie *et al.* (1976). The carboxylation of the vitamin K-dependent proteins and synthetic peptide substrates was carried out under the conditions described by Suttie *et al.* (1976), except that the ATP-generating system was omitted, and 60 μ M-dithiothreitol was added to the incubation mixture.

The synthetic pentapeptide substrates were prepared as follows. For the preparation of L-phenylalanyl-L-leucyl-L-glutamyl-L-glutamyl-L-leucine, *N*-*t*-butoxycarbonyl- γ -benzyl-L-glutamyl-L-leucine benzyl ester was prepared by the reaction of *N*-*t*-butoxycarbonyl- γ -benzyl-L-glutamic acid *N*-hydroxysuccinimide ester with L-leucine methyl ester. The *N*-*t*-butoxycarbonyl group was removed by trifluoroacetic acid, and the product was allowed to react with *N*-*t*-butoxycarbonyl- γ -benzyl-L-glutamic acid *N*-hydroxysuccinimide ester. The *N*-*t*-butoxycarbonyl group was removed from the tripeptide derivative with trifluoroacetic acid, and the product allowed to react with *N*-*t*-butoxycarbonyl-L-leucine *N*-hydroxysuccinimide ester. The *N*-*t*-butoxycarbonyl group was removed from the tetrapeptide with trifluoroacetic acid, and the product allowed to react with *N*-benzyloxycarbonyl-L-phenylalanyl-L-leucyl- γ -benzyl-L-glutamyl- γ -benzyl-L-glutamyl-L-leucine benzyl ester, which after hydrogenation gave the final product. L-Phenylalanyl-L-leucyl-L-glutamyl-L-glutamyl-L-valine was prepared as above, except that L-valine benzyl ester was used in the first step.

Both pentapeptides gave the correct amino acid analysis and were homogeneous on t.l.c. with two developing solvents.

Superoxide formation by normal rat liver microsomes was followed by a modification of the method of Bartoli *et al.* (1977), except that the microsomes were prepared as for the carboxylation experiments and then resuspended in 0.25M-sucrose/0.15M-KCl/50mM-Tris/HCl buffer, pH7.5. After 30min centrifugation at 105000g the microsomal pellet was surface-washed and resuspended in 0.15M-KCl/50mM-Tris/HCl buffer, pH7.5. The superoxide production was measured in an incubation mixture containing 2ml of O₂-saturated 0.15M-KCl/50mM-Tris/HCl buffer, pH7.5, 0.1ml of normal rat liver microsomes, 0.1ml of adrenaline (4mg/ml) and 0.05ml of NADH (10mg/ml) with an Aminco DW2 (American Instrument Co., Silver Spring, MD 20910, U.S.A.) dual-wavelength spectrophotometer at 480–575nm. The vitamin K epoxide formed in various incubation mixtures was assayed by Dr. M. G. Townsend by the procedure of Elliott *et al.* (1976).

Vitamin K (Aquamephyton) was obtained from Merck, Sharpe and Dohme, Hoddesdon, Herts., U.K., and the quinol was prepared by reduction of the quinone with sodium dithionite. Superoxide dismutase, prepared from bovine erythrocytes, was a gift from Dr. J. V. Bannister (Department of Physiology and Biochemistry, University of Malta, Msida, Malta), and catalase was obtained from Boehringer Corp. (London) Ltd., Lewes, Sussex, U.K. NaH¹⁴CO₃ was from The Radiochemical Centre, Amersham, Bucks., U.K., and adrenaline was from BDH Chemicals, Poole, Dorset, U.K.

Results and Discussion

The incubation of liver microsomes from vitamin K-deficient rats with NaH¹⁴CO₃, O₂ and either the leucine C-terminal peptide (leucine-peptide) or the valine C-terminal peptide (valine-peptide) in the

presence of vitamin K quinol resulted in almost the same degree of incorporation of ¹⁴C into each peptide.

This is an unsuspected result, since both Rich *et al.* (1977) and Houser *et al.* (1977) have shown that at a peptide concentration of 2mM, as used in our experiments, both the leucine- and isoleucine-peptides were better substrates than the valine-peptides, and at present we have no explanation for this.

