THE INHIBITION OF VITAMIN K-DEPENDENT CARBOXYLASE BY CYANIDE

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1. Introduction

The formation of γ-carboxyglutamic acid residues from glutamic acid residues is a vitamin K-dependent carboxylation reaction. The reaction has been demonstrated in various species and tissues [1,2], but the most extensive studies have been performed in the microsomal fraction of rat liver. It is generally assumed that the carboxylation reaction is coupled to the epoxidation of reduced vitamin K [3,4], but the reaction mechanism is still unclear. The purification of the carboxylating enzyme (carboxylase) from rat liver seems to be difficult and has not been reported until now. The possible involvement of other cofactors, such as haem groups [5–7] is therefore still a matter of dispute. The main argument for the involvement of haem in the carboxylation reaction was the observation that cyanide inhibits rat carboxylase [5,8]. This inhibition has not been further analyzed, however, whereas other common haem ligands such as azide and carbon monoxide do not inhibit the vitamin K-dependent carboxylation [1].

We have developed a carboxylating enzyme system from the livers of warfarin-treated cows and obtained a 100-fold purification of the microsomal enzyme by immunospecific adsorption onto antibodies against the endogenous substrate [9]. This partly purified enzyme preparation is attached to Sepharose beads and is called solid-phase carboxylase (SP-carboxylase). The enzymatic activity of SP-carboxylase is strictly dependent on the presence of phospholipids and in the presence of an excess of exogenous substrate (Phe–Leu–Glu–Glu–Leu), the carboxylation rate was constant for at least 3 h at 25°C [10]. Here, we describe the results of some more detailed investigations concerning the inhibition of the SP-carboxylase catalyzed reaction by NaCN.

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2. Materials and methods

Unless indicated otherwise, all chemicals were from Merck. All buffers were made free of CO_2 by boiling flushing with nitrogen gas. One-year-old cows were treated with warfarin (Sigma) for 1 week (10 mg/kg daily) and SP-carboxylase was prepared from the livers of these cows [9]. The vitamin K-dependent incorporation of $^{14}CO_2$ was performed in reaction mixtures (0.25 ml), containing 0.1 ml SP-carboxylase slurry (80 μg protein), 150 mM NaCl, 50 mM Tris–acetate (pH 7.0), 2 mM Phe–Leu–Glu–Glu–Leu (Vega Fox), 2 mM dithiothreitol (Sigma), 0.2 mM EDTA, 20 μCi $NaH^{14}CO_3$ (New England Nuclear, 40 Ci/mol) and 0.2 mM vitamin K hydroquinone, which was added in the form of mixed micelles with phosphatidylcholine and Triton X-100 [10]. The reaction mixtures were incubated for 90 min at 25°C and the reaction was stopped by adding 2 ml ice-cold trichloroacetic acid. The supernatants were degassed at 80°C and counted. Counting was performed in a Packard Tricarb scintillation counter using Picofluor-15 (Packard) as a scintillation liquid. The vitamin K-dependent carboxylation is expressed as the amount of CO_2 (nmol) incorporated into Phe–Leu–Glu–Glu–Leu per mg Sepharose-bound protein.

The *t*-butylhydroperoxide-driven carboxylation was measured under similar conditions as described for the vitamin K-dependent reaction, except for the fact that *t*-butylhydroperoxide (5 mM) was added instead of vitamin K hydroquinone. Experiments in which we checked the eventual incorporation of $^{14}CN^-$ were performed in CO_2-free buffers under similar

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conditions as described for the carboxylation reaction except for the fact that 100 μCi $Na^{14}CN$ (New England Nuclear) was added instead of $NaH^{14}CO_3$. The reaction was stopped by adding 100 mM non-labeled NaCN and non-bound label was removed by dialysis. The low molecular mass Phe–Leu–Glu–Glu–Leu was bound to DEAE-Sephadex (Pharmacia) before dialysis.

Vitamin K epoxide was determined by extracting the reaction mixtures with isoproponal/hexane and analyzing the extract with the aid of high-performance liquid chromatography [11].

