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Stimulation of the dithiol-dependent reductases in the vitamin K cycle by the thioredoxin system

Strong synergistic effects with protein disulphide-isomerase

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It has been shown previously that the thioredoxin system (thioredoxin + thioredoxin reductase + NADPH) may replace dithiothreitol (DTT) as a cofactor for vitamin KO and K reductase in salt-washed detergent-solubilized bovine liver microsomes. Here we demonstrate that the system can be improved further by adding protein disulphide-isomerase (PDI) to the components mentioned above. Moreover, NADPH may be replaced by reduced RNAase as a hydrogen donor. In our *in vitro* system the various protein cofactors were required at concentrations 2–5 orders of magnitude lower than that of DTT, whereas the maximal reaction rate was about 3-fold higher. PDI stimulated the thioredoxin-driven reaction about 10-fold, with an apparent K_m value of 8 μM. These data suggest that in the *in vitro* system the formation of disulphide bonds is somehow linked to the vitamin K-dependent carboxylation of glutamate residues. *In vivo*, both disulphide formation and vitamin K-dependent carboxylation are post-translational modifications taking place at the luminal side of the endoplasmic reticulum of mammalian secretory cells. The possibility that the reactions are also coupled *in vivo* is discussed.

INTRODUCTION

Most secretory proteins undergo extensive post-translational processing before they are secreted into the extracellular environment. Although more than 100 post-translational modifications are presently known [1], most of these reactions have been studied in less detail than transcription and translation, the earlier steps in protein biosynthesis. In this paper we focus our attention on two of these post-translational modifications: the conversion of glutamate (Glu) into γ-carboxyglutamate (Gla) residues, and the formation of the disulphide bonds.

The formation of Gla residues is accomplished during a carboxylation reaction, which is catalysed by the microsomal enzyme γ-glutamyl carboxylase (hereafter termed carboxylase). Vitamin K hydroquinone (KH_2) functions as a cofactor in this reaction [2,3]. The oxidation of KH_2 into an epoxide (KO) provides the energy required for the carboxylation step. In two successive reduction steps (see Fig. 1), KO is subsequently recycled into KH_2. *In vitro*, the synthetic dithiothreitol (DTT) as well as reduced thioredoxin may serve as a reducing cofactor for KO and K reductase [4,5]. In its reduced form thioredoxin (12 kDa) is a powerful protein disulphide-reductase and acts as a hydrogen donor for the enzymic reduction of ribonucleotides and methionine sulphoxide [6,7]. Dihydrolipoic acid [8] and dihydrolipoamide (H. H. W. Thijssen, personal communication) also have reductase cofactor activity. The origin and the nature of the physiological reduction system is still a matter of debate, however [9].

Nascent polypeptide chains entering the lumen of the endoplasmic reticulum contain free SH groups, which are transformed into native disulphide bonds by net oxidation and the suggested catalytic action of an enzyme called protein disulphide-isomerase (PDI, EC 5.3.4.1; for a review see [8]). PDI is a 57 kDa acidic protein present in the lumen of the endoplasmic reticulum possessing two thioredoxin-like domains with the active site sequence Trp-Cys-Gly-His-Cys. Recently it was demonstrated that the thioredoxin-like domains of oxidized PDI are substrates for NADPH and mammalian thioredoxin reductase (TR; EC 1.6.4.5), and that PDI has thioredoxin-like activity in the reduction of insulin disulphides [10]. PDI and thioredoxin thus share a similar mechanism of action, summarized in eqns. (1) and (2):

$$\text{PDI-S}_2 + \text{NADPH} + \text{H}^+ \xrightarrow{\text{TR}} \text{PDI-(SH)}_2 + \text{NADP}^+ \quad (1)$$

$$\text{PDI-(SH)}_2 + \text{insulin-S}_2 \xrightarrow{\text{spontaneously}} \text{PDI-S}_2 + \text{insulin-(SH)}_2 \quad (2)$$

PDI-S_2 is also readily reduced by reduced thioredoxin. Hence PDI has a higher redox potential and is a much weaker reductant

