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VITAMIN K-DEPENDENT OXYGENASE/CARBOXYLASE; DIFFERENTIAL INACTIVATION BY SULFHYDRYL REAGENTS

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SUMMARY: **Inhibition of vitamin K-dependent carboxylase and oxygenase by sulfhydryl reagents was compared. Formation of vitamin K epoxide and vitamin K-dependent carboxylation are both strongly (>90%) inhibited by 1 mM p-hydroxymercuribenzoate, and this inhibition is reversed by dithiothrietol. Both activities are also effectively inhibited by N-ethylmaleimide (NEM). Preincubation with vitamin K hydroquinone prevents NEM inhibition of epoxide formation but not of carboxylation. These data argue that separate active sites are required to support vitamin K-dependent epoxide formation and carboxylation and that the binding site for vitamin K oxygenase contains an active thiol group.** © 1987 Academic Press, Inc.

INTRODUCTION: Liver microsomal membranes contain an enzyme complex which carboxylates protein glutamyl residues to yield gamma-carboxyglutamyl (gla) residues. Concomitant with the formation of gla is the oxygenation of vitamin K hydroquinone to vitamin K epoxide. Vitamin K epoxide is then reduced by one or more reductases to oxidized vitamin K, completing a group of reactions known as the vitamin K cycle. At present, the consensus of most investigators is that the monooxygenase which catalyses the incorporation of oxygen into vitamin K hydroquinone to form vitamin K epoxide is coupled in vivo to vitamin K-dependent carboxylase. However, direct evidence for separate enzyme activities which catalyse the oxygenase and carboxylase reactions has not previously been provided (1,2).

The stimulation of vitamin K-dependent carboxylation with dithiothreitol (DTT) and its inhibition by thiol modifiers (2-6) have been interpreted as evidence for an essential sulfhydryl group in the mechanism of vitamin K-dependent carboxylation. However, in spite of the preponderance of evidence supporting the coupling of vitamin K epoxide to carboxylation (2,6-8), most mechanistic studies of the carboxylation reaction have been limited to studies of gla formation. Thus, assuming epoxide formation is required for carboxylation, the present data demonstrating the effects of metabolic inhibitors or stimulators on vitamin K-dependent carboxylation are insufficient to determine whether the observed effect was on the oxygenase or carboxylase portion of the reaction. For example, sensi-

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tivity of carboxylation to sulfhydryl reagents has been documented by several laboratories (2-5); however, the effects of sulfhydryl reagents on formation of vitamin K epoxide have not been previously reported. The present study was undertaken to gain insight into the role of sulfhydryl(s) in vitamin K-dependent carboxylation and epoxide formation as well as to investigate the coupling of the oxygenase and carboxylase reactions. We report here a comparison of the inhibition by sulfhydryl reagents of vitamin K-dependent carboxylation and epoxide formation. In addition, the ability of vitamin K hydroquinone to protect against inhibition of vitamin K-dependent carboxylation and epoxide formation was investigated.

MATERIALS AND METHODS: Preparation and assay of vitamin K-dependent carboxylase/oxygenase have been described elsewhere (4,8,9). Since vitamin K-dependent carboxylase rapidly loses activity in the absence of DTT, buffers used for enzyme purification contained 1 mM DTT. After purification, enzyme preparations were dialysed against 0.33M K_2HPO_4, 0.2% Renex 30, 10% glycerol and 1 mM DTT. Reaction mixtures (0.5 ml) contained 0.05 mM phe-leu-glu-glu-ileu (peptide substrate) and 0.35 to 0.50 mg carboxylase/oxygenase protein preparation in 0.33 M K_2HPO_4, pH 7.2. Unless otherwise indicated, thiols or sulfhydryl reagents were added in minimum volumes of absolute ethanol and reaction mixtures preincubated for specified intervals as indicated. Enzyme reactions were initiated by the addition of 20 nmols of vitamin K hydroquinone. For assay of vitamin K-dependent carboxylation, 10 microcuries of $NaH^{14}CO_3$(58 mCi/mMol) was added immediately after the hydroquinone. Carboxylation was measured as the acid-soluble, non-volatile incorporation of $^{14}CO_2$ into synthetic peptide. Vitamin K epoxide was extracted into chloroform (2:1, v/v) and analyzed by HPLC as previously described (9).

