A Novel Fluorescence Assay To Study Propeptide Interaction with γ-Glutamyl Carboxylase†

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ABSTRACT: The vitamin K-dependent gamma-glutamyl carboxylase catalyzes the posttranslational modification of select glutamate residues of its vitamin K-dependent substrates to gamma-carboxyglutamate. In this report, we describe a new fluorescence assay that is sensitive and specific for the propeptide binding site of active carboxylase. We employed the assay to make three important observations: (1) A tight binding fluorescein-labeled consensus propeptide can be used to quantify the active fraction of the enzyme. (2) The off-rate for a fluorescein-labeled factor IX propeptide was 3000-fold slower than the rate of carboxylation, a difference that may explain how carboxylase can carry out multiple carboxylations of a substrate during the same binding event. (3) We show evidence that substrate binding to the active site modifies the propeptide binding site of carboxylase. The significant (9-fold) differences in off-rates for the propeptide in the presence and absence of its co-substrates may represent a release mechanism for macromolecular substrates from the enzyme. Additionally, sedimentation velocity and equilibrium experiments indicate a monomeric association of enzyme with propeptide. Furthermore, the carboxylase preparation is monodisperse in the buffer used for our studies.

The vitamin K-dependent γ-glutamyl carboxylase is an integral membrane enzyme located in the endoplasmic reticulum. It catalyzes the posttranslational modification of selected glutamate residues of vitamin K-dependent proteins to γ-carboxyglutamate (Gla).[1] In addition to carbon dioxide and a glutamate-containing substrate, carboxylase utilizes vitamin K hydroquinone and oxygen as cosubstrates. The products of the reaction are Gla, vitamin K 2,3-epoxide, and H_2O (*1*−*3*). The oxidation of vitamin K hydroquinone to epoxide is closely coupled to the γ-carboxylation reaction during substrate turnover; however, the mechanism of this coupling is still poorly understood (*1*, *4*). γ-Carboxylation was first found to be an essential modification for the function of prothrombin, a crucial hemostatic protein (*5*, *6*). The list of vitamin K-dependent proteins, however, is rapidly expanding to include those of diverse function (*3*, *7*).

Carboxylase is thought to recognize most protein substrates at the propeptide binding site, which is spatially distinct from the site of catalysis. Recognition usually occurs via an ≈18 amino acid "propeptide" found on all protein substrates to date (*3*). Possible exceptions to this occur for two substrates: matrix gla protein, where the recognition sequence is found in the middle of the mature protein sequence, and osteocalcin, whose propeptide has a very poor affinity for carboxylase (*8*, *9*). The strength of the interaction between the propeptide and carboxylase depends on the specific amino acid sequence of the propeptide; affinities have been found to vary more than 100-fold (*10*). The propeptide of a protein substrate also acts as an allosteric modulator of the active site. Suttie and co-workers demonstrated that saturating concentrations of synthetic human factor X propeptide caused a 9-fold decrease of the K_m of the carboxylase toward the substrate BocMeEEL (*11*, *12*). It has been reported that a free propeptide does not affect the K_m of vitamin K hydroquinone binding to carboxylase in a BocMeEEL- or FLEEL-based reaction (*12*, *13*). However, when a glutamate-containing substrate with a covalently attached propeptide is utilized, the K_m of vitamin K hydroquinone for carboxylase decreases 7−20-fold compared to a FLEEL-based reaction (*9*, *13*).

In this report, we describe a new fluorometric assay that is sensitive and specific for the propeptide binding site of active forms of the γ-glutamyl carboxylase. The assay allows measurement of the number of propeptide binding sites, the equilibrium dissociation constant for a propeptide−carboxylase interaction, and the off-rate of a propeptide from the enzyme. Interestingly, the off-rate for a labeled factor IX propeptide was found to be 3000-fold slower than the rate

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[1] Abbreviations: Gla, γ-carboxyglutamic acid; BocMeEEL, the peptide *tert*-butoxycarbonyl-Glu-Glu-Leu methyl ester; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; FLEEL, the pentapeptide Phe-Leu-Glu-Glu-Leu; FLDDL, the pentapeptide Phe-Leu-Asp-Asp-Leu; dioleoyl-PC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; KH_2, vitamin K_1 hydroquinone; BSA, bovine serum albumin; Cbx, human vitamin K-dependent γ-glutamyl carboxylase; FpCon, the 19mer peptide modified with 5(6)-carboxyfluorescein at the N terminus (fluorescein-GAV-FLSREQANQVLQRRRR); FpFIX, the 18mer peptide modified with 5(6)-carboxyfluorescein at the N terminus (fluorescein-TVFLD-HENANKILNRPKR); pCon, the 18mer peptide AVFLSREQANQV-LQRRRR; pFIX, the 18mer peptide TVFLDHENANKILNRPKR.

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of turnover of FLEEL. This rate difference provides compelling evidence that carboxylase carries out processive catalysis of the factor IX substrate. This report also provides the first evidence that binding of substrates to the active site modifies the propeptide binding site of carboxylase. This form of regulation may provide, at least in part, a release mechanism for macromolecular substrates from the enzyme.

MATERIALS AND METHODS

Materials. Peptides labeled with 5(6)-carboxyfluorescein at the amino terminus and unlabeled peptides (FpCon, FpFIX, pCon, and pFIX)[1] with sequences based on the human propeptide sequences were synthesized by Chiron Mimotopes (Clayton, Victoria, Australia) and characterized as described (*10*). FLEEL was from Bachem (Philadelphia, PA). FLDDL was chemically synthesized and purified by RP-HPLC by the UNC Protein Chemistry Laboratory (UNC—Chapel Hill). The purity of all peptides was approximately 95%, and stock concentrations were determined ($n = 4$, SD = 3−4% of concentration determination) by amino acid analysis performed by the Keck Microchemical Facility at Yale University. 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (dioleoyl-PC) was from Avanti Polar Lipids (Alabaster, AL). Vitamin K_1 (250 mg; Sigma, St. Louis, MO) was dissolved in 300 μL of 100% benzyl alcohol (Aldrich, Milwaukee, WI), diluted to a final concentration of 10 mg/mL in 70 mg/mL Alkamuls EL-620 [a nonionic surfactant composed of 99% castor oil ethoxylates (Rhodia, Spartanburg, SC)], and sonicated briefly to clarify the solution. The concentration of the oxidized vitamin was confirmed by UV absorbance [$\epsilon_{248} = 18\,900$ M^{-1} cm^{-1} (*14*)] and was reduced to the hydroquinone (KH_2) as previously described (*15*). BSA (fraction V, heat shock) was from Boehringer Mannheim. All other materials were from Sigma.