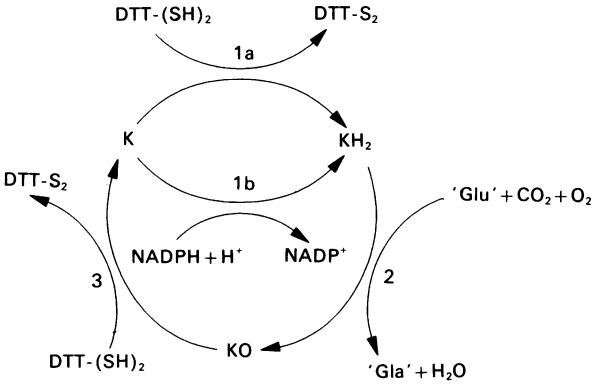

Fig. 1. The vitamin K cycle

Enzymes catalysing the various steps are: 1a, dithiol-dependent vitamin K reductase; 1b, NAD(P)H-dependent K reductase; 2, γ-glutamylcarboxylase; 3, dithiol-dependent KO reductase. 'Glu' stands for peptide- or protein-bound Glu residues.

Abbreviations used: DTT, dithiothreitol; Gla, γ-carboxyglutamate; TR, thioredoxin reductase; PDI, protein disulphide-isomerase.

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than is thioredoxin [10]. This suggests that PDI is reduced by SH groups in the nascent proteins according to eqn. (3):

$$\text{PDI-S}_2 + \text{protein-(SH)}_2 \rightleftarrows \text{PDI-(SH)}_2 + \text{protein-S}_2 \quad (3)$$

Obviously the PDI-bound sulphydryl groups should be oxidized somehow, but nothing is known about the nature and origin of the oxidizing cofactor(s) involved [11].

The common aspects for the vitamin K-dependent carboxylation and the disulphide bond formation are as follows. (1) Both reactions take place at the luminal side of the rough endoplasmic reticulum (similarity of location). (2) Both reactions occur during the early steps of post-translational modification (similarity in time). (3) Both involve thiol–disulphide exchange reactions, but their redox cofactors have remained obscure despite substantial amounts of research (similarity of problem). Because of all of these similarities we have investigated in an *in vitro* system whether these two reactions are coupled, so that disulphide bond formation provides the reducing equivalents required for the reduction of KO and K, i.e. whether the reducing equivalents produced during the formation of the cystine bonds may be oxidized via the vitamin K cycle. The results of these investigations are presented in this paper.

EXPERIMENTAL

Materials

Vitamin K_1 was obtained from Merck (Darmstadt, Germany) and converted to its epoxide form (KO) according to the procedure of Fieser *et al.* [12]. The various forms of vitamin K were added to the reaction mixtures as an ethanol solution. $NaH^{14}CO_3$ (55 Ci/mol) was from Amersham International (Amersham, Bucks., U.K.) and the pentapeptide Phe-Leu-Glu-Glu-Leu (FLEEL) was from Vega Biochemical Co. (Tucson, AZ, U.S.A.). DTT, CHAPS, lipoamide (reduced form) and glutathione (reduced form) were purchased from Sigma (St. Louis, MO, U.S.A.). All other chemicals were of analytical grade or better.

Proteins

The following proteins were prepared from bovine tissues according to previously described methods: PDI [10], thioredoxin [13] and TR [14,15]. Glutaredoxin was prepared from *Escherichia coli* [16]. *E. coli* thioredoxin and TR [6] were from Imco (Stockholm, Sweden). Glutathione reductase (from yeast; EC 1.6.4.2) was purchased from Boehringer (Mannheim, Germany), and insulin (from pig pancreas) and RNAase (from bovine pancreas; EC 3.1.27.5) were from Sigma. RNAase was reduced in 100 mg quantities by incubating the enzyme for 30 min in the presence of 200 mM-DTT and 8 M-urea in buffer C (Tris-buffered saline at pH 8.5) and 37 °C. After removal of the DTT and urea by size-exclusion chromatography in buffer C on a PD 10 column (Pharmacia), the samples were flushed with nitrogen gas and kept in sealed tubes at 4 °C until use. Salt-washed detergent-solubilized microsomes were used as a source of the carboxylase/reductase complex [17].