The FAB mass spectra were obtained using a Kratos MS-50 triple analyzer (10). The samples were dissolved in ethanol (1 ul) and added to a drop of DTT/DTE matrix on the end of the gold target of the FAB direct insertion probe. The sample was bombarded with 8KeV Argon atoms. For accurate mass determinations, the mass spectrometer was tuned to obtain a mass resolving power = 10,000. The ion due to protonated matrix trimer less 2H was used as a mass standard m/z 461.02886). A high voltage divider was used to center m/z 467.35232 in the high mass channel. A mass window of 300 ppm was used. Data were obtained on a Nicolet signal averager and transferred to a Data General Nova 4X computer. Gaussian fits and peak centroids were determined using software written at the Midwest Center for Mass Spectrometry at the University of Nebraska.

Chemicals and reagents were obtained as previously indicated (9).

RESULTS AND DISCUSSION: The data in Table 1 show that vitamin K-dependent carboxylation of synthetic peptide and epoxide formation are co-inhibited by sulfhydryl reagents. In agreement with others (3,5,6) vitamin K-dependent carboxylation is strongly inhibited by 1 mM p-hydroxymercuribenzoate (PHMB). In addition, vitamin K epoxide formation is completely inhibited by this concentration of PHMB. Inhibition of both carboxylation and epoxide formation are effectively reversed by preincubation with 10 mM DTT. Higher concentrations of DTT resulted in comparable reversal of inhibition (data not presented). The reversibility of this inhibition by DTT provides evidence that the inhibition by PHMB is due to mercaptide formation and is not the result of irreversible denaturation or reaction of PHMB with groups other than sulfhydryl in the enzyme (12). These data are consistent with earlier reports of reversibility by DTT of PHMB inhibition of carboxylation (3,5,13).

TABLE I

EFFECTS OF THIO-GROUP REAGENTS ON VITAMIN K-DEPENDENT CARBOXYLATION AND EPOXIDE FORMATION[1]

ADDITIONS:	EPOXIDE % CONTROL	CARBOXYLATION % CONTROL
A) NONE	100	100
B) N-ETHYL MALEIMIDE		
1 MM	63	52
10 MM	0	20
C) PHMB		
1 MM	0	2
1 MM + 10 MM DTT[2,3]	56	83
D) IODOACETIC ACID		
10 MM	80	75

[1] Enzymatic reactions were performed as described in experimental section. Unless otherwise indicated, all numbers are averages of at least three experiments performed on separate days and which agreed within 10%. Representative control values were 0.35 nmol vitamin K epoxide/mg enzyme preparaton/min and 1,500 dpm $^{14}CO_2$ incorporated into peptide substrate/mg enzyme preparation/min.

[2] Enzymatic reaction mixtures were preincubated at 25° C for 10 min. after addition of PHMB in glycyl-glycine, DTT was added and mixtures again incubated for 10 min. at 25° C. Reactions were then initiated by addition of vitamin K hydroquinone.

Both vitamin K-dependent carboxylation and epoxide formation are effectively inhibited by 10 mM N-ethylmaleimide (NEM). Limited solubility in aqueous solvents likely explains the lack of inhibition of carboxylase by NEM reported earlier (13). In agreement with others (13), iodoacetic acid (IAA) was a weak inhibitor of either epoxide formation or carboxylation. Similarly, IAA is a poor inhibitor of vitamin K epoxide reductase (14). However, considering the polarity of IAA and the charge development required for reaction, it is not surprising that alkylation of the enzyme does not occur under our reaction conditions (15).

An attractive hypothesis for the role of an essential sulfhydryl in vitamin K-dependent carboxylation is that vitamin K binds via a sulfhydryl to an enzyme in the complex. In fact, napthoquinones have been shown to react with thiol groups of sulfhydryl enzymes(16). The possibility that vitamin K could protect against inhibition of vitamin K-dependent carboxylation by NEM was tested. As might be expected in these experiments in which a relatively insoluble inhibitor (NEM) is added to a crude enzyme preparation, there was variation in the results. Thus, to control for variability

TABLE II

EFFECTS OF HYDROQUINONE PREINCUBATION ON NEM INHIBITION OF VITAMIN K-DEPENDENT CARBOXYLATION AND EPOXIDE FORMATION[1]

ADDITIONS:	EPOXIDE % CONTROL	CARBOXYLATION % CONTROL
NONE	100.0	100.0
10 mM NEM	32.3 ± 3.4(7)	20.0 ± 3.6(6)
10 mM NEM + 0.040 mM VITAMIN K HYDROQUINONE	79.7 ± 13.2(7)	25.5 ± 2.6(4)