3. Results

Bovine vitamin K-dependent carboxylase was inhibited by NaCN in the solubilized microsomal fraction as well as in SP-carboxylase. The nature of the cyanide inhibition was studied only in the more purified system. In dose–response studies in which the carboxylation reaction was performed at various NaCN concentrations, we observed a parabolic relation between the NaCN concentration and the inverse of the reaction rate (fig.1), indicating that the inhibition is of the non-linear type [12]. Inhibition of 50% was obtained at 1 mM cyanide. When *t*-butylhydroperoxide was used as a coenzyme for SP-carboxylase instead of vitamin K hydroquinone, the carboxylation reaction was 28 000 dpm . mg $protein^{-1}$. h^{-1}, which is ~10% of its normal rate. In this system the inhibition of the carboxylation occurred at similar [NaCN] as in the vitamin K-dependent reaction. We also found the cyanide inhibition to be reversible. After incubation of SP-carboxylase with 100 mM NaCN for 1 h at 25°C, the enzyme activity could be restored up to 80% by subsequent dialysis.

To determine whether the inhibition by cyanide could be counteracted by increasing the concentration of one of the participants of the carboxylation reaction, we varied the concentrations of exogenous substrate, vitamin K hydroquinone, O_2 and CO_2, whereas [cyanide] was kept constant. These experiments were performed at 0.025, 1 and 2 mM NaCN and they showed that the cyanide was competitive with CO_2 (fig.2). No competition was observed with one of the other reaction components. As CO_2 is the material incorporated in the vitamin K-dependent reaction, it might be that after adding NaCN, the

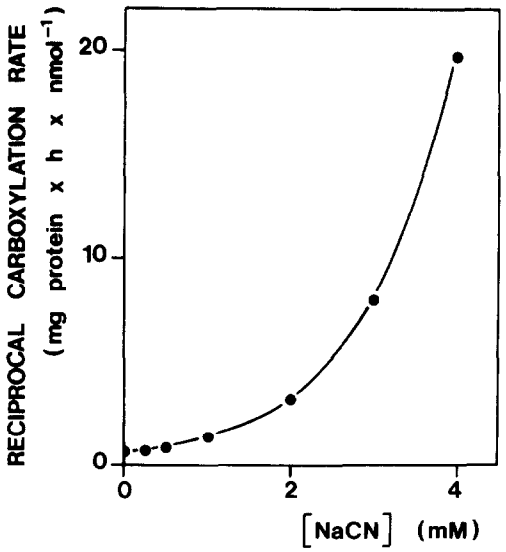

Fig.1. The inhibition of SP-carboxylase by NaCN. The vitamin K-dependent carboxylation was measured in the presence of NaCN as in section 2.

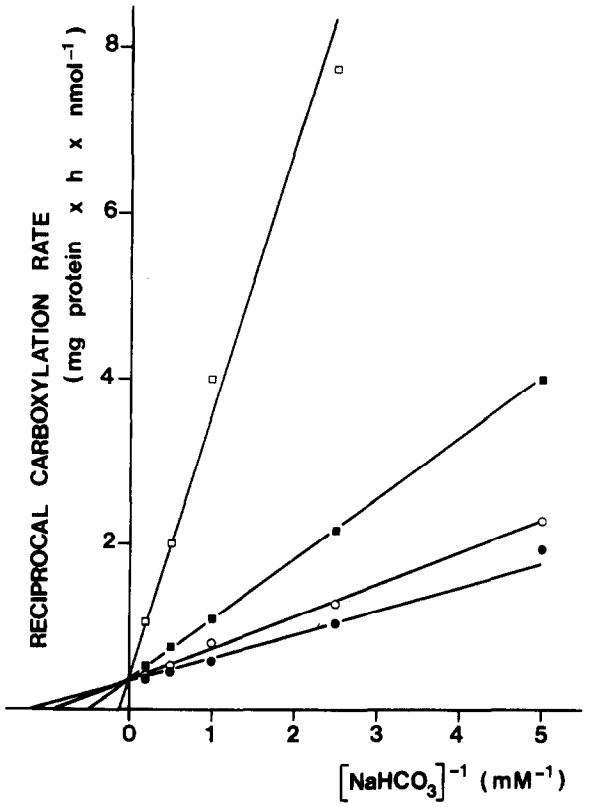

Fig.2. Double reciprocal plots of the vitamin K-dependent incorporation of $^{14}CO_2$ at various NaCN concentrations. The rate of CO_2 incorporation at a number of $NaH^{14}CO_3$ concentrations was measured in the absence of NaCN (●——●), at 0.25 mM NaCH (○——○), 1 mM NaCN (■——■) and 2 mM NaCN (□——□).