Sadowski *et al.* (1976) have shown that the incorporation of ¹⁴C from NaH¹⁴CO₃ into rat prothrombin was dependent on molecular oxygen. We have repeated this experiment with the leucine-peptide and found that the amount of ¹⁴C incorporated in the apparent absence of O₂ is only 12% of that found when O₂ is present, thus confirming the requirement for O₂ in the carboxylation reaction. If the O₂ required for incorporation of ¹⁴C into the leucine-peptide is being converted into superoxide, it should be possible to inhibit ¹⁴C uptake in the presence of a specific scavenger for superoxide. In the experiment shown in Table 1 the effect of adding superoxide dismutase on the uptake of ¹⁴C into the leucine-peptide is investigated. The results show that superoxide dismutase markedly inhibits ¹⁴C incorporation. This effect is not seen if albumin (10mg/ml) is added instead of the dismutase, indicating that inhibition of ¹⁴C incorporation is not a non-specific protein effect. Superoxide dismutase also inhibits the incorporation of ¹⁴C into the trichloroacetic acid-insoluble fraction of microsomes prepared from vitamin K-deficient rats. This fraction contains the prothrombin precursor (Shah *et al.*, 1973). Table 1 also shows that the addition of catalase (2mg/ml) potentiates the inhibition of ¹⁴C incorporation caused by superoxide dismutase. Control experiments showed that catalase alone caused no inhibition. The concentration of superoxide dismutase used in these experiments is high, and it is possible that the site of superoxide generation may not be readily accessible to the enzyme.

Bartoli *et al.* (1977) have shown that rat liver

Table 1. *Effect of superoxide dismutase (SOD) on the incorporation of ¹⁴C into pentapeptide and the trichloroacetic acid-insoluble fraction of liver microsomes of vitamin K-deficient rats*

Vitamin K was added as the quinol in the experiment with the pentapeptide and as the quinone for the protein incorporation. The results were obtained from duplicate incubations. The leucine pentapeptide was present at a final concentration of 2mM and was omitted from the protein incorporation experiment. The radioactivity counting efficiency was 75%.

	Peptide incorporation (c.p.m./ml)	Percentage of control	Protein incorporation (c.p.m./ml)	Percentage of control
Microsomes alone	1440	—	2000	—
+Vitamin K	27600	100	4843	100
+2mg of SOD	19530	68	3934	82
+5mg of SOD			3469	71
+10mg of SOD	11320	36	3161	65
+20mg of SOD	12290	39		
+20mg of SOD+2mg of catalase	8870	26		

Table 2. *Production of superoxide by normal rat liver microsomes*

The incubation mixture contained 2ml of O₂-saturated buffer, 0.1ml of microsomal suspension, 0.1ml of adrenaline (4mg/ml), 0.05ml of NADH (10mg/ml), 0.1ml of vitamin K (10mg/ml) and superoxide dismutase (SOD) (10 µg/ml).

	Superoxide produced (pmol/min per ml)
Microsomes	0
Microsomes+NADH	60
Microsomes+NADH+vitamin K	230
Microsomes+NADH+vitamin K+SOD	0

microsomes produce superoxide by an NADPH-dependent pathway; superoxide was detected by the oxidation of adrenaline to adrenochrome. We have repeated their experiments using normal rat microsomes and adding NADH and vitamin K. Table 2 shows that the normal rat microsomes produce superoxide at a low rate, which is considerably enhanced after the addition of vitamin K. If the microsomes were prepared in sucrose, as in the carboxylation experiments, the rate of superoxide release was only 21% of that seen in Table 2. This suggests that, under the conditions used to study the carboxylation, the superoxide does not readily escape from the microsomes.

Superoxide dismutase at a concentration of 10 µg/ml inhibits adrenochrome formation, but the dismutase at concentrations of 10mg/ml is required to cause appreciable inhibition of the carboxylation reaction. This apparent inefficiency of the superoxide dismutase in inhibiting the carboxylation reaction could be explained by the relative inaccessibility of the superoxide-generating site to the dismutase.