After the extensive washing procedure, the PDI content of these microsomes was determined using the micromethod of insulin reduction (see below), and was estimated at 3.5 μM. The microsomes contained neither thioredoxin nor TR in detectable amounts (by Western blot analysis).

Various assays

Vitamin K-directed carboxylase activity was determined as described earlier [18], but with some modifications to allow for a maximal activity of the PDI/thioredoxin system. Reaction mixtures contained 25 mM-Tris/HCl, pH 7.5, 100 mM-NaCl, 5 mM-EDTA, 0.4% (w/v) CHAPS, 0.1 mM-vitamin K quinone, 5 μCi of $NaH^{14}CO_3$ and 1 mg of microsomal proteins; the incubation temperature was 20 °C. Reducing cofactors were added as indicated. Initial reaction rates were calculated from time courses obtained after incubations for 0, 5, 10, 20 and 30 min. Each experimental point was determined in duplicate, and blank values (no vitamin K added) were subtracted throughout.

KO reductase activity was assayed in 0.25 ml reaction mixtures of a similar composition as described for the carboxylase, but the samples were preincubated at 20 °C for 3 min and the reaction was started by adding KO instead of K. Aliquots (50 μl) were taken after incubation for 2.5, 5 and 10 min; during this period the reaction rates were linear. Vitamin K metabolites were extracted from the reaction mixtures and analysed by h.p.l.c. as described by de Boer-van den Berg *et al.* [19].

Kinetic data were calculated by the non-linear least-squares technique [20], in which we used the initial reaction rates at six to eight different substrate concentrations, ranging from 0.25 to 4 times the apparent K_m value of the compound tested. The data are presented as the means ± S.E.M. of three independent experiments.

The enzymic activities of PDI, thioredoxin, TR reductase and glutaredoxin were tested by methods described previously [10,15]. Protein concentrations were assessed according to Sedmak & Grossberg [21].

RESULTS

Cofactors for vitamin K-dependent carboxylase

In a first set of experiments we compared synthetic DTT with seven natural reducing agents for their ability to stimulate the vitamin K-dependent carboxylase reaction *in vitro* (Table 1). A low but distinct activity was found in the presence of NADH and NADPH, but the reaction was insensitive to warfarin up to concentrations as high as 100 μM. In contrast, the reactions stimulated by the other cofactors were all highly sensitive to inhibition by warfarin, with apparent K_i values below 1 μM. This demonstrates once more that the NAD(P)H-dependent reduction of vitamin K is accomplished via an alternative pathway [22], which will not be discussed in this paper. From Table 1 it appears that DTT, thioredoxin and lipoamide are all capable of stimulating the vitamin K-dependent carboxylase reaction, but that thioredoxin differs from the other two cofactors by its low apparent K_m. Therefore we regard thioredoxin as a good candidate for being a natural cofactor for the reductases involved in the vitamin K cycle. Maximal activity was only obtained in the presence of the NADPH-dependent enzyme TR, suggesting that in our system oxidized thioredoxin is recycled via this pathway.

Additional cofactors for (vitamin K + thioredoxin)-dependent carboxylase

Because the maximal reaction rate of the DTT-stimulated reaction was still 3-fold higher than that of the thioredoxin-catalysed reaction, we investigated whether the latter reaction could be stimulated further by one of the other cofactors mentioned above. As is shown in Table 2, only PDI had a measurable effect, and it is notable that the combination of PDI + thioredoxin resulted in a 3-fold higher reaction rate than was obtained with DTT. The apparent K_m (8 μM) for PDI was about 12-fold higher than the concentration of endogenous PDI (0.7 μM; see the Experimental section) in our reaction mixtures, which explains the stimulatory role of added PDI.

We have also compared the characteristics of a system containing thioredoxin and TR from *E. coli* with a system in which all cofactors were of bovine origin. As can be seen from Table 3,

Table 1. Reducing agents as cofactors for vitamin K-dependent carboxylase

All measurements were performed under the standard conditions described in the Experimental section. Both thioredoxin and PDI were tested in the presence of an excess of TR (0.2 μM) and NADPH (4 mM). PDI (up to 24 μM) was also tested in the absence of these compounds. Glutaredoxin (highest concentration 65 μM) was assayed in the presence of an excess of glutathione (4 mM), glutathione reductase (10 μg/ml) and NADPH (4 mM). The effect of PDI was checked in the presence of 4 mM-NADPH. Oxidation of the protein dithiols was retarded by the presence of 10 μM-DTT in all reaction mixtures. Both thioredoxin and TR were from *E. coli*. n.d., not detectable.

