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Vitamin K-dependent carboxylase

Possible role for thioredoxin in the reduction of vitamin K metabolites in liver

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In the liver vitamin K epoxide, which is produced during the posttranslational carboxylation of protein-bound glutamic acid residues, is recycled by the action of one or more dithiol-dependent reductases. In vitro synthetic dithiols may serve as a cofactor for these enzymes, but the physiological reductant has not yet been found. In this paper we report that in vitro the commercially available thioredoxin/thioredoxin reductase from *E. coli* can replace the synthetic dithiols during the various reactions of the vitamin K cycle. Based on the assumption that in vivo thioredoxin also plays a role in the regeneration of vitamin K hydroquinone from the epoxide, an extension of the generally accepted vitamin K cycle is proposed.

Vitamin K; γ-Carboxyglutamic acid; Thioredoxin; Carboxylase; Warfarin

1. INTRODUCTION

Vitamin K-dependent carboxylase is a microsomal enzyme system involved in the formation of γ-carboxyglutamic acid (Gla) residues [1–3]. The energy required for the carboxylation reaction is generated by the simultaneous oxidation of the coenzyme vitamin K hydroquinone (KH_2) into vitamin K epoxide (KO). The epoxide may then be recycled again to the hydroquinone by one or more reductases, possibly via vitamin K quinone (K) as an intermediate.

In the literature much confusion exists about the number and the nature of the reducing enzyme system(s). Dithiol-dependent enzymes have been described which convert KO to K [4,5] and K to KH_2 [6,7] and the question of whether these two activities are carried out by the same enzyme has not yet been answered satisfactorily. Also, an NADH- or NADPH-dependent reductase has been reported, which is able to generate KH_2 from K but which cannot recycle KO [8,9]. It has been suggested [10] that the latter enzyme may be similar to the cytosolic DT-diaphorase (EC 1.6.99.2). In contrast to the dithiol-dependent reductases, the NAD(P)H-dependent enzyme seems to be insensitive to vitamin K antagonists of the dicoumarol type (warfarin, acenocoumarol, phenprocoumon). The concentration of NAD(P)H-dependent reductase in washed microsomes is relatively low, however. Two conclusions may be drawn from the data reported until now: (i) dithiols like dithiothreitol are much better cofactors for the in vitro reductase(s) than NAD(P)H and (ii) the physiological counterpart of these dithiols has not yet been identified.

Thioredoxin is a protein which has been described as acting as a dithiol in many biochemical reactions and occurs in organisms differing as much as *E. coli* and mammals [11,12]. Also, in calf liver its presence has been established [13]. In its oxidized form the protein requires an enzyme nam-

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ed thioredoxin reductase (EC 1.6.45) and NADPH to be converted into its reduced form. In this way thioredoxin may be efficiently recycled in vivo. We have tested whether the commercially available thioredoxin/thioredoxin reductase system from *E. coli* would be able to replace dithiothreitol in the carboxylase/reductase enzyme system from bovine liver. The results of these experiments are presented here.

2. MATERIALS AND METHODS

2.1. *Chemicals*

Vitamin K_1, warfarin, dithiothreitol and 3-([3-cholamidopropyl]dimethylammonio)-1-propanesulfonate (CHAPS) were obtained from Sigma (St. Louis, USA) and the synthetic pentapeptide Phe-Leu-Glu-Glu-Leu (FLEEL) from Vega Biochemical (Tucson, USA). Vitamin K_1 epoxide was prepared as in [14]. Thioredoxin and thioredoxin reductase were purchased from IMCO (Stockholm, Sweden) and $NaH^{14}CO_3$ (40 Ci/mol) from Amersham International (England). Atomlight was from New England Nuclear (Dreiech, FRG) and NADPH from Boehringer (Mannheim). All other chemicals were of the highest purity commercially available.