A similar situation arises in cytochrome *P*-450-dependent hydroxylation reactions, which are not inhibited by superoxide dismutase if intact liver microsomes are used, but are inhibited when a reconstituted hydroxylation system is studied (Strobel & Coon, 1971). Richter *et al.* (1977) have shown that the cytochrome *P*-450-dependent hydroxylation reactions in the presence of intact microsomes can be inhibited by a copper-tyrosine complex, and they conclude that only small hydrophobic compounds can successfully reach the superoxide-generating site. Similarly, Miller & Macdowall (1975) found that superoxide dismutase was not as effective as adrenaline or ascorbate in obscuring the formation of Tiron (4,5-dihydroxy-1,3-benzenedisulphonic acid) semiquinone by the attack of superoxide. The superoxide was generated in this instance by the illumination of spinach chloroplasts.

Table 3. *Effect of catalase and superoxide dismutase (SOD) on the formation of vitamin K epoxide by liver microsomes of vitamin K-deficient rats*

The incubations were carried out under the same conditions as for the experiment shown in Table 1, except that warfarin (40 µg/ml) was added and NaH¹⁴CO₃ was omitted. Vitamin K was present at a final concentration of 5 µg/ml.

	Molar ratio vitamin K oxide/vitamin K
Control	1.89
+SOD (10mg/ml)	0.88
+Catalase (1mg/ml)	0.50
+SOD (10mg/ml)+catalase (1 mg/ml)	0.48

Willingham & Matschiner (1974) have proposed that the carboxylation of prothrombin is accompanied by the conversion of vitamin K into the epoxide; however, Sadowski *et al.* (1977) have examined the conditions under which the epoxidation occurs and point out that, whereas the synthesis of prothrombin requires bicarbonate, the formation of epoxide does not, and, further, that epoxidation can occur in the absence of carboxylation.

It is possible, however, that vitamin K epoxide could arise indirectly as a consequence of the formation of O₂⁻. We have examined the effect of both superoxide dismutase and catalase on the formation of vitamin K epoxide (Table 3). Our results show that, like the carboxylation reaction, the production of vitamin K epoxide is inhibited by superoxide dismutase (10mg/ml). However, the epoxidation reaction is also inhibited by catalase (1mg/ml), which in the absence of superoxide dismutase has no effect on the rate of carboxylation. This supports the idea that vitamin K epoxide is formed as a consequence of the production of superoxide, rather than as a by-product of the carboxylation reaction.

The oxidation of quinols and semiquinones to yield superoxides has been described (Cadenas *et al.*, 1977; Lorentzen & Ts'o, 1977; Patel & Willson, 1973), but the involvement of vitamin K in the production of superoxide for the carboxylation reaction has not been considered. As in many reactions in which superoxide is involved, it is difficult to identify the reacting species, because of the possible intervention of reactions such as:

Reaction (1) may be spontaneous (Bielski & Gebicki, 1970) or be catalysed by superoxide dismutase (McCord & Fridovich, 1969). There is evidence that a metal-catalysed Haber-Weiss reaction (reaction 2)

may also be important in biological systems (Cohen, 1977; Kellog & Fridovich, 1977). In addition, various reactions have been proposed between $\text{OH}^\cdot$ or $\text{O}_2^{\cdot-}$ and CO_2 , and bicarbonate, yielding the active carbon species $\text{CO}_3^{\cdot-}$ and $\text{CO}_2^{\cdot-}$ (Hodgson & Fridovich, 1976; Pujet & Michelson, 1976; Michelson & Durosay, 1977; Stauff *et al.*, 1973). However, superoxide and CO_2 also are known to react in the presence of catalytic amounts of water to yield peroxocarbonates HCO_4^- and $\text{C}_2\text{O}_6^{2-}$ (Mel'nikov *et al.*, 1962; Firsova *et al.*, 1963).

Thus it is possible that the generation of $\text{O}_2^{\cdot-}$ during the oxidation of a reduced species of vitamin K leads via a reaction with CO_2 to the formation of at least four active carbon intermediates, one of which is subsequently involved in the carboxylation of specific glutamic acid residues of the vitamin K-dependent coagulation factors.

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