Compound added	$K_m^{app.}$ (mM)	$V_{max.}^{app.}$ (relative rate)
DTT	0.8±0.2	100±18
NADH	1.5±0.3	7±2
NADPH	1.1±0.3	8±3
Glutathione	n.d.	n.d.
Thioredoxin+TR+NADPH	0.022±0.005	32±7
Glutaredoxin	n.d.	n.d.
PDI	n.d.	n.d.
PDI+TR+NADPH	n.d.	10±2
Lipoamide	0.6±0.1	21±4

Table 2. Stimulation of (thioredoxin + vitamin K)-dependent carboxylase

All reaction mixtures contained thioredoxin (0.2 mM), TR (0.2 μM) and NADPH (4 mM). Further details are as described in the legend to Table 1. n.d., not detectable.

Compound added	$K_m^{app.}$ (mM)	$V_{max.}^{app.}$ (relative rate)
None	–	32±5
DTT	0.8±0.3	100±24
Glutathione	–	29±4
Glutaredoxin	–	30±3
Lipoamide	n.d.	36±7
PDI	0.008±0.002	335±66

Table 3. Apparent K_m values for cofactors of vitamin K-dependent carboxylase

All measurements were performed under standard conditions. Trx, thioredoxin. If added in excess, the concentration of the various reaction components was 10 times the K_m value. Further details are as described in the legend to Table 1.

Reaction component	$K_m^{app.}$ (μM) in system with Trx/TR from: *E. coli*	Calf thymus
Trx	20±6	21±5
TR	0.019±0.006	0.081±0.012
PDI	8.1±1.8	7.9±2.4

the apparent K_m values for thioredoxin and PDI were similar in both systems, whereas in the homologous system that for TR was slightly (4-fold) higher than in the heterologous system. This may be due to the rather high sensitivity of mammalian thioredoxin/TR systems to irreversible inactivation by molecular oxygen [23], or to the fact that bovine TR has a broader substrate specificity than the *E. coli* enzyme [6].

Table 4. Reduced RNAase as a cofactor for vitamin K-dependent carboxylase

All incubations were performed for 30 min under standard conditions, and the results are expressed as d.p.m. incorporated per mg of microsomal protein. Each experimental point was determined in triplicate, and the values are means±S.E.M. Trx, thioredoxin. The various reaction components were used in the following concentrations: DTT, 4 mM; *E. coli* thioredoxin, 0.2 mM; *E. coli* TR, 0.2 μM; PDI, 24 μM; NADPH, 4 mM; reduced RNAase, 0.2 mM. Further details are as described in the legends to Tables 1 and 2.

System used	$^{14}CO_2$ incorporated (d.p.m./mg)
DTT	5419±1877
Trx/TR+NADPH	2864±724
Trx/TR+PDI+NADPH	16320±4518
Trx/TR+reduced RNAase	92±25
Trx/TR+PDI+reduced RNAase	12941±2932

Table 5. Cofactor activity of various reductants for KO reductase

All measurements were performed under standard conditions; the concentrations of the various components (if added) were 10 times the $K_m^{app.}$ values found for the vitamin K-dependent carboxylation reaction. The results are expressed as pmol of vitamin K formed/min per mg of microsomal protein. Each experimental point was determined in triplicate, and the values are means±S.E.M. Trx, thioredoxin. Further details are as in the legend to Table 1.