Preparation and Characterization of Carboxylase. Expression of recombinant human carboxylase in insect cells and purification of the enzyme to apparent homogeneity (purity >98%) were performed essentially as described (*10*). To concentrate the enzyme (to ≈2 mg/mL), it was batch bound to SP-Sepharose (Pharmacia) for 2 h at 4 °C. The resin was then loaded into a glass column, thoroughly washed with buffer (25 mM MOPS, 75 mM NaCl, 10% glycerol, 1 mM DTT, 0.1% dioleoyl-PC, 0.28% CHAPS, 100 μM EDTA, pH 7.0), and eluted with the same buffer, but including 500 mM NaCl (at pH 7.4), at 4 °C. The concentration of the purified protein was determined from absorbance measurements of samples at 280 nm using a calculated extinction coefficient [152 640 M^{-1} cm^{-1} (*16*)] for recombinant carboxylase + tags. The NH_3^+ tag sequence is DYKDDDDK; the COO^- tag sequence is EDQVDPRLIDGK (*10*). An extinction coefficient calculated from the amino acid sequence of a protein has been shown to be nearly the same as the true ϵ value for the protein. This conclusion was based on the observation that the average deviation of the calculated ϵ value from the an ϵ value based on the most reliable measurements of protein concentration for 80 sample proteins was found to be at 3.8% (*16*). The mass of glycosylation of the recombinant enzyme product (12 800 Da) was determined with Endo H digestion of enzyme samples as previously described (*17*). The rate of turnover of FLEEL in turnover buffer at 10.5 °C was measured by including 5 μCi of $NaH^{14}CO_3$ (specific activity, 54 mCi/mmol; ICN Corp.) in the buffer (described below, total 3.0 mM HCO_3); incorporation of $^{14}CO_2$ into FLEEL was measured as previously described (*15*).

Fluorescence Conditions. Standard buffer was the following: 100 mM MOPS, 180 mM NaCl, 3.3% glycerol, 6.3 mM DTT, 66 μM EDTA, 0.1% dioleoyl-PC, 0.28% CHAPS, and 0.4% BSA, pH 7.3. BSA (which was found neither to act as a substrate nor to inhibit FLEEL carboxylation) was added to this buffer because it greatly reduced the standard deviation of fluorescence measurements. Turnover buffer was the same but included 25 mM sodium benzoate, 5.0 mM FLEEL, 130 μM KH_2 in Alkamuls detergent solution (final concentration of Alkamuls in turnover buffer 1.4 mg/mL), and 3.0 mM $NaHCO_3$. Excessive addition of Alkamuls to the final titration solution was found to destabilize carboxylase, so the highest usable concentration of KH_2 was 130 μM. This concentration was optimal but nonsaturating; the concentrations of KH_2, FLEEL, and $NaHCO_3$ utilized correspond to approximately 60%, 80%, and 100% saturation of carboxylase, respectively, as determined by the carboxylation assay. Control buffer conditions were the same as standard buffer but included 25 mM sodium benzoate, 5.0 mM FLDDL, 1.4 mg/mL Alkamuls, and 3.0 mM $NaHCO_3$.

Fluorescence Data Collection. Samples of carboxylase and propeptide were preincubated in 300 μL of buffer for 1.5 h at the desired temperature to allow the mixture to reach equilibrium. A sample was then added to a microcuvette, and its fluorescence was measured using an SLM-Aminco 8100 spectrofluorometer (*18*) with the sample chamber cooled to either 4.5 or 10.5 °C (accuracy of ±0.1 °C). Excitation and emission wavelengths were set to 495 and 525 nm, respectively. For *anisotropy titrations*, excitation and emission slits were at 8 nm band-pass. For each sample, the plane-polarized fluorescence at four Glan-Thompson polarizer settings [I_{VV}, I_{VH}, I_{HV}, I_{HH} (*19*)] was measured. Each measurement of an 8 s duration was repeated six times. The fluorescence of the buffer alone was subtracted from each measurement, and the anisotropy of the sample was calculated (*19*). For *intensity titrations*, the polarizers were removed, and the slits were set at 2 and 8 nm, respectively. Three intensity measurements for each sample were made (F_A, F_B, and F_C, where F_A = buffer + propeptide + Cbx, F_B = buffer + propeptide, and F_C = buffer only), and F/F_o was calculated (*18*). Each data point represents an independent sample in all titration figures. For a *time course* experiment, all data were collected from a single sample. Slits were at 2 and 32 nm, respectively (polarizers present). After carboxylase and labeled propeptide reached equilibrium, a 350-fold excess of the unlabeled propeptide was added, and the time course was begun. I_{HV} and I_{HH} were measured immediately to determine the *G* factor. At 30 s intervals, measurements of I_{VV} and I_{VH} were made (13 s duration each); these values were corrected for buffer fluorescence; and the anisotropy was calculated for that time point. The carboxylase was stable for up to 18 h under the conditions of the experiment (including excess propeptide), and no noticeable photobleaching of fluorescein occurred during the time course.