[1] Experimental conditions were as described in Table 1 except that vitamin K hydroquinone was added to reaction mixtures and preincubated 2 minutes prior to addition of NEM. Epoxide formed prior to addition of NEM (≤17%) has been subtracted. Values are the average ± SEM of the number of experiments shown in parentheses.

in enzyme preparations, epoxide formation and carboxylation were usually assayed from the same reaction tubes. As shown in Table II, vitamin K hydroquinone does not protect against inhibition of peptide carboxylation by NEM. In contrast, preincubation of vitamin K hydroquinone with the enzyme prior to addition of NEM protects against inhibition of vitamin K epoxide formation.

When expressed as a percentage of control, the amount of epoxide produced is approximately doubled when vitamin K hydroquinone is added prior to the addition of NEM. In previous experiments (8), the ratio of epoxide to gla formed was 3.5:1. In the positive controls, approximately 5 nmols vitamin K epoxide/mg protein is formed. Thus in the presence of 10 mM NEM, approximately five times as much epoxide/mg protein is formed as gla (1.6 nmols epoxide and ~0.3 nmols of gla). Conversely, when 0.04 mM vitamin K hydroquinone is added prior to the addition of NEM, the ratio of epoxide to gla produced is approximately doubled (approximately 4.0 nmols epoxide but < 0.4 nmols gla). Epoxide produced in the presence of vitamin K hydroquinone and NEM has been corrected for the amount of vitamin K epoxide produced during preincubation (17% of control). Thus, vitamin K hydroquinone appears to protect the active site for catalysis of vitamin K epoxide production against inhibition with NEM. Conversely, inhibition of vitamin K carboxylase appears to be at a separate thiol of unknown function.

In similar experiments, another enzyme in the vitamin K cycle, epoxide reductase, when pretreated with vitamin K epoxide is also protected from inhibition with NEM (14). In the presence of

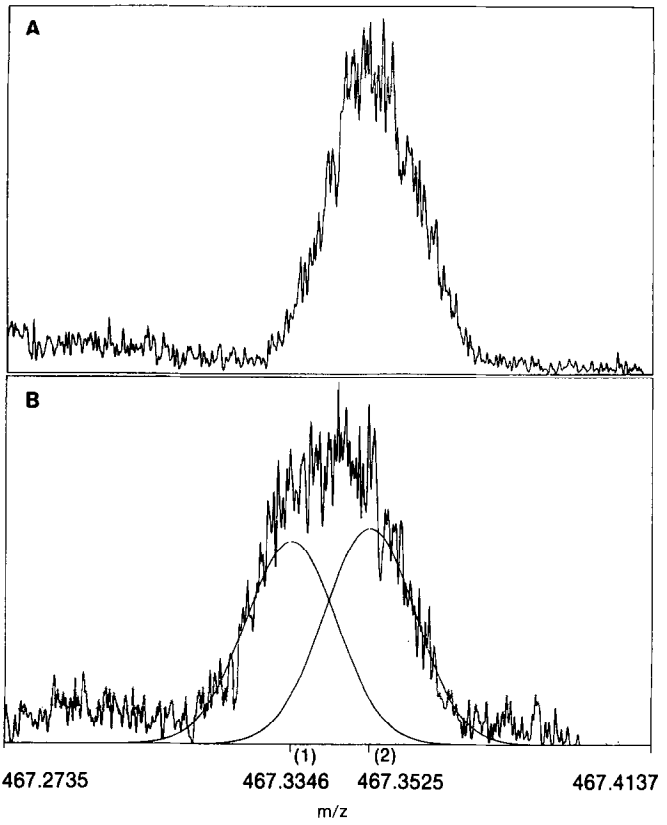

Figure 1 Mass spectral analyses of vitamin K epoxide formed in the presence of NEM and vitamin K hydroquinone.

A. Authentic vitamin K epoxide

B. Vitamin K epoxide producted in enzymatic reaction mixture preincubated with vitamin K hydroquinone prior to addition of NEM.

vitamin K epoxide and NEM, approximately twice as much vitamin K was produced as in the presence of NEM alone. As experimental conditions were different, our results cannot be directly compared, however, given the difference in the two systems, the extent of protection was remarkably similar. Thus, binding of vitamin K to thiol may be characteristic of vitamin K-dependent enzymes.

