Effect of Vitamin K-dependent Protein Precursor Propeptide, Vitamin K Hydroquinone, and Glutamate Substrate Binding on the Structure and Function of γ-Glutamyl Carboxylase*

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The γ-glutamyl carboxylase utilizes four substrates to catalyze carboxylation of certain glutamic acid residues in vitamin K-dependent proteins. How the enzyme brings the substrates together to promote catalysis is an important question in understanding the structure and function of this enzyme. The propeptide is the primary binding site of the vitamin K-dependent proteins to carboxylase. It is also an effector of carboxylase activity. We tested the hypothesis that binding of substrates causes changes to the carboxylase and in turn to the substrate-enzyme interactions. In addition we investigated how the sequences of the propeptides affected the substrate-enzyme interaction. To study these questions we employed fluorescently labeled propeptides to measure affinity for the carboxylase. We also measured the ability of several propeptides to increase carboxylase catalytic activity. Finally we determined the effect of substrates: vitamin K hydroquinone, the pentapeptide FLEEL, and NaHCO3 on the stability of the propeptide-carboxylase complexes. We found a wide variation in the propeptide affinities for carboxylase. In contrast, the propeptide tested had similar effects on carboxylase catalytic activity. FLEEL and vitamin K hydroquinone both stabilized the propeptide-carboxylase complex. The two together had a greater effect than either alone. We conclude that the effect of propeptide and substrates on carboxylase controls the order of substrate binding in such a way as to ensure efficient, specific carboxylation.

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4 The abbreviations used are: KH2, vitamin K hydroquinone; MOPS, 3-[N-morpholino]propanesulfonic acid; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate; PC, 1,2-dioleoyl-sn-glycerol-3-phosphocholine.
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lization of the propeptide-carboxylase complex by FLEEL a
general phenomenon among propeptides, and do substrates
other than FLEEL affect the stability of the complexes alone or
in combination? 3) Is the substrate effect on the propeptide-
carboxylase complex similar for all propeptides? 4) Is the effect
on small substrate carboxylation similar for all propeptides as
suggested for the propeptides of factors IX and X and pro-
thrombin? 5) Are there amino acid residues necessary for these
functions other than those already recognized as important for
binding to carboxylase, and finally, how do these effects con-
tribute to enzyme specificity and/or efficiency?

EXPERIMENTAL PROCEDURES

Materials—All of the chemicals used were reagent grade.
The pentapeptide substrate, FLEEL, was purchased from Bachem
(Philadelphia, PA). Propeptides labeled with 5,6-carboxyfluor-
rescein on the amino terminus, based upon the sequences of the
human, mouse, and pufferfish propeptides, were synthesized by
Mimotopes (Clayton, Victoria, Australia). All of the peptides
were confirmed to be at least 95% pure using high performance
liquid chromatography and mass spectrometry analysis. Label-
ing of the peptides with 5,6-carboxyfluorescein did not have an
effect on the propeptide-carboxylase interaction (6). 1,2-Dio-
leoyl-sn-glycero-3-phosphocholine was purchased from Avanti
Polar Lipids (Alabaster, AL). Vitamin K₁ was purchased from
Abbott Laboratories (Chicago, IL) and was reduced to KH₂ as
described previously (11). Alkamuls EL-620 was graciously
donated by Rhodia (Cranbury, NJ). NaH¹⁴CO₃ was from ICN
pharmaceuticals (Costa Mesa, CA).

Carboxylase was produced using Sf9 insect cells and stan-
dard molecular biology methods and purified as described (12).
The concentration of the active enzyme was determined as
described previously (6). All of the experiments were per-
formed at 4°C unless described otherwise.

Data Analyses—Kinetic and dissociation constants were esti-
mated using nonlinear least squares analyses of multiple data
sets in SigmaPlot version 9.0 (Systat Software, Inc.). The Kₐ
d values for the various propeptides were estimated using the
numerical integration software Dynafit (BioKin, Ltd.) (13). We
also modeled the propeptide dissociation data mechanistically
using Dynafit.

Fluorescence Anisotropy Measurements—The studies to
determine propeptide Kₐ and kₐ values were performed essen-
tially as described previously (6) with excitation at 490 nm and
emission measured using 515-nm cut-off filters. The only dif-
fERENCE was that we used an SLM 8000 spectrofluorometer
modified by the OLIS Corporation (Athens, GA). Anisotropy
values were converted to concentration of bound propeptide
or fraction bound as described (6). This method of analysis
weights the anisotropies accounting for the intensity differ-
ences between the free and bound propeptide. In all cases,
unless otherwise stated, references to propeptides are to those
that are fluorescein-labeled.