Sedimentation Data Collection. All experiments were done at the Macromolecular Interactions Facility (UNC—Chapel Hill) using a Beckman XL-A analytical ultracentrifuge equipped with absorbance optics. For velocity studies, two

Table 1: Fitted Equilibrum Parameters for the Interaction of Fluorescein-Labeled Propeptides with Recombinant Human Carboxylase

peptide	buffer	temp (°C)	observable	stoichiometry propeptide−Cbx	K_d (nM)	Δr or R
FpCon	standard	4.5	anisotropy	0.50 ± 0.01[a]	nd	0.179 ± 0.006[a]
FpFIX	standard	4.5	anisotropy	0.53 ± 0.01[b]	4.4 ± 0.3[b]	0.165 ± 0.001[b]
FpCon	control	10.5	anisotropy	0.50[c]	nd	0.180[c]
FpCon	turnover	10.5	anisotropy	0.49[c]	nd	0.177[c]
FpFIX	control	10.5	anisotropy	0.69 ± 0.02[b,d]	3.9 ± 0.3[b]	0.157 ± 0.001[b]
FpFIX	turnover	10.5	anisotropy	0.71 ± 0.02[b,d]	1.2 ± 0.1[b]	0.147 ± 0.001[b]
FpFIX	control	10.5	intensity	0.50 ± 0.02[b,d,e]	4.7 ± 0.7[b]	0.820 ± 0.003[b]
FpFIX	turnover	10.5	intensity	0.50 ± 0.02[b,d,e]	1.5 ± 0.4[b]	0.866 ± 0.003[b]

[a] Uncertainty is the standard deviation of three independent experiments. [b] Uncertainties are standard error based on the data of two titrations fitted simultaneously. [c] Average of two independent experiments. [d] The apparent increase of stoichiometry of the FpFIX-Cbx interaction as measured by anisotropy (≈0.70) beyond that seen for that measured by intensity (0.50) is statistically significant. The increase was found to be solely surfactant dependent; it is likely that the surfactant affects the tumbling of (a) species involved in the FpFIX−Cbx equilibrium (which would affect anisotropy) without affecting the intensity. [e] For intensity data, the stoichiometric parameter was fitted to both control and turnover data sets simultaneously to allow a more accurate estimation of binding parameters from these data, based on the assumption that FpFIX binds Cbx in control and turnover buffer with the same stoichiometry (explained in detail in Materials and Methods).

1.6 μM samples of carboxylase were pre-equilibrated with or without a 5-fold excess (8 μM) of pCon propeptide in standard buffer (without BSA but including 10% glycerol) at pH 7.3. Samples (410 μL) were loaded into two-sector cells and centrifuged at 40 000 rpm for 18 h at 4.5 °C, with 280 nm absorbance scans recorded every 20 min. For equilibrium studies, enzyme at three concentrations (4.1, 2.7, and 1.4 μM) was preequilibrated with either a 5-fold excess of pCon or a 1.5-fold excess of FpCon for each enzyme concentration. Samples (110 μL) were centrifuged at 18 000 rpm at 4.5 °C, and absorbance scans were recorded every 2 h, until equilibrium was reached.

DATA ANALYSIS

Analysis of Binding of FpCon to Carboxylase. Anisotropy data were converted to the fraction of the ligand (FpCon) bound using the equation (*19*):

$$f_b = \frac{r_{obs} - r_0}{(r_{obs} - r_0) + R(r_{max} - r_{obs})} \quad (1)$$

where f_b is the fraction of the ligand bound and r_{obs}, r_0, and r_{max} are the observed anisotropy, the anisotropy of the free ligand, and the anisotropy of the ligand when bound to saturating amounts of carboxylase, respectively. R is the fluorescence intensity of the fluorophore saturated with carboxylase divided by the intensity of the free fluorophore (0.70, 0.68, and 0.70 for FpCon in standard, control, and turnover buffer, respectively) and is included to properly weight the anisotropy of the different species (*20*). This correction, while of marginal significance for modeling of the FpFIX data (described below), affects the determination of stoichiometry ($\Delta i = 0.08$) from the FpCon data beyond the uncertainty of this parameter (SD ± 0.01, Table 1).

Analysis of Binding of FpFIX to Carboxylase Using Anisotropy as an Observable. Our model assumed that FpFIX peptide binds reversibly to carboxylase at multiple, identical, independent, and noninteracting sites. Titration data were fitted to an equation (eq 2) which describes this model:

$$r_{obs} = \frac{f_b(r_0 - Rr_0 - R\Delta r) - r_0}{f_b(1 - R) - 1} \quad (2A)$$

where

$$f_b = \{(i[C_t] + [F_t] + K_d) - \sqrt{(i[C_t] + [F_t] + K_d)^2 - 4i[C_t][F_t]}\}/2[F_t] \quad (2B)$$

and $[F_t]$ and $[C_t]$ are the total concentrations of FpFIX and carboxylase, respectively. K_d is the dissociation constant describing the equilibrium, and i is the number of moles of propeptide combining per mole of carboxylase. Δr is the total anisotropy change ($r_{max} - r_0$), and r_{obs}, r_0, and R are as defined above. R for FpFIX was 0.85, 0.82, and 0.865 in standard, control, and turnover buffers, respectively. The derivation and assumptions of equations analogous to eq 2B are described in detail in previous references (*21*, *22*). Equation 2A accounts for the fluorescence intensity change observed for FpFIX upon binding to carboxylase. The parameters i, K_d, and Δr were fitted simultaneously to data sets determined at two different propeptide concentrations. This (nonlinear and unweighted) regression and all other regressions (except Figure 3) were implemented by Sigma Plot version 5.0 (SPSS Software, Chicago, IL).

Analysis of Competition of pFIX vs FpFIX for Binding to Carboxylase. The equations used to model propeptide competition for a binding site are analogous to the equations used to describe a similar model developed by Olson et al. (*23*). In our model, the labeled (FpFIX) and unlabeled (pFIX) propeptides can reversibly bind carboxylase at the same multiple, identical, independent, and noninteracting sites:

$$lC + F \underset{K_d}{\rightleftharpoons} C_lF \quad (3)$$

$$nC + P \underset{K_{d2}}{\rightleftharpoons} C_nP \quad (4)$$

where l and n moles of carboxylase (C) combine per mole of FpFIX (F) and pFIX (P), respectively. K_d is as defined above, and K_{d2} is the dissociation constant describing the equilibrium between pFIX and carboxylase. This model can be described by a trinomial equation:

$$D_1[f_b]^3 + D_2[f_b]^2 + D_3[f_b] + D_4 = 0 \quad (5)$$

where

$$D_1 = l[F_t]\left(\frac{K_{d2} - K_d}{K_d}\right)$$

$$D_2 = [C_t]\left(\frac{K_d - K_{d2}}{K_d}\right) + l[F_t]\left(\frac{K_d - 2K_{d2}}{K_d}\right) + K_d - K_{d2} - n[P_t]$$

