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Chemistry

Mechanism of cyanide inhibition of the blood-clotting, vitamin K-dependent carboxylase

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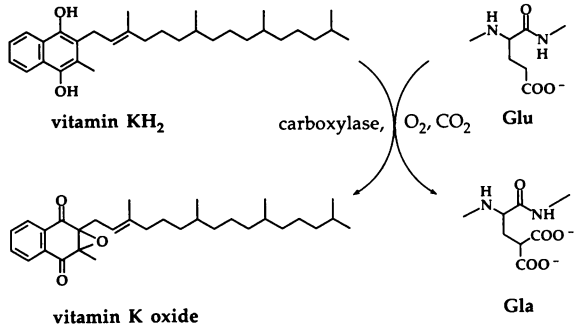
ABSTRACT **Cyanide is a competitive inhibitor of carbon dioxide in the vitamin K-dependent glutamate carboxylase system, which plays a central role in the function of the blood clotting cascade. The mechanism of cyanide inhibition has been obscure for some time. At pH 7.2, cyanide (pK_a = 9.21) will exist in solution as hydrogen cyanide to the extent of 99%. Hydrogen cyanide is a linear triatomic molecule able to serve as a surrogate for carbon dioxide at the enzyme active site. Hydrogen cyanide is an acid; it will quench the deprotonated glutamate carbanion precursor to γ-carboxyglutamate, resulting in inhibition of the carboxylation sequence.**

Cyanide inhibits the crucial vitamin K-dependent carboxylase (1–5) that transforms the N-terminal glutamic residues to γ-carboxyglutamates (6–9) in eight proteins of the blood clotting cascade. In the course of the carboxylation reaction (Glu → Gla) the biologically active hydroquinone form of vitamin K, vitamin KH_2, is oxidized to vitamin K oxide (Scheme I). DeMetz *et al.* (2) and Olson *et al.* (4) showed that cyanide causes the extent of carboxylation to decrease in approximately linear fashion, while vitamin K oxide formation increases in the compensating sense (Fig. 1). Cyanide, in effect, uncouples vitamin K oxide formation from carboxylation.



Scheme I

Inhibition by cyanide suggested the possible involvement of a heme prosthetic group or other metal-containing cofactor at some stage in the vitamin K-dependent carboxylation sequence (2, 10–13). DeMetz *et al.* (2) could detect no heme absorption at 415 nm in a partly purified carboxylase preparation and succeeded in setting a lower limit of <0.05 nmol of heme per mg of protein. The conclusion that there probably is no heme prosthetic group associated with the carboxylase is supported by the mechanistic analysis presented here.

DeMetz *et al.* (2) established that cyanide is a reversible, competitive inhibitor of carbon dioxide in the carboxylation step. Direct covalent attachment of cyanide to glutamate was ruled out by demonstrating that [^{14}C]cyanide was not incorporated into glutamate-containing peptide substrate in place of carbon dioxide (2). In spite of the observation of competitive carbon dioxide inhibition, it was concluded that cyanide and carbon dioxide probably do not occupy the same or (even) partially the same enzyme binding site (2), in part because the neutral, electrophilic carbon dioxide is chemically very different from the negatively charged, nucleophilic cyanide ion and in part because of the apparent nonlinearity of the inhibition by cyanide (2). As a consequence, the role of cyanide and the mechanism of its inhibition of glutamate carboxylation in the proteins of the blood clotting cascade have remained obscure.

