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Vitamin K-dependent γ-carbon-hydrogen bond cleavage and nonmandatory concurrent carboxylation of peptide-bound glutamic acid residues

(mechanism/carboxylation reaction)

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ABSTRACT The pentapeptide Phe-Leu-Glu-Glu-Leu, tritiated at the γ carbon of each Glu residue, has been synthesized. In a system using microsomal preparations derived from rat liver, vitamin K-dependent tritium release from the L-Glu residues of this substrate can occur without the concurrent γ-carboxylation of Glu. This tritium release reaction, which indicates cleavage of the γC-H bond, although easily uncoupled from CO_2-dependent γC carboxylation, does require the reduced (hydroquinone) form of vitamin K and oxygen. The data argue against a concerted mechanism for the cleavage of the γC-H bond and carboxylation and against a mechanism in which the vitamin functions solely to transfer or activate CO_2. Although the tritium release is related clearly to the oxidation of vitamin KH_2, it is not yet established how the subsequent carboxylation proceeds. However, two carboxylation mechanisms compatible with the results are discussed.

The detailed mechanism by which vitamin K supports the posttranslational conversion of specific glutamic acid (Glu) residues to γ-carboxyglutamic acids (γ-CGlu) in certain proteins remains unknown. The vitamin K-dependent carboxylating enzyme system, which is situated in the endoplasmic reticulum (1), can be solubilized partially with nonionic detergents, but it has not yet been extensively purified; hence, detailed mechanistic studies have been impossible. The basic requirements of the enzyme system in rat liver microsomal preparations include oxygen, CO_2 (2) or bicarbonate, and the hydroquinone form of vitamin K; neither ATP nor biotin is involved (3–6). The enzyme system will carboxylate appropriate endogenous protein substrates as well as certain added oligopeptides (7) such as the pentapeptide Phe-Leu-Glu-Glu-Leu, which corresponds to amino acid residues 5–9 of rat prothrombin.

In recent experiments related to biotin-dependent carboxylations, Stubbe and Abeles (8) demonstrated that with β-fluoropropionyl coenzyme A as the substrate, the biotin-dependent enzyme propionyl-CoA carboxylase catalyzed the formation of ADP with the elimination of fluoride ion. In this study, the rate of fluoride release, indicative of the minimal rate of abstraction of the α-proton, was 6 times that of ADP formation which is also equivalent to the rate of formation of biotin-CO_2. This indicated that hydrogen abstraction from the substrate could occur without concomitant CO_2 transfer from biotin to the substrate. These data strongly suggest that proposals of concerted mechanisms (9, 10) of proton abstraction and carboxylation for biotin-dependent carboxylations are not feasible.

A similar approach also could be used with a vitamin K-dependent carboxylating enzyme system if an appropriate substrate were available. Thus, we have synthesized the pentapeptide substrate, Phe-Leu-Glu-Glu-Leu, which is tritiated at the γ carbon of both Glu residues. We report here that the vitamin K-dependent enzymatic cleavage of the C-H bond in the γ position of the glutamate in this substrate can proceed in the absence of carboxylation.

MATERIALS AND METHODS

Preparation of Tritium-Labeled Pentapeptide. Because L-Glu specifically tritiated at the γ carbon was unavailable, L-Glu (specific activity 50 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) tritiated at both the β and γ carbons (55% β and 45% γ as determined by tritium NMR analysis, with a random distribution between the *R* and *S* positions at each carbon) was obtained from New England Nuclear. Unlabeled L-Glu (1 g) was added to radioactive Glu (25 mCi), and the amino acid was benzyl-esterified specifically at the γ position (11). The γ-benzylglutamate was treated with *t*-butoxycarbonyl (*t*-Boc) azide in triethylamine (12), and the resulting protected amino acid was used in the solid phase synthesis of the pentapeptide Phe-Leu-Glu-Glu-Leu. Boc-L-Leu resin (3.5 g, 0.41 meq/g), prepared by reaction of the chloromethyl resin with Boc-L-Leu and triethylamine, was used. All four amino acids were N^{α}-Boc protected; each amino acid coupling step was repeated three times. The tritiated Boc-L-Glu-γ-benzyl ester (3.1 meq) was dissolved in methylene chloride, divided in half, and used only for the first coupling at each Glu addition in an amount equivalent to the Boc-Leu on the resin; all other couplings were with a 3-fold excess of the appropriate protected amino acid.