$$D_3 = [C_t]\left(\frac{2K_{d2} - K_d}{K_d}\right) + l[F_t]\left(\frac{K_{d2}}{K_d}\right) + K_{d2} + n[P_t]$$

$$D_4 = -[C_t]\left(\frac{K_{d2}}{K_d}\right)$$

and where $[C_t]$, $[F_t]$, and f_b are defined as described previously and $[P_t]$ is the total concentration of unlabeled propeptide (pFIX). The derivation and assumptions of equations analogous to eq 5 are described in detail in previous references (*23*, *24*). The collected anisotropy data were first converted to the fraction bound using eq 1, setting $r_0 = 0.092$, $r_{max} = 0.257$ [determined via FpFIX titration of carboxylase (Figure 2)], and $R = 0.85$. This model assumes that the receptor (carboxylase) is univalent. For this reason, $[C_t]$ was set to the active enzyme concentration [for example, 65 nM total carboxylase is 34.7 nM (65 × 0.53) active carboxylase], and l was set to 1.0 in the parameter fit. The parameters K_d, K_{d2}, and n were fitted simultaneously to the three data sets using SCIENTIST (MicroMath Software, Salt Lake City, UT).

Analysis of Binding of FpFIX to Carboxylase Using Intensity as an Observable. Intensity data were modeled with the equation:

$$\frac{F}{F_0} = f_b R \quad (6)$$

where f_b and R are defined above. Anisotropy data are more precise than intensity data (Figures 6 and 7); therefore, K_d and R for both turnover and control conditions were fitted simultaneously to two data sets, while i was fitted simultaneously to all four data sets. This is based on the assumption that FpFIX binds carboxylase with identical stoichiometry in both turnover and control buffers, which is well supported by the anisotropy results for FpFIX−Cbx and FpCon−Cbx equilibriums (Table 1).

Analysis of Time Course Data. Anisotropy measured at each time point was converted to the fraction of FpFIX bound (eq 1) and fitted to an equation describing a single exponential decay:

$$f_{bt} = (f_{bi} - f_{bf}) \exp(-k^*t) \quad (7)$$

where f_{bt}, f_{bi}, and f_{bf} are the fraction bound at time t, initial time, and final (infinite) time, respectively, k is the first-order rate constant, and t is time. For control buffer experiments, data were fitted to eq 7 by minimizing the parameters f_{bi}, f_{bf}, and k. For turnover buffer, data were fitted to f_{bi} and k, while f_{bf} was set to the optimized value determined from the control buffer.

Analysis of Analytical Centrifugation Data. For sedimentation equilibrium experiments, absorbance profiles taken at three protein concentrations were individually fitted to a model describing the exponential concentration distribution of a single, homogeneous species at equilibrium (*25*):

$$c_r = c_m \exp\left(\frac{M(1 - \bar{v}\rho)\omega^2(r^2 - r_m{}^2)}{2RT}\right) + \text{base} \quad (8)$$

where c_r and c_m are the concentrations of the complex at a radial position (r) from the center of rotation and at the meniscus (r_m), M is the molecular mass, $\bar{v}$ is the partial specific volume of the complex, ρ is the solvent density, base is the absorbance due to nonsedimenting material, and ω, R, and T are previously defined (*25*). Data were fitted to the model using c_m, $M(1 - \bar{v}\rho)$ and base as adjustable parameters. M was calculated from $M(1 - \bar{v}\rho)$ using a value of $\bar{v}$ (0.718 $cm^3 \cdot g^{-1}$) calculated for the 1:1 propeptide−enzyme complex at 4.5 °C. $\bar{v}$ was calculated from the amino acid composition of the complex (*26*) and was adjusted to account for the 12.1% glycosylation (by weight) of the recombinant carboxylase [using a $\bar{v}$ value for a carbohydrate residue of 0.63 $cm^3 \cdot g^{-1}$ (*26*)] and for the temperature of the experiment (*27*). The buffer density (1.043 $g \cdot cm^{-3}$) was determined by repeated ($n = 15$, SD = 0.005) weighing of an ice-chilled 1.0 mL of buffer volume samples dispensed from a calibrated pipetman. Velocity data were analyzed using the Beckman XL-A/XL-I Data Analysis Software, Version 4.0.

RESULTS

Binding of FpCon to Carboxylase. We first sought to develop an assay to determine the number of propeptide binding sites in our purified carboxylase preparation. Previous work from our laboratory indicated that a "consensus" propeptide binds very tightly to purified carboxylase in vitro [$K_i \leq 430$ pM (*28*)], so we titrated our enzyme preparation against fluorescein-labeled consensus propeptide (FpCon) at 4.5 °C (Figure 1). FpCon appeared to bind stoichiometrically to carboxylase up to 0.70 fraction bound (Figure 1). The equivalence point of carboxylase concentration was determined from the intersection of a line determined from the linear regression of the data points up to 0.70 f_b with a line drawn at $f_b = 1$. An apparent FpCon−Cbx stoichiometry of 0.50 ± 0.01 was determined for the carboxylase preparation used in these studies.

Binding of FpFIX to Carboxylase. To confirm that different propeptides bind with the same stoichiometry, we next titrated carboxylase against a fluorescein-labeled factor IX propeptide (FpFIX) at 4.5 °C. Two titration data sets utilizing different propeptide concentrations were adequately fit to a binding model of multiple, identical, independent, and noninteracting sites (Figure 2). The K_d for the FpFIX−Cbx interaction was 4.4 ± 0.3 nM, while the value for i (0.53 ± 0.01, Figure 2, Table 1) is very close to the value determined for the FpCon−Cbx interaction (0.50 ± 0.01, Figure 1, Table 1). From these results we conclude that FpFIX and FpCon bind carboxylase with the same stoichiometry. To determine if FpFIX binding to carboxylase was reversible and specific for the propeptide binding site, we competed FpFIX against increasing concentrations of pFIX for carboxylase binding (Figure 3). The K_d value determined for the FpFIX−Cbx interaction in this assay (5.7 ± 0.6 nM) is nearly the same as the K_d value determined for the pFIX−Cbx interaction (7.8 ± 1.0 nM, Figure 3). This indicates that addition of fluorescein to the amino terminus of the factor IX propeptide has little effect on its affinity for the