2.2. *Carboxylase assay*

Salt-washed microsomes and partly purified carboxylase were prepared from the livers of normal cows [15]. Unless indicated otherwise, the vitamin K-dependent incorporation of $^{14}CO_2$ was measured in reaction mixtures (0.125 ml) containing 0.3 mg partly purified carboxylase, 0.15 M NaCl, 1 M $(NH_4)_2SO_4$, 20 mM Tris-HCl, pH 7.5, 8 mM $MnCl_2$, 5 μCi $NaH^{14}CO_3$, 4 mM FLEEL, 0.16% (w/v) CHAPS, 0.4 mg/ml phosphatidylcholine (added as mixed micelles with cholate in a 1:1 (w/w) ratio) and 0.4 mM of either vitamin K quinone or vitamin K epoxide. The vitamin was solubilized by mixing it with the phosphatidylcholine before preparing the mixed micelles according to De Metz et al. [16]. Dithiothreitol, NADPH, thioredoxin and thioredoxin reductase were added as indicated. The reaction mixtures were incubated at 25°C and the reaction was stopped by adding 1 ml of 5% (w/v) trichloroacetic acid. The samples were subsequently boiled for 1 min and counted in Atomlight. All measurements were made in duplicate and mean values are given throughout this paper.

2.3. *Other assays*

Protein concentrations were determined as described by Sedmak and Grossberg [17].

3. RESULTS

The three reactions which give rise to the 'vitamin K cycle' are depicted in fig.1. The formation of Gla residues occurs in reaction 1 by the enzyme carboxylase, which utilizes vitamin K hydroquinone (KH_2) as a coenzyme. The progress of the reaction may be measured by the incorporation of $^{14}CO_2$ into a suitable substrate. Reactions 3 + 1 may be measured by starting the same carboxylase assay with vitamin K quinone (K) instead of KH_2. The reduction of K into KH_2, which precedes the carboxylation reaction, is accomplished by K reductase. Either dithiols (e.g. dithiothreitol) or NAD(P)H may be used as a reducing cofactor, but it was found that at saturating concentrations (5 mM) dithiothreitol is about 20-fold more effective than NADPH. The NADPH-driven reaction reaches a plateau level at about 2 mM NADPH and the apparent K_m for this cofactor was calculated to be 0.5 mM. All experiments described below were performed in the absence of dithiothreitol, but in the presence of an excess of NADPH, i.e. 20-times the K_m value (10 mM).

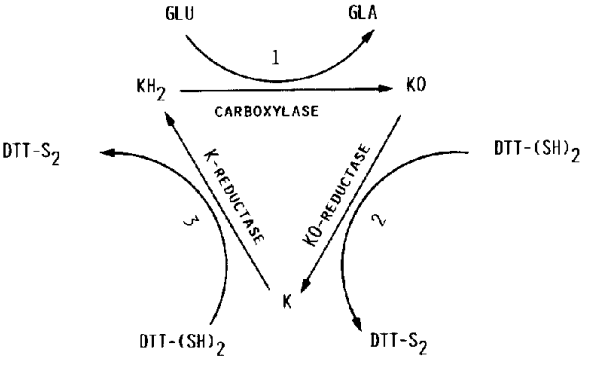

Fig.1. The vitamin K cycle. It is generally assumed that vitamin K is recycled in the liver by the action of three enzymes: carboxylase, KO reductase and K reductase. It is not certain whether the two reducing steps are accomplished by one or by two enzymes.