Eighty percent of the added tritiated Boc-L-Glu-γ-benzyl ester was coupled at each step. The finished peptide was cleaved from the resin by treatment with HBr in trifluoroacetic acid at room temperature for 90 min (13); after the resin was filtered and washed with trifluoroacetic acid, the combined filtrates were evaporated at reduced pressure. The residue was redissolved and evaporated four times from $HOAc/H_2O$, 3:1 (vol/vol) and finally dissolved in $HOAc/H_2O$, 1:1 (vol/vol). The pentapeptide was purified by gel filtration over a Bio-Gel P-2 column equilibrated with 1% HOAc in H_2O, on which the elution volume of unlabeled Phe-Leu-Glu-Glu-Leu (Vega-Fox Biochemicals, Tucson, AZ) had been determined. The radioactive peak eluting in this volume was dried under reduced pressure. The peptide, which contained 10% of the original radioactivity in Glu, was 95% pure, as judged by thin-layer chromatography (7). The amino acid composition was: Phe,

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Abbreviations: Boc *t*-butoxycarbonyl; γ-CGlu, γ-carboxyglutamic acids.

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1.00; Leu, 2.08; Glu, 2.20. Tritium exchange with solvent was followed at each step in the preparation and was negligible. The specific activity of the peptide was 9345 dpm/nmol.

The peptide obtained after gel filtration was purified to homogeneity (as judged by amino acid composition) by exchange chromatography on QAE-Sephadex. Because the impurities removed by the QAE-Sephadex were substrates for neither the tritium release nor the carboxylation reactions and because the purification step resulted in a further decrease in recovery of product, we have used the peptide recovered from gel filtration for our studies.

For assay, peptide was dissolved in a minimal volume of 1 M KOH. This resulted in a solution of neutral pH which was then brought to the desired volume with H_2O; it was stored at 4°C and prior to assay was evaporated at reduced pressure to remove any traces of tritiated H_2O generated on storage.

Preparation of Vitamin K-Dependent Carboxylating System. A microsomal pellet, obtained from the livers of vitamin K-deficient rats (4), was extracted twice with a 40-fold excess (wt/vol) of cold acetone at 0°C in a Potter–Elvehjem homogenizer. After each extraction, the suspension was centrifuged at 7500 × *g* for 10 min at 4°C; the final pellet was dried under reduced pressure. The resulting powder was stored desiccated at 4°C; prior to assay, powder (50 mg/ml) was resuspended with a Potter–Elvehjem homogenizer in 0.25 M sucrose/25 mM imidazole·HCl, pH 7.2/2 mM dithiothreitol. This powder retains 70% of the carboxylating activity of the microsomal pellet, and the activity remains during storage (>75% after 2 weeks).

Conditions for Assay. The powder was incubated with the tritiated peptide in a total volume of 0.125 ml as described (4), with one modification. Pyridoxal 5′-phosphate (2 mM), reported by Suttie *et al.* (14) to stimulate carboxylation, has been included. After incubation, one aliquot of the deproteinized reaction mixture was desiccated at acid pH in a scintillation vial to remove remaining $NaH^{14}CO_3$ and then assayed for ^{14}C in a Searle Mark III liquid scintillation counter with a program that measured only 0.2% of the ^3H radioactivity present while retaining 70% efficiency for ^{14}C.

To measure ^3H release, we placed an aliquot in the side arm of a Thunberg vessel and froze it as the vessel was evacuated. The tube was placed in acetone/dry ice, and the ^3H in the water that lyophilized to the tube was measured at 40% efficiency.