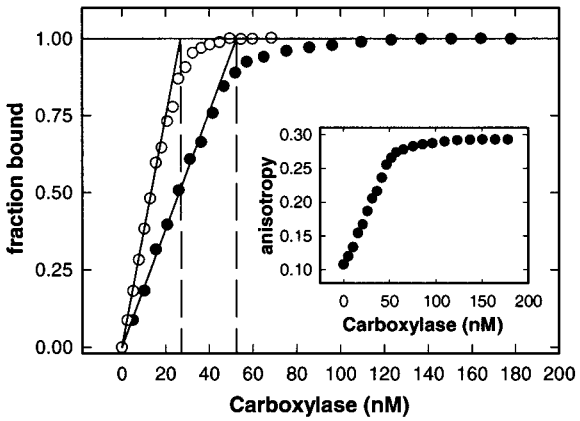

FIGURE 1: Titration of purified carboxylase against a constant concentration of a fluorescein-labeled consensus propeptide (FpCon) in standard buffer at 4.5 °C. The fraction of FpCon bound was calculated from anisotropy of samples using eq 1 as described in Materials and Methods. The points represent samples containing varying concentrations of purified carboxylase and either (○) 13.8 or (●) 27.7 nM FpCon. Sloped solid lines represent linear regressions of data points up to 0.70 fraction bound. Dashed lines indicate the equivalence point of the total carboxylase concentration binding to the entire concentration of FpCon. The FpCon−Cbx stoichiometry was determined to be 0.50 ± 0.01 (n = 3). Inset: Anisotropy data for the 27.7 nM titration.

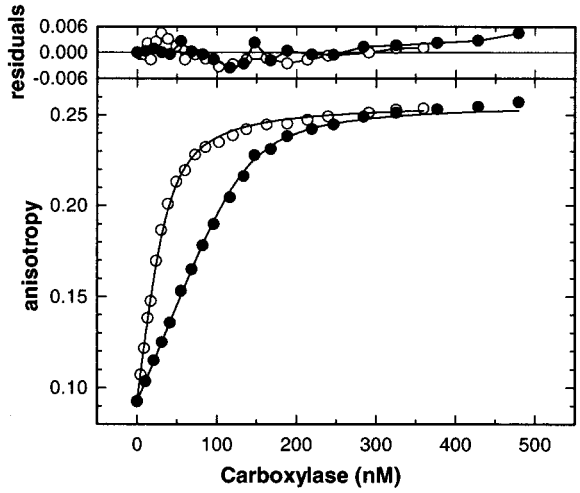

FIGURE 2: Titration of purified carboxylase against a constant concentration of fluorescein-labeled factor IX propeptide (FpFIX) in standard buffer at 4.5 °C. The points represent samples containing varying concentrations of purified carboxylase and either (○) 17.1 or (●) 68.4 nM FpFIX. Solid lines represent a binding model of reversible association of propeptide with carboxylase (eq 2) fitted simultaneously to both data sets, with optimized parameters (K_d = 4.4 ± 0.3 nM, i = 0.53 ± 0.01) fully listed in Table 1.

carboxylase. Additionally, the former value agrees very well (within 1.3-fold) with the K_d value of 4.4 nM for the FpFIX−Cbx interaction determined by titration.

Evidence for a Monomeric Association of Carboxylase with Propeptide. The apparent propeptide−carboxylase stoichiometry (of about 0.5:1) as determined via the fluorescence assay could indicate that two enzymes bind per molecule of propeptide. A more likely possibility is that the stoichiometry of the propeptide−carboxylase interaction is 1:1 and that approximately half of our enzyme preparation is comprised of a form (or forms) that cannot bind propeptide. This possibility is reasonable, as the stability of carboxylase (an integral membrane protein) is known to be very sensitive to

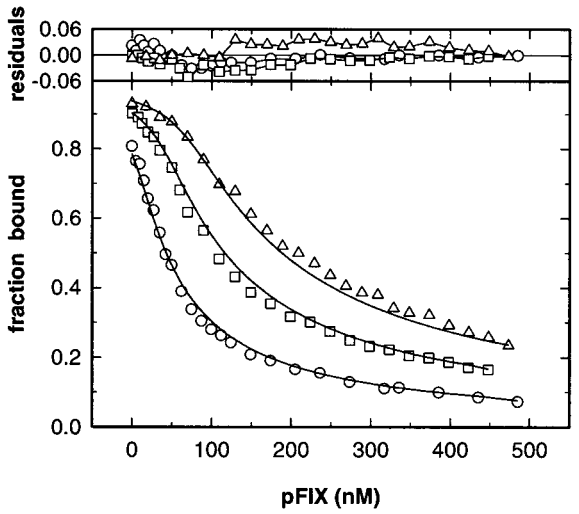

FIGURE 3: Competition of the unlabeled factor IX propeptide (pFIX) against FpFIX for the propeptide binding site on carboxylase in standard buffer at 4.5 °C. Samples were preincubated for 4 h at 4.5 °C before anisotropy measurements were taken. The points represent samples containing varying concentrations of pFIX, 17.1 nM FpFIX, and either (○) 34.7, (□) 68.8, or (△) 102.4 nM active carboxylase. The lines represent a binding model of labeled and unlabeled peptides competing reversibly for the same site on carboxylase (eq 5) fitted simultaneously to the three data sets, with the following optimized parameters: K_d (FpFIX−Cbx interaction) = 5.7 ± 0.6 nM; K_d (pFIX−Cbx interaction) = 7.8 ± 1.0 nM, n = 1.11 ± 0.10. l (stoichiometry of FpFIX−active Cbx interaction) was set to 1.00 in the model fit (see Materials and Methods). Uncertainties are 95% confidence limits.