Since NADPH alone seems to be a poor cofactor for vitamin K reductase, we have tried to stimulate reactions 1 + 3 by adding the dithiol protein thioredoxin and/or the reducing enzyme system thioredoxin reductase. As is shown in fig.2 the two proteins alone have no effect on the carboxylase activity, but the combination of the two proteins resulted in a 7-fold stimulation of the NADPH-driven carboxylation reaction. The results shown in this figure were obtained with partly purified carboxylase, but similar data were observed if salt-washed solubilized microsomes were used as a source of carboxylase (not shown). Under our experimental conditions saturating concentrations of thioredoxin reductase were reached at 0.6 μM (fig.2A) and all experiments described below were performed therefore at 1 μM thioredoxin reductase. Under these conditions the carboxylase reaction could be stimulated 7-fold by adding thioredoxin (fig.2B). From the data shown in fig.2B we have calculated the apparent K_m for thioredoxin, which was 20 μM. For the rest of our experiments we have worked at a thioredoxin concentration of 150 μM.

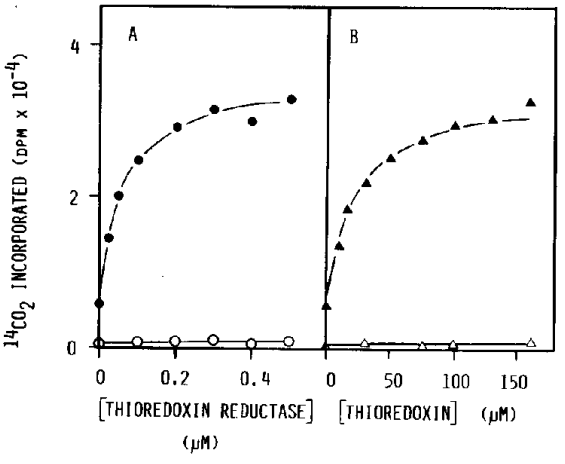

Fig.2. Effects of thioredoxin reductase and thioredoxin on the carboxylation reaction. All reaction mixtures contained vitamin K quinone as a sole source of vitamin K and were incubated for 1 h at 25°C. (A) Increasing amounts of thioredoxin reductase were added to standard reaction mixtures containing 150 μM thioredoxin. (B) Increasing amounts of thioredoxin were added to standard reaction mixtures containing 1 μM thioredoxin reductase. All experiments were performed in the presence (closed symbols) and absence (open symbols) of 10 mM NADPH.

To investigate reactions 1 + 2 + 3 (fig.1) in one system, the carboxylation reaction has to be started with vitamin K epoxide (KO). We have checked if the thioredoxin/thioredoxin reductase system could be used as a sole source of reducing cofactor (thioredoxin-$(SH)_2$) for both vitamin K reductases. The results of these experiments are summarized in table 1 and it is clear that all reactions of the vitamin K cycle are catalyzed by the thioredoxin/thioredoxin reductase system. Moreover, in the presence of KO as well as in that of K the thioredoxin-stimulated reaction rate amounted to about 40% of that of the DTT-stimulated one.

It is well known that vitamin K antagonists (warfarin) inhibit the DTT-dependent K and KO reductases and not the NADH-dependent K reductase. We have determined to what extent the thioredoxin-dependent KO and K reductase activities were inhibited by warfarin. As shown in table 2 the KO-dependent carboxylation reaction is completely inhibited by 10 μM warfarin thus indicating that also with thioredoxin-$(SH)_2$ as a cofactor, KO reductase is inhibited by vitamin K antagonists. A similar concentration also inhibited the main part of the K-dependent carboxylation

Table 1

Peptide carboxylation in the presence of various cofactors

Cofactors used	Initial carboxylation rate (dpm per min)
K + DTT	2173
K + NADPH	106
K + NADPH + thioredoxin	857
KO + DTT	1601
KO + NADPH	22
KO + NADPH + thioredoxin	645

In the cases in which thioredoxin-$(SH)_2$ was used, thioredoxin reductase (1 μM) was also present in the reaction mixtures. The initial carboxylation rates were obtained from time course experiments and were constant for the first 30 min or more. Results are expressed as the amount of $^{14}CO_2$ (dpm) incorporated per min under our experimental conditions. Blank values (obtained in the absence of either K or KO) were subtracted