Determination of CO_2 Content of Samples. The titration method described by Rose (15) was used with the following modifications. Incubations and titration were carried out in the sealed vial under N_2 that had been bubbled through KOH. $Ba(OH)_2$ was 10 mM; HCl was 10 mM. These modifications allowed detection of as little as 50 nmol of CO_2 with a reproducibility of ±10%.

RESULTS AND DISCUSSION

The tritiated peptide and commercially prepared unlabeled peptide were compared for their capacities to be carboxylated and were identical. Although ^3H should be released during these incubations from one or both Glu residues, there was no way to predict whether it would be recovered in water or whether the release would be vitamin K dependent. However, as we show in Table 1, 10 times more tritiated water was recovered from an incubation of the peptide with enzyme and vitamin K than was recovered when either the acetone powder or the vitamin was omitted, when the enzyme preparation was heated to 100°C, or when the complete system was assayed without incubation. The vitamin K-dependent tritium release is a reflection of γC-H bond cleavage, which could either precede or occur in concert with carboxylation. Although approximately 0.3% of the tritium on the peptide was recovered as water, the precise number of γC-H bonds cleaved during incubation cannot be determined without additional information: the isotope effect at the γ carbon is not known and, although it is quite unlikely, it is impossible to exclude the possibility that vitamin K-dependent tritium release occurs from both the β and γ carbons. In addition, only a fraction of the tritium released may be recovered in water. To evaluate this last possibility, we incubated pentapeptide labeled with [^{14}C]phenylalanine (New England Nuclear, [1-^{14}C]alanine, specific activity 50 Ci/mol) with the ^3H-labeled pentapeptide. The $^{14}C/^3H$ ratio remains the same as that prior to incubation or after incubation in the absence of vitamin K. In this experiment, a release of less than 5% of the tritium would not have been detected as a change in the $^{14}C/^3H$ ratio and, thus, release of as much as 10% of the tritium at the γ carbon could have escaped detection. However, we have failed to detect vitamin K-dependent tritium transfer to molecules other than water in the following experiment. Incubations of tritiated pentapeptide with and without vitamin K were terminated with perchloric acid as described in Table 1. When the pellets, obtained after centrifugation of these acidified reaction mixtures, were dissolved with 1 M KOH and assayed for vitamin K-dependent tritium transfer from the peptide to the pellet, none was found. Furthermore, when the residue remaining in the side arm of the Thunberg vessel after lyophilization of an incubation mixture that contained vitamin K was dissolved in water and chromatographed on a Bio-Gel P-2 column, the only radioactive column fractions were those containing the pentapeptide.

Table 1. Vitamin K-dependent ^3H release

Condition	cpm
Complete system	1694
Omit acetone powder	94
Omit vitamin K	140
Boiled acetone powder	97
Complete system, no incubation	92

Incubations were for 30 min in room air at 27°C in a total volume of 130 μl. The reaction mixture contained 100 μl of resuspended acetone powder, 2 mM NADH, 2 mM pyridoxal 5′-phosphate, 1.6 mM tritiated pentapeptide (5×10^5 cpm), and either 5 μl of ethanol or 5 μg of vitamin menaquinone-3 (MK_3) in 5 μl of ethanol (penultimate addition). $NaHCO_3$ (0.5 μmol) was added last, and the tubes were capped during the incubation. Reactions were terminated by addition of 270 μl of 10% perchloric acid, and the mixtures were centrifuged to remove precipitated protein. An aliquot of the supernatant solution (200 μl) was assayed for tritiated water. Results are expressed as cpm for the entire 130-μl incubation mixture and are the average of triplicate determinations which differed by less than 15%.