temperature, especially in the absence of propeptide (Figure 5A inset and ref *12*). Nevertheless, we determined if the propeptide induces oligomerization of the carboxylase. First, we carried out sedimentation velocity studies on our preparation in the absence and presence of saturating amounts of the unlabeled consensus propeptide (pCon) in conditions similar to those of the standard buffer at 4.5 °C. Data from both samples were adequately fitted to a model of a single species sedimenting at 4.5 $S_{20,wat}$ without propeptide and 4.7 $S_{20,wat}$ with propeptide (n = 2, Table 2); hence, the propeptide appears not to alter the aggregation state of carboxylase. Next, we carried out sedimentation equilibrium studies to determine the molecular weight of the carboxylase in complex with either pCon or FpCon. The data for either complex fit a model describing a single homogeneous species (Figure 4) with a molecular mass of ≈109 300 ± 3000 Da (Table 2). The mass predicted for a 1:1 propeptide−carboxylase complex is ≈105 100 Da, a value that includes the mass for the enzyme based on its amino acid sequence + tags (89 904 Da) + glycosylation (12 800 Da) + one bound propeptide [either 2643 Da (FpCon) or 2227 Da (pCon)]. The experimentally determined molecular mass is very close (within ≈4200 Da) to the mass expected for a monomeric association of carboxylase with propeptide. The small amount of additional mass could be due to a small number of bound detergent and/or phospholipid molecules in the complex [phospholipid has been shown to be required for carboxylase activity (*29*)]. The determined mass is, however, inconsistent with the mass predicted for a dimeric carboxylase assembly on the propeptide (≈207 800 Da). A concentration determination from the absorbance profiles of the samples at equilibrium indicates that greater than 95%

Table 2: Sedimentation Analysis of Recombinant Human Carboxylase

Svedberg constant	Velocity Data: complex pCon−Cbx	Velocity Data: complex Cbx
$s_{4,buf}$[a]	1.9	1.8
$s_{20,wat}$[b]	4.7	4.5

parameter	[Cbx] (μM)	Equilibrium Data: FpCon−Cbx (Da)	[Cbx] (μM)	Equilibrium Data: pCon−Cbx (Da)
$M(1 - \bar{v}\rho)$[c]	1.4	28530 ± 620 (113 610)	1.4	28210 ± 460 (112 330)
	2.7	26500 ± 750 (105 530)	2.7	26830 ± 490 (106 840)
	4.1	27100 ± 570 (107 910)	4.1	27460 ± 400 (109 380)
M_{av}[d] (measured)		109100 ± 3400		109500 ± 2200
M[e] (predicted)		105300		104900

[a] Average of two independent determinations. [b] Calculated from $s_{4,buf}$ values from a determination of buffer viscosity to be 1.59 cP at 20 °C. [c] Uncertainty is standard error. M (in parentheses) is calculated from $M(1 - \bar{v}\rho)$, where $\bar{v}$ is set to 0.718 $cm^3 \cdot g^{-1}$. [d] Average of the three molecular mass determinations ± SD. [e] Predicted molecular mass is that for a 1:1 propeptide−enzyme complex, based on the sequence and the weight of glycosylation of the recombinant carboxylase complex.

of the enzyme preparation was found in solution. This indicates that the buffer conditions utilized to study carboxylase contained minimal amounts of large soluble aggregates (e.g., large micelles) or insoluble precipitates of enzyme. From this result, we conclude that our preparation is monodisperse in the buffer conditions utilized in this study. In sum, the sedimentation velocity and equilibrium data are consistent with a monomeric association of enzyme with propeptide, and we conclude that the apparent substoichiometric value of this interaction is due to the presence of forms of carboxylase that cannot bind propeptide. This model is further supported by the considerable variability of the fraction of enzyme that contains propeptide sites found between different enzyme preparations; three additional preparations were titrated against FpCon and found to contain 62%, 45%, and 30% binding sites.

Specificity of the Assay for Active Forms of the Carboxylase. To determine if labeled propeptides bound specifically to active forms of the enzyme, we studied the relationship between catalytic activity and the number of propeptide binding sites of a carboxylase preparation. We incubated enzyme at 20 °C for over 5 h to induce activity loss (Figure 5A inset). Aliquots of enzyme removed during the time course were tested for carboxylation activity and were titrated against FpCon to determine the number of binding sites, as described in Materials and Methods. Figure 5A shows the excellent correlation ($r^2 = 1.00$) between the number of propeptide binding sites and the catalytic activity of a carboxylase sample. The plot also indicates that carboxylase that does not bind propeptide has only 1.5% of the original catalytic activity (Figure 5A). Similar results were obtained with a duplicate experiment but using a fluorescein-labeled human factor X propeptide to titrate binding sites (data not shown). The nearly perfect correlation between the existence of a functional propeptide binding site and carboxylase activity (Figure 5A) suggests that a determination of the propeptide−carboxylase stoichiometry also determines the