Determination of the Kₐ Values of Propeptides—All of the
samples were prepared in the following buffer: 100 mM MOPS,
PH 7.5, 180 mM NaCl, 3.3% glycerol, 6.3 mM DTT, 66 μM EDTA,
0.1% 1,2 dioleoyl-sn-glycero-3-phosphocholine, 0.28% CHAPS,
and 0.4% BSA. Propeptides (4 nM) were incubated with various
concentrations of carboxylase (2 h), and the anisotropy was
determined.

We also determined the Kₐ values for human factors X
and IX and prothrombin in the presence of 2.4 mM FLEEL.
The FLEEL was added first to the carboxylase and then the
propeptide.

Determination of Dissociation Rate Constants of Propep-
tides from Carboxylase—We measured the dissociation of the
propeptides of prothrombin, protein C, and factor IX from car-
boxylase. Fluorescence experiments were performed in the fol-
lowing buffer: 50 mM MOPS, pH 7.5, 500 mM NaCl, 5% glycerol,
6.3 mM DTT, 66 μM EDTA, 0.16% PC, 0.16% CHAPS, and 0.2%
BSA. The propeptide and enzyme (20 nm fluorescein-labeled
propeptide and 100 nm carboxylase) were incubated for 1 h. To
initiate dissociation of the propeptide, we added 4 μM unlabeled
factor IX propeptide. Anisotropy measurements were taken
every 2 s.

To measure the effect of FLEEL, NaHCO₃, and/or KH₂ on
kₐoff, we incubated them either together or in various combina-
tions with the carboxylase and propeptide prior to the start of
the experiment. Final concentrations were 4.8 mM FLEEL, 160
μM KH₂, and 1.4 mM NaHCO₃. We measured dissociation as
described above.

Because KH₂ is hydrophobic, it is dissolved in a viscous emul-
sifier, Alkamuls EL-620. Alkamuls alone had a small effect on
kₐoff, so we included it in all of the dissociation experiments. The
final concentrations were 0.50 mg/ml Alkamuls EL-620, 0.27
mg/ml dextrose, and 0.066 mg/ml benzyl alcohol, identical to
the concentrations in experiments with KH₂.

Measuring the Effect of Various KH₂ Concentrations
on Propeptide Dissociation from Carboxylase—We measured
the dissociation of the propeptide of factor IX from wild-type
carboxylase at various KH₂ concentrations. The experiments
were performed as described above for measuring kₐoff.

Measurement of the Effect of Propeptides on FLEEL Carbo-
xylation—We measured the effect of 10 propeptides on the rate
of FLEEL carboxylation. Activity was determined using ¹⁴CO₂
incorporation into FLEEL as described (14) in the presence of
80 μM propeptide. We also determined the Kᵣ and kᵣ values
of carboxylase for FLEEL in the presence of human factor IX, pro-
tein C, and prothrombin propeptides (80 μM). At this high
concentration of peptides, essentially all of the carboxylase will be
in complex with the propeptide.

RESULTS

Kₐ Values of Various Carboxylase-Propeptide Complexes—
The carboxylase Kₐ values for the 11 human (plus consensus),
three mouse, and two pufferfish propeptides are summarized in
Figs. 1 and 2. For comparison, our Kₐ values are 4–7-fold lower
than the previously reported Kₐ values for several of the human
propeptides (12). However, the relationship among the Kₐ and
Kᵣ values are similar. In other words, as with the Kᵣ values, the
Kₐ of factor X was less than that of factor IX, which was less than
that of prothrombin.

The difference in the Kₐ values versus the Kᵣ values may be
due to several factors involved with different methods and con-
ditions. For example, the Kᵣ values were determined under cat-
Analytic conditions and at 20 °C versus 4 °C, different buffers, and competition versus direct binding.

In Fig. 2 the propeptides of mouse and pufferfish are compared with their human counterparts. The most striking differences are between mouse and pufferfish factor X and mouse and human prothrombin.

Results of single binding experiments with factor IX and prothrombin propeptides are shown (Fig. 3). The analysis with Dynafit (see “Experimental Procedures”) estimated the \( K_d \) and the propeptide concentration; we fixed propeptide-carboxylase stoichiometry at 1. Analysis of the shown factor IX data yielded a \( K_d \) of 4.95 nM and a propeptide concentration of 4.37 nM. The intended propeptide concentration was 4 nM. The shown prothrombin data yielded a \( K_d \) of 26.3 nM and a propeptide concentration of 4.35 nM.