To determine that the product formed in the presence of NEM and vitamin K hydroquinone was vitamin K epoxide, the product was collected and mass spectral analyses performed. These data are shown in Fig.1. The FAB spectrum shows a broad peak about twice the width of the epoxide standard. Computer-generated gaussian fits showed two ions at m/z 467.3346 and 467.3525. A major ion fits to the expected mass for vitamin K epoxide (m/z 467.3525) with an error of 3.0 parts per million. The other component in this region, (m/z 467.3346) of undetermined composition was present in control

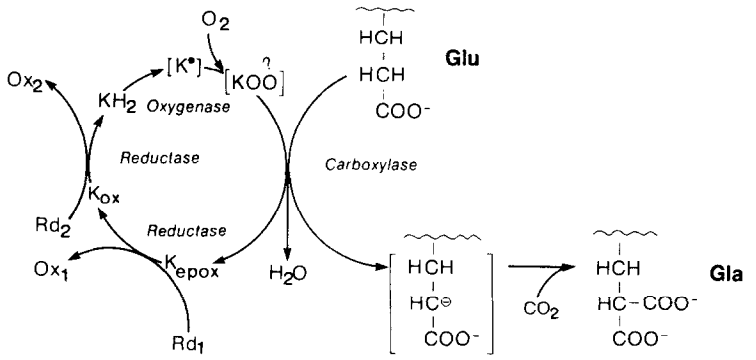

Figure 2 Vitamin K Oxygenase/Carboxylase

Rd_1 = NAD(P)H or $FADH_2$, Kox = Oxidized vitamin K, KH_2 = vitamin K hydroquinone, Kepox = vitamin K epoxide; $K^\bullet$ = vitamin K semiquinone, KOO = oxygenated vitamin K intermediate, gla = gamma carboxyglutamic acid.

samples not containing vitamin K. Thus the only product of the reaction which was detected, either by HPLC or mass spectroscopy is vitamin K epoxide.

We have previously demonstrated that epoxide formation and gla production are not tightly coupled, in that the ratio of epoxide to gla is 3.5 (8). Similar uncoupling has been seen with a number of other monooxygenases, including flavin-dependent hydroxylases (17), pterin-dependent hydroxylases (18) and α-ketoglutarate dependent hydroxylases. In those cases where the protein involved has been purified, there is a single active site. In contrast, the differential effect of vitamin K on inactivation of epoxide and gla formation suggests that two separate active sites are involved in this case. The two sites may be on the same peptide chain or on two separate chains. In either case, the results presented here are the first evidence for such separate active sites in the vitamin K-dependent carboxylation reaction.

Taken together, these data argue that vitamin K hydroquinone binding to an essential sulfhydryl in the enzyme is required for formation of vitamin K epoxide, presumbly via an oxygenated intermediate required for the carboxylation reaction (20). The present data are consistent with the mechanism outlined in Fig.2. In this mechanism, vitamin K oxygenase catalyzes the addition of oxygen to vitamin K semiquinone (22) to produce an oxygenated vitamin K intermediate, possibly a peroxide (20). Binding of vitamin K to this enzyme is to a thiol site which is competed by NEM. An oxygenated vitamin K intermediate or its product, hydroxide ion, then reacts at the active site of the carboxylase to

facilitate production of the carbanion. Carbon dioxide is finally added to produce gla. This hypothesis is consistent with our recent studies demonstrating different oxygen requirements for vitamin K-dependent carboxylation and epoxide formation (8) and also with the work of others showing uncoupling of epoxide formation from vitamin K-dependent carboxylation by cyanide (7) and insensitivity of oxygenase activity to CO_2 concentrations (21). In this mechanism, the oxygenase and carboxylase could be separate sites on a single enzyme or separate enzymes. The available data are insufficient to distinguish between these possibilities. However, in either case, a stepwise reaction mechanism would be predicted; there are no known examples of concerted reactions in monooxygenases.

Further work to determine the mechanism of vitamin K binding to the oxygenase sulfhydryl and its role in vitamin K-dependent carboxylation will require a source of purified enzyme. In spite of the efforts of a number of laboratories (2), enzyme preparations with the activity required for rigorous mechanistic studies are not yet available. The present studies suggest that these difficulties in part may have resulted from separation of the oxygenase and carboxylase activities during purification. In addition, the differential protection by vitamin K of radiolabelled NEM binding to thiol sites in this enzyme complex could prove useful in further purification studies. This approach has been useful in purification of proteins in the vitamin K epoxide reductase system (22).

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