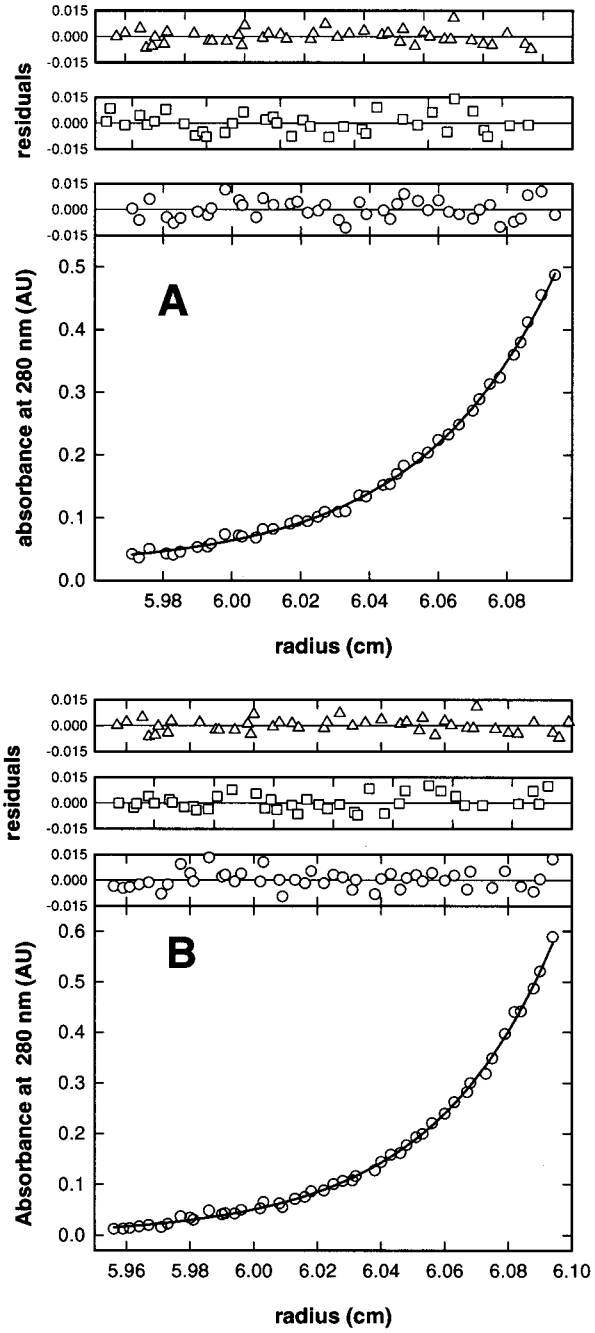

FIGURE 4: Determination of the buoyant molecular weight of the carboxylase in complex with either FpCon or pCon by sedimentation equilibrium. For all panels, carboxylase at (○) 4.1, (□) 2.7, and (△) 1.4 μM was preequilibrated with either a 1.5-fold excess of FpCon (panel A) or a 5-fold excess of pCon (panel B) for each enzyme concentration. Samples were centrifuged at 18 000 rpm at 4.5 °C, and absorbance scans for the 4.1μM samples at 20 h are presented here, after equilibrium was reached. Residuals for all samples are shown [first tick on abscissa is 6.50 cm for (□) and 6.96 cm for (△), respectively, with succeeding ticks every 0.02 cm]. The model lines describe a single homogeneous species fitted to the data shown with a buoyant molecular mass [$M(1 - \bar{v}\rho)$] of 27 100 ± 570 Da (panel A) and 27 460 ± 400 Da (panel B).

catalytically active fraction of the carboxylase preparation. Thus, the preparation used in this study contained about 50% active carboxylase. There is no reason to expect that inactive carboxylase will affect the apparent stoichiometry of the propeptide-active carboxylase interaction (Figure 5A). However, to confirm that inactive forms of the enzyme do not

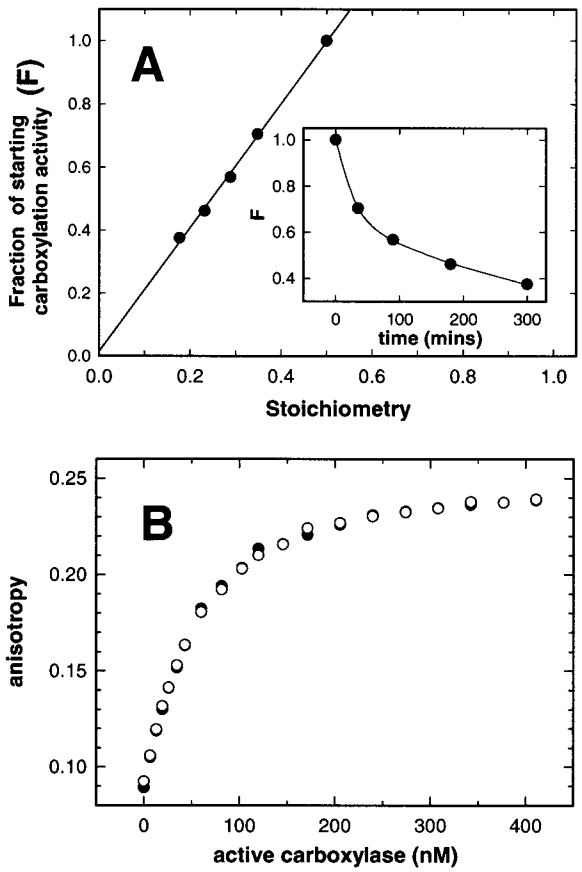

FIGURE 5: Specificity of fluorescence assay for active forms of the carboxylase. (A) A stock of purified carboxylase was incubated at 20.0 °C for 5 h in standard buffer, during which time samples were removed for determination of carboxylation activity and number of propeptide sites via titration against 13.8 nM FpCon (carried out as seen in Figure 1). The plot shows the correlation between carboxylation activity and the number of propeptide binding sites. The correlation constant of linear regression is 1.00. The inset shows the time course of the loss of carboxylation activity of carboxylase upon incubation at 20.0 °C. (B) Comparison of carboxylase binding to FpFIX in standard buffer at 4.5 °C in the absence and presence of excess denatured enzyme. The points represent samples containing varying concentrations of active carboxylase preparation, 8.6 nM FpFIX, and either the absence (●) or presence (○) of a concentration of denatured carboxylase 8-fold greater than the active carboxylase in the sample.

affect the K_d of the interaction, we performed a full carboxylase titration against 8.6 nM FpFIX ([FpFIX] ≈2-fold above K_d) in the absence and presence of a 4-fold excess of denatured enzyme (thermally inactivated in excess CHAPS). We found that excess inactive carboxylase has no effect on the FpFIX−Cbx equilibrium (Figure 5B).

Binding of FpFIX to Carboxylase during Turnover and Control Conditions. Previous work from several laboratories indicates linkage between the propeptide and active sites of γ-glutamyl carboxylase (*9*, *11*, *30*, *31*). For example, early studies have indicated that saturating amounts of the propeptide allosterically modify the active site (*11*, *12*). We hypothesized that the reciprocal effect would occur; that is, substrates binding to the active site would modify the propeptide binding site of carboxylase. To test our hypothesis, we first titrated carboxylase against FpFIX in the presence of substrates (Figure 6). The buffer utilized in this experiment included FLEEL [a peptide that is turned over by carboxylase and does not occupy the propeptide binding