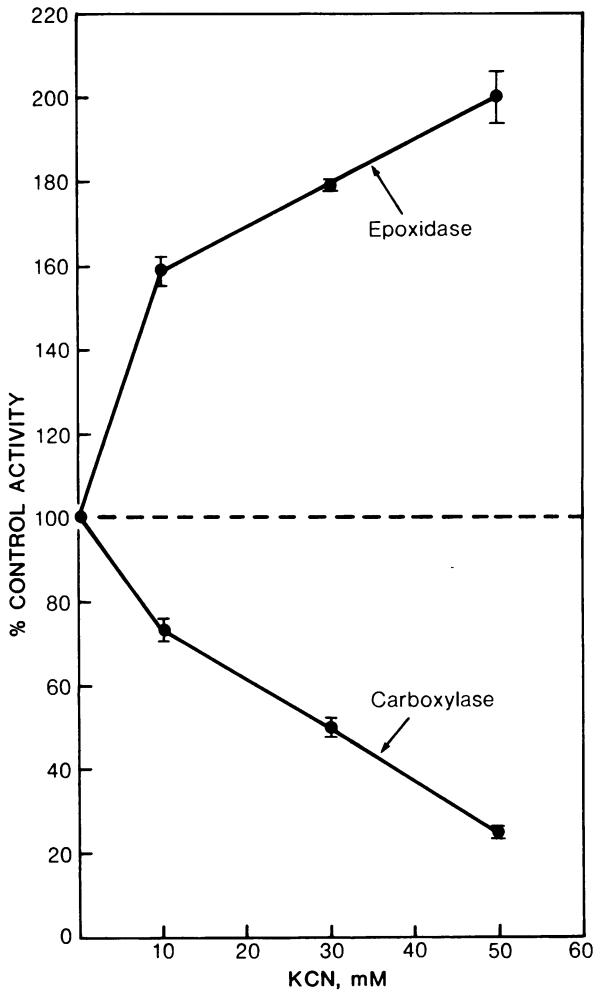


FIG. 1. Effect of cyanide ion on the epoxidation and carboxylation activities of the vitamin K-dependent carboxylase. This figure is reproduced from ref. 4 with permission (copyright Royal Swedish Academy of Sciences).

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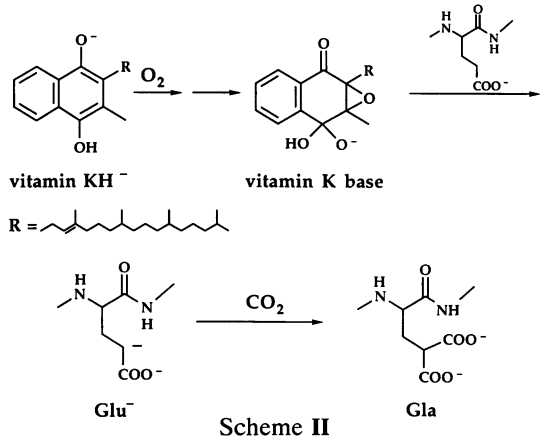
Mechanism of Action of Vitamin K

What is the nature of the reactive glutamate intermediate that leads to carboxylation? Arguments have been presented in favor of carbanion (2, 14–16) and free radical (17) intermediates. Free radical carboxylation is unattractive from a thermochemical standpoint, but the difficulty associated with base-promoted abstraction of the γ proton adjacent to the carboxylate in glutamate ($pK_a \approx 25$) under normal biochemical circumstances has encouraged exploration of alternative hypotheses, such as that involving free radicals (18).

A model has recently been developed for the vitamin K-dependent carboxylation that supports the carbanion hypothesis (19, 20). The model suggests that the formation of vitamin K oxide occurs by spontaneous oxygenation of vitamin KH^- and that this transformation provides the energetic driving force to produce the strong base (vitamin K base) required to remove the γ proton of glutamate (Scheme II) (19, 20).



Scheme II

In this proposed scheme for the carboxylase promoted carboxylation, a key intermediate is the carbanion (Glu^-) formed by deprotonation of glutamate (3, 19, 20). Although it is expected to be highly reactive, the Glu^- intermediate is apparently protected to some degree against protonation at the active site by the surrounding enzyme and by the reactivity of the normal substrate, carbon dioxide. In the presence of high concentrations of carbon dioxide, low levels of tritium from tritiated water are incorporated into glutamate (3). When carbon dioxide is depleted from the reaction system, higher levels of tritium from tritiated water are incorporated into the γ position of glutamate under the influence of the carboxylase (21).

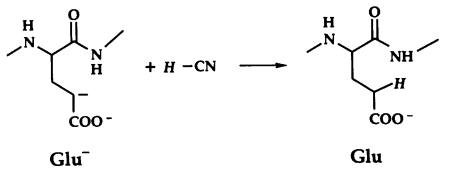
By contrast, removal of tritium from γ-tritiated glutamate in peptide substrates is a ready reaction dependent on the concentrations of oxygen and vitamin KH_2, but independent of the CO_2 concentration. This is also consistent with the mechanism outlined in Scheme II.

A Mechanism for Cyanide Inhibition

The *in vivo* glutamate carboxylation reaction is carried out at pH 7.2, where cyanide (pK_a 9.21) will be 99% in its protonated form, hydrogen cyanide. Like carbon dioxide, hydrogen cyanide is a linear triatomic molecule and can, therefore, fit readily into the carboxylase enzyme active site in place of carbon dioxide. Carbon dioxide may be involved in hydrogen bonding at the enzyme active site (Scheme III). If so, hydrogen cyanide also has a lone pair of electrons available for hydrogen bonding to the enzyme (Scheme III). The highly polar character of hydrogen cyanide (dipole moment, 2.98 D) will contribute to hydrogen bonding (22, 23). Thus, size, shape, and polarity can combine to make hydrogen cyanide an effective active site competitor of carbon dioxide.