As shown in Fig. 1, time courses of tritium release from, and carboxylation of, the tritiated peptide paralleled each other throughout a 180-min incubation. If after 3 hr the acetone powder is centrifuged and resuspended with fresh reactants, no significant additional tritium release or carboxylation occurs during further incubation. When we added known inhibitors of carboxylation to standard reaction mixtures or omitted compounds known to be required for carboxylation, we demonstrated a striking correlation between tritium release and carboxylation in every experiment (Table 2). Of particular note are the observations that tritium release and carboxylation required both the hydroquinone form of the vitamin and O_2. A recent study (16) suggests that a vitamin K hydroperoxide may be generated from the hydroquinone in the presence of O_2 and that this putative intermediate may support carboxylation. The 2,3-epoxide is ultimately formed from the hydroperoxide. Our

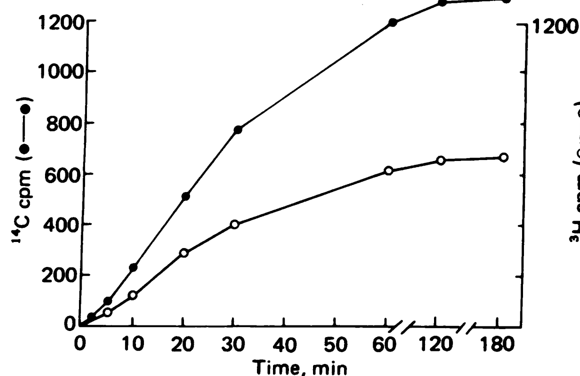


FIG. 1. Time courses for carboxylation and tritium release. Incubations were for the times indicated; other conditions were as in Table 1. The last addition was 0.5 μmol of $NaH^{14}CO_3$ (specific activity 4000 cpm/nmol). One aliquot (100 μl) of the deproteinized reaction mixture was assayed for ^{14}C; a second (100 μl) was assayed for release of ^{3}H from the pentapeptide substrate (specific activity 3738 cpm/nmol). The results are the average of duplicate determinations from which controls incubated without vitamin K have been subtracted and show the cpm measured in the respective aliquots.

data are compatible with the requirement for the formation of this intermediate to catalyze tritium release. Pyridoxal 5'-phosphate, but not pyridoxine, stimulated ^{3}H release to the same degree as it stimulated carboxylation; whether this stimulation reflects an integral step in the carboxylation mechanism remains to be determined.

Although these data demonstrate a correlation between tritium release and carboxylation, they do not prove that a carboxylation must accompany each γC-H bond cleavage. If the specific activities of $NaH^{14}CO_3$ in the reaction mixtures were measured, the amounts of CO_2 fixed in incubations in which the bicarbonate concentrations are varied could be determined and compared with the recovery of tritiated water. When fixing of CO_2 and tritium release were measured over a range of bicarbonate concentrations, there was essentially no change in the amounts of vitamin K-dependent ^{3}H released in experiments in which the amounts of CO_2 fixed varied by more than 10-fold (Table 3). If we assume that there is no ^{3}H released at the β position, that all ^{3}H released is recovered as water, and that the enzyme acts chirally as would be anticipated in these reactions, about 1950–2300 pmol of Glu undergo γC-H bond cleavage. This value, which is already 24-fold more than the amount of γ-CGlu formed at the lowest bicarbonate concentration, would be further increased if a significant isotope effect existed or if there were ^{3}H transfer to molecules other than water. Conversely, in the unlikely event that vitamin K were catalyzing the release of all the ^{3}H from Glu, γC-H bond cleavage of 490–575 pmol of Glu would have occurred. Since time courses (data not shown) of ^{3}H release at the bicarbonate concentrations used in Table 3 are identical, the results cannot be explained by a prolongation of ^{3}H release at the lower bicarbonate concentrations. Rather, this experiment demonstrates that γC-H bond cleavage and carboxylation can be uncoupled. A concerted mechanism requiring an active form of the vitamin, enzyme, substrate, and CO_2 is not possible, and the data are incompatible with the concept that the vitamin functions solely to activate or transfer CO_2.