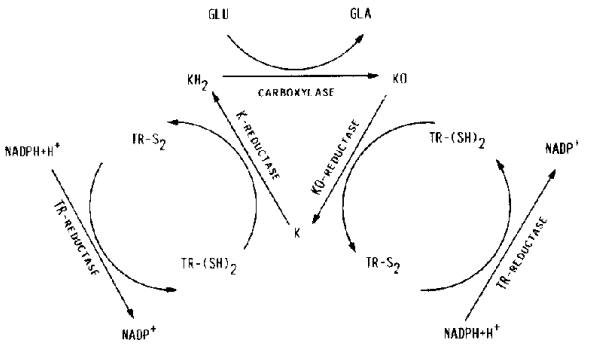

Fig.3. Proposed extension of the vitamin K cycle. The synthetic dithiols from fig.1 have been replaced by thioredoxin-$(SH)_2$ and the thioredoxin-$(SH)_2$ generating system (thioredoxin reductase + NADPH). TR, thioredoxin.

reaction, but a residual activity of about 7% was found, even if the warfarin concentration was raised to 0.1 mM. A comparable activity was obtained in the absence of thioredoxin and thioredoxin reductase, so that we must conclude that the warfarin-insensitive activity originates from the NADH-dependent K reductase, which has been reported earlier to be insensitive for coumarin derivatives [9].

4. DISCUSSION

In vitro the conversion of vitamin KO to vitamin K has been shown to be absolutely dependent on synthetic dithiols such as dithiothreitol [2]. Also, for the reduction of vitamin K the dithiol-dependent reductase is by far the most effective enzyme system [14]. Although KO reductase was discovered more than 10 years ago [19], the physiological counterpart of its in vitro cofactor has never been discovered.

Here, we have shown that the thioredoxin/thioredoxin reductase from *E. coli* is able to replace dithiothreitol during the in vitro carboxylation reaction. The activity obtained was about 40% of that in the presence of dithiothreitol. These experiments indicate that possibly the thioredoxin/thioredoxin reductase system is the physiological system which produces the reducing equivalents required for the reduction of vitamin KO and vitamin K in vivo. It is not unlikely that the thioredoxin/thioredoxin reductase system from bovine liver will prove to be an even more effective system for bovine liver carboxylase, since a substantial species specificity of thioredoxin has been reported [12]. In fact it is surprising that bovine vitamin KO reductase and vitamin K reductase both recognize *E. coli* thioredoxin-$(SH)_2$.

Based on our experiments we postulate an extension of the vitamin K cycle in which the oxidation of dithiothreitol is replaced by that of thioredoxin, whereas the oxidized thioredoxin is recycled via thioredoxin reductase + NADPH (fig.3).

A practical aspect of our findings is that the thioredoxin/thioredoxin reductase provides a good alternative for the strongly reducing conditions (caused by the synthetic dithiols) during the in vitro carboxylation of disulfide-containing substrates. This opens up the possibility of starting experiments in which protein substrates such as blood coagulation factor precursors [either present as an endogenous substrate complexed to carboxylase from warfarin-treated animals or added as an exogenous substrate (e.g. obtained via recombinant-DNA technology)] are carboxylated in vitro without reducing the disulfide bonds in these proteins. This is a first requirement before the in vitro carboxylation of, for instance, blood coagulation factor IX precursors may be expected to increase the procoagulant activity of this protein.

Table 2

Inhibition of peptide carboxylation by warfarin

Cofactors used	Warfarin concentration (mM)	Initial carboxylation rate (dpm per min)
K	0	203
K + thioredoxin	0	1840
K	0.01	137
K + thioredoxin	0.01	138
KO	0	13
KO + thioredoxin	0	1556
KO	0.01	0
KO + thioredoxin	0.01	9

All reaction mixtures contained 10 mM NADPH. In those cases in which thioredoxin was used, thioredoxin reductase (1 μM) was also present in the reaction mixtures. Further details as described in the legend to table 1

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