Next, to expand on our previous studies on the propeptide of factor IX, we examined the effect of glutamate substrates on the carboxylase \( K_d \) values of the factor X and prothrombin propeptides, in addition to that of factor IX (data not shown). As with the propeptide of factor IX, the other propeptides had lower \( K_d \) values in the presence of FLEEL.

The Effect of Substrates on the Dissociation Rate Constants of Propeptides from Carboxylase—In previous studies from this laboratory, the results showed that under catalytic conditions or with FLEEL, the \( k_{\text{off}} \) for the propeptide of factor IX was slower than when the propeptide was bound to carboxylase in the absence of substrates (6, 7). To determine whether this is a general phenomenon, we determined the \( k_{\text{off}} \) values for the propeptides of protein C and prothrombin as well as that of factor IX in the presence and absence of FLEEL.

In all cases the data described below were normalized to one for maximum bound propeptide. The actual maximum bound propeptide varied from 80% for the propeptide of prothrombin in the absence of substrate to increasing values for the higher affinity situations with substrates and for factor IX and protein C propeptides.

We previously showed that a slow loss of carboxylase activity occurs at 20 °C (6). In our present study (results not shown), we found that the biphasic curves were not caused...
by an active and inactive form of carboxylase. On incubation at 20 °C for up to 6 h, the amount of propeptide-bound gradually decreased. However, inactivation did not affect the rate of dissociation of the propeptide of prothrombin nor the biphasic nature of the data.

With FLEEL the dissociation data fit a single exponential for all three propeptides. The $k_{off}$ values for the three propeptides were all slower than in the absence of FLEEL (Fig. 4 and Table 1).

KH$_2$ with NaHCO$_3$ decreased the $k_{off}$, and in all cases the data fit a single exponential equation (Fig. 4). To determine whether both KH$_2$ and NaHCO$_3$ were necessary for the change in the $k_{off}$, we performed additional experiments in the presence of either KH$_2$ or NaHCO$_3$ with the propeptide of protein C. The results (results not shown) indicate that NaHCO$_3$ alone has no effect on $k_{off}$.

We also used this technique to measure the previously unknown affinity of vitamin K for carboxylase. The change in $k_{off}$ for the carboxylase–propeptide dissociation with increasing KH$_2$ was what one would expect from saturable binding (Fig. 5). The apparent $K_d$ of 19 mM is similar to the reported $K_m$ for KH$_2$ (9, 11, 15).

**Substrate Binding Effects in γ-Glutamyl Carboxylase**

**TABLE 1**

| Addition(s) | Factor IX | Protein C | Prothrombin |
|-------------|-----------|-----------|-------------|
| None        | 2.5 (1)   | 3.7 (1)   | 12 (1)      |
| KH$_2$      | 1.0 (0.40)| 2.1 (0.57)| 4.8 (0.40)  |
| FLEEL       | 0.36 (0.14)| 1.1 (0.30)| 3.2 (0.27)  |
| KH$_2$ + FLEEL | 0.24 (0.10)| 0.52 (0.14)| 1.3 (0.11)  |

* The values within parentheses denote the ratios of $k_{off}$ in the presence of KH$_2$ and/or FLEEL to $k_{off}$ for propeptide alone.

* Alkamuls EL-620 was added to samples with no KH$_2$.

**Effect of Propeptides on FLEEL Carboxylation**—Next we investigated whether there is a correlation between FLEEL carboxylation rate and the affinity or sequence of the propeptide used to stimulate that carboxylase activity. The activities are similar but do vary (Table 2). However, the rates do not correlate with the propeptide affinity. Although the two with the highest activity are consensus and factor X, which have the lowest $K_d$ values, the next highest stimulation occurs with protein C and prothrombin. The latter two have the highest $K_d$ values.

To further test this, we determined the $K_{cat}$ and $k_{cat}$ for FLEEL in the presence of high concentrations of factor IX, protein C, and prothrombin propeptides (Table 3). These results are consistent with the previous experiment in that the kinetics are similar despite a range of $K_d$ values from 5 to 25 mM. We measured the rates and kinetic constants at propeptide concentrations (>3000 times $K_d$) ensuring that almost all carboxylase is bound to each propeptide.