Cofactor(s)	Source of Trx/TR	Rate of KO reduction (pmol/min per mg)
DTT	–	25.6±3.2
PDI	–	0
Trx	*E. coli*	13.6±2.9
Trx	Bovine thymus	9.7±2.7
Trx+PDI	*E. coli*	57.1±14.8
Trx+PDI	Bovine thymus	49.8±11.5

In all experiments mentioned thus far, NADPH was used as a hydrogen donor for the PDI-containing systems; the physiological reductant of PDI is probably not NADPH but more likely a reduced protein. Therefore we have measured the kinetic properties of the (PDI + thioredoxin)-driven carboxylase reaction in the presence of either NADPH or reduced RNAase (see Table 4). No differences between the two systems were observed, except that the RNAase-directed reaction could be blocked completely by 100 μM-warfarin, whereas in the presence of NADPH the inhibition by warfarin was only 92%. The residual activity probably originated from the warfarin-insensitive NADPH-dependent vitamin K reductase mentioned earlier in this paper.

Cofactors for KO reductase

The reductases of the vitamin K cycle may be tested directly by extraction of the various vitamin K metabolites from the reaction mixtures, followed by h.p.l.c. analysis. At rate-limiting concentrations of the various cofactors this method was not sufficiently accurate to yield reproducible kinetic data. Therefore we have measured the effects of the various cofactors for KO reductase under non-limiting conditions only. The results are summarized in Table 5, and demonstrate the same tendency as was found for vitamin K-dependent carboxylase. PDI alone is not a cofactor for the enzyme, the cofactor activity of thioredoxin+TR is 30–40% that of DTT, and the combination of thioredoxin, TR and PDI may stimulate the reaction to a level which is several-

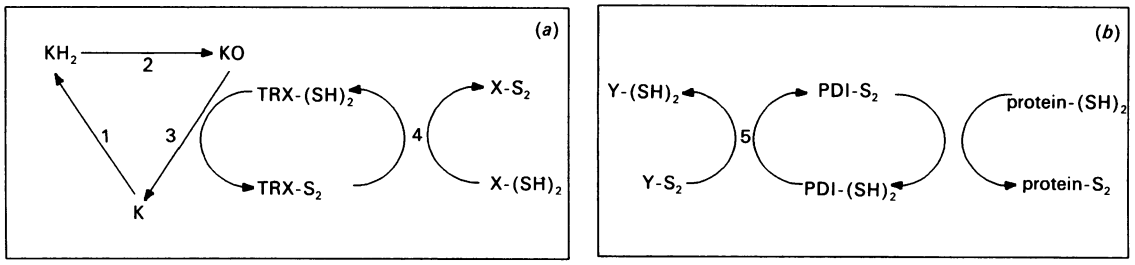

Fig. 2. Oxidoreductases involved in the reduction of KO and K (*a*) and possible redox carriers for the oxidation of protein-bound cysteine SH groups (*b*)

Both reactions 4 and 5 may occur spontaneously, but are catalysed by TR. X and Y stand for hypothetical redox cofactors (not yet identified). The possibility that the recycling of PDI is directly coupled to that of thioredoxin and vitamin K is discussed in the text.

fold higher than that reached with DTT alone. As in the case of vitamin K-dependent carboxylase, the data obtained with thioredoxin and TR from *E. coli* were slightly better than those obtained with the all-bovine system.

DISCUSSION

Despite considerable efforts, neither KO reductase nor vitamin K reductase have yet been purified to homogeneity. Therefore we have used the rather crude system of salt-washed detergent-solubilized microsomes for all experiments described in this paper. It was shown that under these experimental conditions the combination of reduced RNAase, PDI and thioredoxin/TR is superior to DTT in providing the reducing equivalents required in the vitamin K cycle. This is demonstrated by the fact that the apparent K_m values for these compounds are between two and five orders of magnitude lower than that for DTT, whereas the maximal reaction rate is about 3-fold higher. These conclusions hold true for homologous systems in which all proteins are of bovine origin, but also for systems in which thioredoxin and TR are from *E. coli*. Advantages of the latter proteins are that they are commercially available, and that they are less sensitive to inactivation by thiol oxidation with molecular oxygen [6]. Our data clearly demonstrate that in an *in vitro* system the process of disulphide bond formation may be coupled to the reductive steps in the vitamin K cycle. All reaction components required for this coupling are natural compounds, the occurrence of which in the endoplasmic reticulum has been demonstrated [24–26]. However, the way in which this coupling is brought about remains uncertain.