Although these results do not allow the formulation of a detailed mechanism, they are compatible with the following interpretation. The initial vitamin K-dependent homolytic cleavage of the γC-H bond proceeds with abstraction of hydrogen atom to generate a substrate radical. Radical formation can occur in two ways:

Table 3. Dissociation of vitamin K-dependent tritium release from carboxylation

HCO_3^-, mM	CO_2 fixed, pmol	[^{3}H]Water recovered, cpm	γC-H bonds cleaved,* pmol
0.2	92	1669	1985
0.5	221	1869	2222
1	390	1816	2160
2	654	1922	2285
4	1028	1642	1952

Incubations were as described in Table 1 with the following exceptions: prior to the addition of bicarbonate, the test tubes were sealed with serum caps and the gas phase was made N_2/O_2 (80:20); reactions were initiated by injecting the indicated amounts of $NaHCO_3$ (containing 1.3×10^6 cpm of $NaH^{14}CO_3$) through the serum caps. Bicarbonate concentrations at zero time were determined by measuring total CO_2. For this, reaction mixtures identical to those that were incubated were made up in the sealed vials used for CO_2 determination. Extensive bubbling of reagent solutions with N_2 and multiple precipitations and resuspensions of the acetone powder in N_2-treated buffer reduced the total CO_2 concentration of reaction mixtures before addition of $NaH^{14}CO_3$ to less than 0.05 mM. No change in the specific activity of the bicarbonate occurred during incubations under these conditions. Results are the average triplicate determinations from which controls without vitamin K have been subtracted.

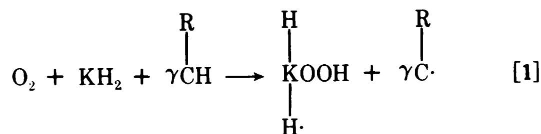
* Calculations are based on the assumptions that no tritium is released from the β carbon, that all tritium released is recovered in water, that a negligible isotope effect exists at the γ carbon, and that the enzyme is acting chirally. See text for details.

Table 2. Simultaneous assay for vitamin K-dependent ^{14}C fixation and ^{3}H release under various conditions

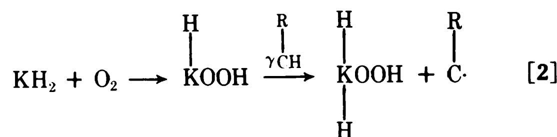
Condition	^{14}C fixed		[^{3}H]Water recovered	
	cpm/incubation	%	cpm/incubation	%
Complete system	3756	100	1522	100
−NADH	404	11	134	9
−NADH, + vitamin K hydroquinone	3380	90	1354	89
−Dithiothreitol	3028	81	1332	88
−Pyridoxal 5'-phosphate	1728	46	684	45
−Pyridoxal 5'-phosphate, + pyridoxine	1368	36	536	35
−Oxygen	44	1	30	2
+ Tetrachloropyridonol (50 μg/ml)	236	6	82	5
+ 2-Chloro-3-phytyl-1,4-napthoquinone (50 μg/ml)	2176	58	926	61
+ Warfarin (15 μM)	3376	90	1358	89

Conditions were as described in Table 1. The last addition was 0.5 μmol of $NaH^{14}CO_3$ (4×10^6 cpm/μmol). The hydroquinone of vitamin MK_3, which was generated from the quinone with $NaBH_4$, was used immediately. Deproteinized reaction mixtures were assayed for ^{14}C and ^{3}H were as described in *Methods*. Results are the average of triplicate determinations from which the appropriate control incubated without vitamin K has been subtracted.

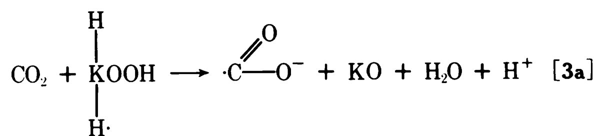
(*i*) The initial reaction is a mixed function oxidation.

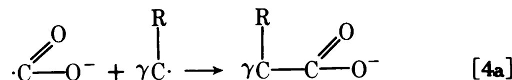


(*ii*) Oxidation of vitamin K hydroquinone to a vitamin K hydroperoxide (or vitamin K semiquinone peroxide adduct) is followed by reaction of the hydroperoxide with the peptide substrate. It has been reported (16) that *tert*-butylhydroperoxide acts as a weak vitamin K analog in the peptide-carboxylating system and that at lower concentrations *tert*-butylhydroperoxide is an apparent competitive inhibitor of the vitamin. Although it would seem premature to eliminate a mixed function oxidation as the initial reaction, the reported effects of *tert*-butylhydroperoxide in this system would favor the second pathway.