Table 1
Effect of NaCN on carboxylase and epoxidase activity

NaCN (mM)	$^{14}CO_2$ incorporation (nmol . mg^{-1} . h^{-1})	Vitamin K epoxide generation (nmol . mg^{-1} . h^{-1})
0	2.5	46
2	0.4	48
20	0	53

The vitamin K-dependent incorporation of $^{14}CO_2$ and the formation of vitamin K epoxide were measured in standard reaction mixtures (1 ml), containing 20 μM warfarin. After incubation for 90 min at 25°C, 0.4 ml was used for determining the amount of incorporated CO_2 and another 0.4 ml was taken for assessing the amount of epoxide

inhibitor was incorporated into glutamic acid residues. Therefore we compared the incorporation of $^{14}CN^-$ and $^{14}CO_2$ by vitamin K-dependent SP-carboxylase. Although $^{14}CO_2$ was incorporated with a rate of 250 000 dpm . h^{-1} . mg $protein^{-1}$, the incorporation of $^{14}CN^-$ could not be demonstrated.

The vitamin K-dependent carboxylation of glutamic acid residues and the epoxidation of vitamin K hydroquinone are 2 closely associated functions. Hence we also studied the influence of cyanide on the epoxidation reaction (table 1). In order to inhibit trace amounts of reductase, which might be present in the system, we added 20 μM warfarin to these reaction mixtures. Whereas the carboxylation is completely blocked at 5 mM NaCN, no inhibition of epoxide formation was observed even at 20 mM NaCN. This clearly demonstrated, that the formation of vitamin K epoxide is not necessarily coupled to the carboxylation reaction.

The inhibition of carboxylase by NaCN might be explained by the binding of CN^- to a functional haem group. Therefore we resolubilized carboxylase from the solid phase with the aid of 6 M urea and 2% SDS. Whereas under similar conditions haem could be detected (as measured by the absorbance at 415 nm) in crude microsomes (2.5 nmol/mg protein), we did not find haem in our purified preparation, which indicates, that the amount of haem in carboxylase is <0.05 nmol/mg protein.

4. Discussion

The inhibition of vitamin K-dependent carboxylase by cyanide was found to be competitive with CO_2. However, it is not probable that cyanide and CO_2 have the same or partially the same binding sites.

(i) The non-linearity of the inhibition by cyanide indicates that >1 binding site for cyanide exists on carboxylase, and that there is a cooperativity in the cyanide binding by carboxylase. On the other hand, we do not have any indication for >1 binding site for CO_2.

(ii) The electrophilic CO_2 is chemically very different from the nucleophilic CN^-. Thus it is not surprising that we did not find a vitamin K-dependent incorporation of CN^-.

A possible involvement of haem-iron is not substantiated by the observed type of cyanide inhibition, since in that case the inhibition would not be competitive with CO_2 but rather with more reducing agents like vitamin K hydroquinone. A second argument for the non-involvement of haem is that haem does not co-purify with carboxylase, but >98% is removed from SP-carboxylase as compared to the crude microsomal solution.

A hydroperoxide intermediate of vitamin K has been postulated as the coenzyme required for the carboxylation event [13]; *t*-butylhydroperoxide supports the carboxylation reaction, even in the absence of vitamin K [13,14]. We observed, that the *t*-butylhydroperoxide-driven carboxylase copurifies with the vitamin K-dependent enzyme and that both activities are blocked by NaCN. This supports the hypothesis that both, the *t*-butylhydroperoxide-driven carboxylation and the vitamin K-dependent carboxylation are accomplished by the same enzyme.

It has been postulated that the vitamin K-dependent carboxylation is coupled to the epoxidation of vitamin K hydroquinone. Carboxylation without epoxidation has never been demonstrated, and inhibitors of the epoxidation reaction (e.g., chloro-K and peroxidases) also inhibit the carboxylation reaction [7,15]. On the other hand, the epoxidation event seems not necessarily to be coupled to the carboxylation event because, on a molar basis, the amount of epoxide formed always exceeds the amount of incorporated CO_2 [3]. Moreover, epoxidation has even been demonstrated in the absence of CO_2 [15]. This uncoupling of the 2 reactions is also illustrated by the fact that cyanide blocks the carboxylation reaction without a simultaneous inhibition of the epoxide formation. Cyanide might therefore be helpful for studies of the epoxidation event in the absence of carboxylation. The question whether epoxidation and carboxylation are both catalyzed by the same enzyme remains to be answered, however.

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