The fact that reduced thioredoxin is the only reaction component displaying cofactor activity by itself for KO and K reductase seems to support the reaction pathway shown in Fig. 2(*a*). This is consistent with results from Gardill & Suttie [27], who demonstrated that *E. coli* thioredoxin may be used as an affinity ligand for the partial purification of KO reductase. On the other hand, it is likely that PDI is involved in disulphide bond formation as shown in Fig. 2(*b*), and it is tempting to speculate about the possibility that both processes are directly coupled (i.e. X = PDI and Y = thioredoxin). However, such a sequence of events is not in line with the respective redox potentials of the proteins involved, thioredoxin being a much stronger reductant than is PDI [10,28]. This was previously also concluded from experiments in which mixtures containing equimolar concentrations of reduced thioredoxin and oxidized PDI were incubated, and in which an equilibrium was reached after 90% of the thioredoxin and 10% of the PDI were in the oxidized state [23]. It should be realized, however, that in the optimal carboxylating system the apparent K_m for PDI is about 3-fold lower than that for thioredoxin (Table 3), and that the concentration of PDI in the lumen of the endoplasmic reticulum is very high [8]. Under these conditions, and provided that most of the PDI occurs in the reduced state, it cannot be excluded that sufficient amounts of thioredoxin are reduced to allow for its involvement in the vitamin K cycle. The likelihood of such a mechanism being operational *in vivo* therefore depends on the ratio between the luminal concentrations of PDI and thioredoxin in the endoplasmic reticulum. However, although with the aid of immunohistochemical techniques the occurrence of thioredoxin in the lumen of the endoplasmic reticulum has been demonstrated unequivocally [26], its local concentration is not known at this time.

An alternative explanation for our data is that in the *in vitro* system, thioredoxin (a powerful protein-disulphide reductase) is required to reduce critical disulphides on the carboxylase, which have artificially formed during the microsome preparation and which inactivate the enzyme. Additionally, both thioredoxin and PDI may be active as reductants supplying electrons during the reduction of KO. This explanation implies that, *in vivo*, PDI-$(SH)_2$ may even function as the only cofactor for KO reductase.

It is well known that the synthesis of Gla (carboxylase activity) and the formation of KO (epoxidase activity) only proceed at equimolar rates at saturating concentrations of CO_2 and carboxylatable substrate [29]. Most probably these conditions are not met *in vivo*, and it is intriguing to speculate why the recycling of vitamin K may be uncoupled from the carboxylase activity, and may exceed by far the rate of Gla formation. A putative relationship between disulphide bond formation and the reductive steps in the vitamin K cycle readily explains the necessity of such an uncoupling; however, *in vivo* the number of disulphide bonds formed during protein synthesis will probably exceed the number of Gla residues; hence the oxidation rate of KH_2 must be higher than the protein carboxylation rate.

An alternative explanation for the putative involvement of PDI in the vitamin K cycle would be that the enzyme has a structural role. In our model all proteins involved in the carboxylation reaction (PDI, TR, thioredoxin and the enzymes of the vitamin K cycle) must be aligned in a complex protein/phospholipid aggregate, which is disrupted by the preparation procedure for the microsomes and by the detergent used in the assay. It is known that PDI is the β-subunit of prolyl hydroxylase, where it serves as a structural component [30], and it might have a similar role in the carboxylase/reductase complex. This hypothesis requires further confirmation, however. Experimental systems should be developed, in which it is possible to demonstrate whether the model proposed in Fig. 2 occurs *in vivo*. If the coupling between disulphide bond formation and vitamin K recycling proves to be a physiological route, an explanation will also have to be found for the mechanism of disulphide bond formation during periods of oral anticoagulant treatment.

The conclusions which can be drawn thus far are that for *in vitro* studies the PDI/thioredoxin system is an improvement over

the commonly used synthetic dithiols such as DTT. Maximal activity is only obtained if both proteins (PDI as well as thioredoxin/TR) are present in the reaction mixture, but the precise mechanism by which they participate in the reduction of KO and K is presently uncertain. The PDI/thioredoxin system is a good candidate to be the physiological 'cofactor' providing the reducing equivalents required in the vitamin K cycle, but whether these dithiol proteins are also involved in the vitamin K cycle *in vivo* remains to be proven.

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