After the CO_2-independent generation of the radical (γC·), the carboxylation event may proceed in one of two ways (a or b):





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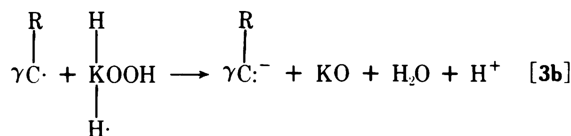

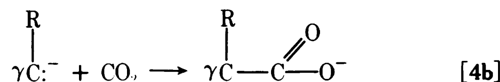

The direct carboxylation of carbanions by CO_2, as in reaction **4b**, is a well-known reaction in synthetic organic chemistry. The donation of an electron from a radical or radical carbanion to another radical to form a new carbanion (reaction **3b**) has less precedent, but it may occur with the vitamin K hydroperoxide intermediate, as it may also in reactions involving ascorbic acid, which can donate hydride and form a very stable radical anion intermediate (17). However, generation of the glutamate substrate carbanion, as in reaction **3b**, would most likely require a prior neutralization of the negatively charged carboxylate ion. This would not be necessary in the radical–radical condensation.

Finally, dissociation of γC-H bond cleavage from carboxylation raises the interesting possibility that vitamin K-dependent γC-H bond cleavage could precede the addition of reactants other than CO_2 to appropriate carbon atoms. Recently, both the vitamin K menaquinone-4 and its 2,3-epoxide have been identified in several invertebrate species in which γ-CGlu has not yet been found (18).

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1. Helgeland, L. (1977) *Biochim. Biophys. Acta* **499,** 181–193.
2. Jones, J. P., Gardner, E. J., Cooper, T. G. & Olsen, R. E. (1977) *J. Biol. Chem.* **252,** 7738–7742.
3. Esmon, C. T., Sadowski, J. A. & Suttie, J. W. (1975) *J. Biol. Chem.* **250,** 4744–4748.
4. Friedman, P. A. & Shia, M. A. (1976) *Biochem. Biophys. Res. Commun.* **70,** 647–654.
5. Jones, J. P., Fausto, A., Houser, R. M., Gardner, E. J. & Olsen, R. E. (1976) *Biochem. Biophys. Res. Commun.* **72,** 589–597.
6. Friedman, P. A. & Shia, M. A. (1977) *Biochem. J.* **163,** 39–43.
7. Suttie, J. W., Hageman, J. M., Lehrman, S. R. & Rich, D. H. (1976) *J. Biol. Chem.* **251,** 5827–5830.
8. Stubbe, J. & Abeles, R H. (1977) *J. Biol. Chem.* **252,** 8338–8340.
9. Retey, S. & Lynen, F. (1965) *Biochem. Z.* **342,** 256–271.
10. Rose, I. A., O'Connell, E. L. & Solomon, F. (1976) *J. Biol. Chem.* **251,** 902–904.
11. Stewart, J. M., Young, J. D., Benjamin, E., Shimizu, M. & Leung, C. Y. (1966) *Biochemistry* **5,** 3396–3400.
12. Carpino, L. A. (1957) *J. Am. Chem. Soc.* **79,** 4427–4431.
13. Stewart, J. M. & Young, J. D. (1969) *Solid Phase Peptide Synthesis* (Freeman, San Francisco), pp. 40–41.
14. Suttie, J. W., Larson, A. E., Canfield, L. M. & Carlisle, T. L. (1978) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **37,** 2605–2609.
15. Rose, J. G. (1976) *Anal. Biochem.* **76,** 358–360.
16. Larson, A. E. & Suttie, J. W. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 5413–5416.
17. Stewart, R. (1964) in *Oxidation Mechanisms,* eds. Breslow, R. & Karplus, M. (Benjamin, New York), pp. 112–113.
18. Burt, V. T., Bee, R. & Pennock, J. F. (1977) *Biochem. J.* **162,** 297–302.