**DISCUSSION**

Our goal in the present study was to further understanding of the structural and functional interactions of carboxylase with its substrates. All indications are that the propeptides of vita-
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![Graph showing relative fraction of wild-type carboxylase bound to KH2 at varying KH2 concentrations](image)

**Table 2**

| Propeptide       | $K_a^{\mu}$ | $1^4CO_2$ incorporated in FLEEL |
|------------------|-------------|---------------------------------|
| Consensus        | 0.08        | 0.238                           |
| Factor X         | 0.59        | 0.236                           |
| Matrix Gla protein| 0.98        | 0.195                           |
| Protein Z        | 1.80        | 0.198                           |
| Factor VII       | 2.34        | 0.198                           |
| PRGP1            | 2.28        | 0.211                           |
| Protein S        | 2.28        | 0.158                           |
| Factor IX        | 4.97        | 0.185                           |
| Protein C        | 18.6        | 0.234                           |
| Prothrombin      | 25.8        | 0.225                           |

*The values are from Fig. 1.

**Table 3**

| Propeptide | $k_{in}$  | $k_{out}$  |
|------------|-----------|------------|
| Factor IX  | 0.29 ± 0.01 | 30 ± 1     |
| Protein C  | 0.36 ± 0.03 | 26 ± 2     |
| Prothrombin| 0.31 ± 0.03 | 33 ± 2     |

*The values are from two determinations.

min K-dependent protein precursors provide most of the energy for their binding to the carboxylase. It is also likely that the propeptide of such a protein constitutes the first site to interact with the enzyme. As the first binding event it most likely sets the stage for binding of the other substrates. In addition, the turnover rate during carboxylation of peptide substrates depends to a certain extent on the propeptide affinity for the carboxylase (7). For these reasons we felt that the carboxylase-bound propeptide would be a good model for the enzyme vitamin K-dependent protein precursor complex. Our idea was to characterize the carboxylase-propeptide interaction without substrates and then study the effect of substrates on the stability of the complex.

As we expected, based on results from previous work, we found the propeptides exhibit a broad range of affinities (12). All have what one would consider a high affinity for the carboxylase, that is, a $K_a$ in the nanomolar range. This is an especially high affinity for a reaction occurring in the endoplasmic reticulum where one would expect high local substrate concentrations. The propeptide residues identified as important earlier (16–18) (residues –6, –9, –10, –15, and –16) tend to be conserved among the propeptides or at least have conservative substitutions. Otherwise there is wide variation in the character of most residues.

In contrast to the variation in affinities, the propeptides we tested all stimulated FLEEL carboxylation to a similar extent. Even for the differences in stimulation there is no correlation between stimulatory effect and propeptide affinity. As with affinity, we were unable to identify the patterns of primary structure necessary for the effect on FLEEL. These results agree with observations regarding the prothrombin and factor X (wild-type and truncated) as well as factor IX propeptides (1–5). The results regarding the relationship between propeptide sequence, affinity for carboxylase, and the ability to stimulate carboxylase activity indicate that binding requires relatively few conserved residues, and once any propeptide binds, the active carboxylase conformations are all similar.

As we discussed in our previous work, at least part of the increase in carboxylase catalytic activity is caused by increased FLEEL binding (19). Because propeptide binding affects FLEEL, FLEEL binding must affect propeptide binding. In fact earlier studies had shown that FLEEL or catalytic conditions slowed FLEEL binding (19). Progressing results indicated that propeptide binding affects FLEEL (19). Dynafit allows testing the fit of data to a given mechanism(s). Our dissociation data fit a single exponential equation. On the other hand, when we added substrate(s) to the propeptide-carboxylase mixture, a single exponential equation adequately fit the dissociation data. KH2 and FLEEL decreased the $k_{out}$ for the prothrombin and protein C propeptides as well as for that of factor IX. The effect of both together was greater than either substrate alone. These results and those from the earlier study suggest that substrate binding to the enzyme-propeptide complex causes a shift in equilibrium to a conformation that has higher affinity for the propeptide, and as a result, higher catalytic activity (20).

To determine whether a multiple conformation model fits our results, we analyzed our data using the software Dynafit (13). Dynafit allows testing the fit of data to a given mechanism(s). Our dissociation data fit a mechanism that includes two forms of carboxylase and a mechanism in which two propeptides bind to one carboxylase. Although an earlier report suggested that carboxylase might bind two propeptide molecules (21), Presnell et al. (6) showed that the carboxylase binds only one propeptide. Therefore we conclude that a model in which at least two forms of carboxylase exist, which have different affinities for propeptide and presumably propeptide-containing substrates, best describes this interaction. Compar-
ing our results to those of earlier studies puts our results into the context of how substrate binding affects carboxylase activity and specificity and may put the earlier studies into the context of how substrate binding affects carboxylase structure.