Scheme III

Hydrogen cyanide is an acid. When bound to the enzyme, it will be poised at the active site to protonate the glutamate carbanion (Glu^-) (Scheme IV). This provides an explanation for the two related observations embodied in Fig. 1. (*i*) Protonation of the glutamate carbanion by hydrogen cyanide will result in decreased production of carboxylated product. (*ii*) At the same time, since the production of vitamin K oxide depends on the concentration of glutamate in the peptide substrate (12), regeneration of starting glutamate will lead to an increased yield of vitamin K oxide.



Scheme IV

As depicted in Scheme IV, hydrogen cyanide also represents a means to breech the barrier to protonation of Glu^- at the active site in competition with carboxylation. Thus, in tritiated water, hydrogen cyanide will undergo rapid isotope exchange with the medium; it can then transfer tritium to Glu^-. The data of McTigue and Suttie (3) indicate that this is indeed the case. Vitamin K-dependent incorporation of tritium from tritiated water into Boc-Glu-Glu-Leu-OMe is shown in Table 1. In the complete system varying amounts of tritium are incorporated into the tripeptide substrate. In each of the four experiments in Table 1 addition of cyanide leads to an average 20% (range, 8–73%) increase in the incorporation of tritium as predicted by the model in Scheme IV. Table 1 also shows that increasing the level of carbon dioxide results in a sharp lowering of the tritium incorporation. It should be noted that this series of experiments was conducted with the idea that addition of cyanide, a known inhibitor of carboxylation, would decrease the rate of the carboxylation step and favor tritium incorporation into the substrate (3). However, no mechanistic explanation was provided to show how or why this was the case.

When an acid such as hydrogen cyanide is incorporated at the active site, protonation of the vitamin K base (Scheme II) can also occur and lead to reduced loss of label from the substrate. This prediction is supported by the results of Suttie and coworkers (5) (Table 2), where addition of cyanide

Table 1. Vitamin K-dependent incorporation of ^3H from ^3H_2O into Boc-Glu-Glu-Leu-OMe

Reaction components	dpm per 100 nmol A	B
Experiments I		
Complete system	2110	1710
Without O_2	190	<100
Without vitamin KH_2	<100	<100
Plus 10 mM KCN	2600	2970
Experiments II		
Complete system	5660	6250
Without vitamin KH_2	<100	<100
Plus 10 mM KCN	6930	6770
Plus 5 mM $NaHCO_3$	1390	1740

The data are taken from ref. 3; A and B are duplicate experiments.

Table 2. Effect of peptide substrate concentration and cyanide on vitamin K epoxidation and release of ^3H from [β,γ-^{3}H]Glu in the substrate Phe-Leu-Glu-Glu-Leu

Addition(s)	Vitamin K epoxide, nmol/ml	^3H released, nmol/ml	Ratio
0.75 mM peptide	10.4	9.3	1.12
5.75 mM peptide	30.7	29.0	1.06
0.75 mM peptide + 10 mM KCN	21.9	6.3	3.48

The data are taken from ref. 5.

results in increased production of vitamin K oxide and diminished removal of tritium from substrate. The effect seen in Table 2 is smaller than that in Table 1 and might profitably be examined over a range of cyanide concentrations.

General Applicability

As far as we have been able to ascertain, hydrogen cyanide has not previously been considered as a competitive surrogate for carbon dioxide.

In addition to the vitamin K-dependent carboxylase, a number of other carboxylases are inhibited by cyanide. These include: ribulose-1,5-bisphosphate carboxylase (24–26), acetoacetate decarboxylase (27), and aminomalonate decarboxylase (28). In these transformations the available evidence supports various cyanohydrin and Schiff base–cyanide adducts as the inhibitory species. However, it may be worthwhile to inquire anew to learn whether hydrogen cyanide might be a competitive inhibitor for carbon dioxide in these systems, in the sense described above. In another context, a central feature of hemoglobin is its capacity to transport carbon dioxide by means of a carbamate linkage (29). It would be interesting to learn whether hydrogen cyanide binds similarly to the protein, in an amidine linkage, in addition to its well known binding to the iron of the heme group.

Conclusion

Arguments have been presented suggesting that cyanide inhibition of the vitamin K-dependent carboxylase be interpreted in terms of the acid–base properties of hydrogen cyanide. This approach lends strong support to the hypothesis that the reactive intermediate in the carboxylation is the glutamate carbanion. In addition, a well-defined acid–base role for hydrogen cyanide weakens the hypothesis that a heme or other metal cofactor is involved in the carboxylation sequence.

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