Li et al. (4) showed that epoxidation and carboxylation can uncouple. In other words, when all substrates other than CO₂ are present, epoxidation of KH₂ occurs without carboxylation. This indicates that if all other substrates are present, the CO₂ is not needed until the last step in carboxylation: addition to the γ-carbanion of glutamate. It is consistent with the prediction of Uotila (22), who concludes from kinetic results that CO₂ was likely the last substrate to react. This also fits logically with our results that indicate bicarbonate does not affect the $k_{\text{cat}}$ of propeptide from the carboxylase.

Sugiura et al. (10) showed that carboxylase does not catalyze epoxidation of KH₂ in the absence of propeptide or FLEEL. The reaction is fastest, ~10-fold faster than with any single component, with both FLEEL and propeptide. FLEEL alone is more effective than propeptide alone. As the authors suggest, this indicates that binding of both or either causes a conformational change that increases the epoxidase activity and/or KH₂ binding.

The experiments of Bouchard et al. (8) showed that N-ethylmaleimide inhibited carboxylase. Glu-containing substrates, such as FLEEL or a peptide containing the propeptide of prothrombin and the first 10 residues of its Gla domain, increased the rate of inactivation, but propeptide alone did not. They also report that KH₂ protects from inactivation only when a Glu-containing substrate is present. These results are in some ways hard to interpret because N-ethylmaleimide is expected to modify Cys residues. It now appears that Cys residues are not directly involved in carboxylase catalysis, as was previously thought. However, these studies are consistent with our hypothesis that substrate binding supports multiple conformations of the carboxylase.

A more recent work by Rishavy et al. (23) implicates two lysine residues (Lys-217 and Lys-218), not cysteines, as important in catalysis. Quantum chemical calculations are consistent with this model, but they also support a second possible mechanism for removal of the KH₂ proton involving only one lysine (24). Because both models are similar for our purposes, we will focus on the two-lysine model in this discussion.

Rishavy et al. (23) propose that Lys-218, in combination with Lys-217, is involved in deprotonating KH₂. According to this model, in the absence of substrates Lys-218(0) is not charged, but Lys-217(+1) is, perhaps because of the proximity of the two lysines to one another. They further suggest that when the Glu substrate approaches the lysine pair, it interacts with Lys-217(+1), thus allowing Lys-218(0) to acquire a proton. Assuming the KH₂ is nearby, Lys-218(0) can then remove the hydroxyl proton that starts the pathway to the vitamin K alkoxyide, the strong base necessary for carbanion formation, and on to carboxylation of the Glu residue.

We interpret our results presented here and those of others described above to support a mechanism that might occur as follows. First, the vitamin K-dependent protein binds to carboxylase through its propeptide. As mentioned above the two lysines (Lys-217 and Lys-218) of carboxylase involved in catalysis are adjacent to one another, allowing one residue to remain uncharged (Lys-218(0)). Therefore, when substrate binds, the glutamate(s) to be carboxylated must not be near Lys-217 and Lys-218. If the glutamate were in proximity to the lysines at this point, then the glutamate with a negative charge and the lysine with a positive charge (Lys-217(+1)) could interact. If that occurred in the absence of KH₂, then the free lysine, Lys-218(0), could acquire a proton from solvent and thus not be available to remove the KH₂ proton necessary for strong base production. Therefore, to set the stage for glutamate interaction with the active site, propeptide binding must first cause the conformational change that promotes KH₂ binding near the lysines. Next KH₂ binding supports a conformation that brings the glutamate substrate in proximity to Lys-217(+1) and Lys-218(0). Now through its negative charge, the glutamate residue binds Lys-217(+1), freeing Lys-218(0), which can then abstract the hydroquinone proton from the nearby KH₂. Glutamate binding to Lys-217(+1) is essential for a second reason; the glutamate negative charge must be neutralized to make its γ-proton removal energetically favorable (25). That step is necessary to produce the carbanion precursor of carboxylation. According to Davis et al. (24), after removal of the proton from the hydroquinone group on KH₂, the rest of the reactions producing γ-carboxyglutamic acid are relatively energetically favorable.

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