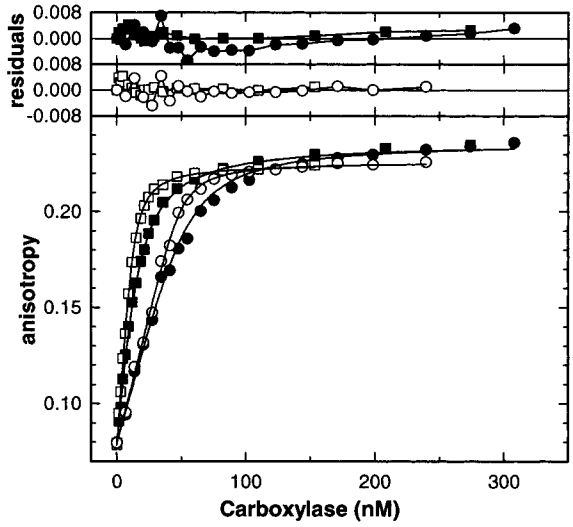

FIGURE 6: Comparison of carboxylase binding of FpFIX in turnover and control buffer conditions at 10.5 °C using fluorescence anisotropy as an observable. The light points represent samples containing varying carboxylase concentrations and either 8.6 (□) or 34.2 nM (○) FpFIX in turnover buffer, which included 130 μM vitamin KH_2, 5 mM FLEEL, and 3 mM $NaHCO_3$. The dark points represent samples containing varying carboxylase concentrations and either 8.6 (■) or 34.2 nM (●) FpFIX in control buffer, which included 5 mM FLDDL and 3 mM $NaHCO_3$. For each condition, a pair of lines represent the binding model (eq 2) fitted simultaneously to both data sets, with optimized parameters (control buffer, K_d = 3.9 ± 0.3 nM; turnover buffer, K_d = 1.2 ± 0.1 nM) fully listed in Table 1.

site (*1*)], $NaHCO_3$, KH_2, and a surfactant to solubilize the reduced naphthoquinone ("turnover buffer"; see Materials and Methods for complete description). Next, titrations of carboxylase against FpFIX were done in "control buffer" that included FLDDL (a peptide that is not turned over by carboxylase nor inhibits FLEEL carboxylation and was added to control for any nonspecific effects due to FLEEL), $NaHCO_3$, and the surfactant, but KH_2 was omitted. The titrations were done at 10.5 °C, a temperature that allowed significant carboxylase catalysis without detectable destabilization of the enzyme during the incubation (Figure 5A inset). The two data sets collected in each buffer utilized propeptide concentrations reasonably near the K_d of the interaction (e.g., 2- and 8-fold greater than K_d for control buffer). The optimized K_d for the FpFIX−Cbx interaction in control buffer was 3.9 ± 0.3 nM. In turnover buffer, the optimized K_d for the interaction was 1.2 ± 0.1 nM, an apparent 3.3-fold affinity increase compared to control buffer (Figure 6, Table 1).

Because FpCon binds very tightly to carboxylase, an accurate determination of K_d for the interaction cannot be made. However, using this peptide, we confirmed that there was no change of stoichiometry of the propeptide−carboxylase interaction in the presence of substrates compared to control conditions. The apparent stoichiometry of FpCon−Cbx was determined to be 0.49 (n = 2) in turnover buffer, and 0.50 (n = 2) in control buffer, by titrating carboxylase against 13.8 nM FpCon in the buffers described above (Table 1, data not shown).

Binding of FpFIX to Carboxylase during Turnover and Control Conditions As Measured by Fluorescence Intensity. We performed independent titrations of carboxylase against FpFIX but monitored changes of fluorescence intensity of

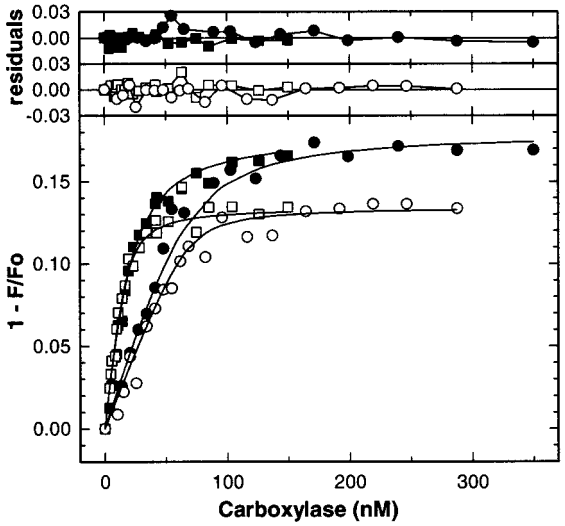

FIGURE 7: Comparison of carboxylase binding of FpFIX in turnover and control buffer conditions at 10.5 °C using fluorescence intensity as an observable. The light points represent samples containing varying carboxylase and either 8.6 (□) or 34.2 nM (○) FpFIX in turnover buffer. The dark points represent samples containing varying carboxylase and either 8.6 (■) or 34.2 nM (●) FpFIX in control buffer. For each condition, a pair of lines represents the binding model (eq 6) fitted as described in Materials and Methods, with optimized parameters (control buffer, $R = 0.820 \pm 0.003$; turnover buffer, $R = 0.865 \pm 0.003$) fully listed in Table 1.

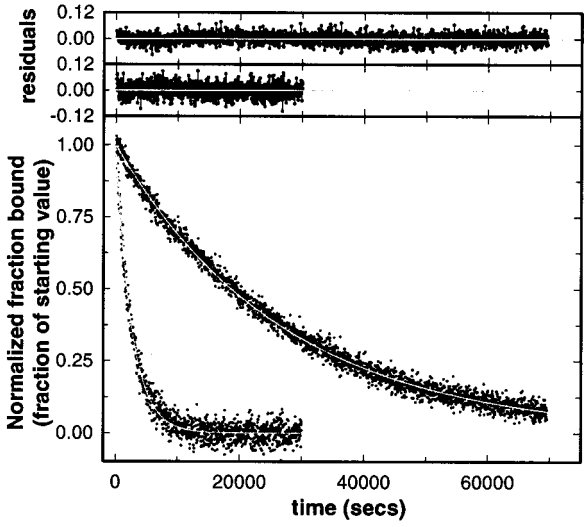

FIGURE 8: Comparison of the off-rate of FpFIX from carboxylase in turnover and control buffer conditions at 10.5 °C. Carboxylase (13 nM) was preincubated with 6.8 nM FpFIX for 90 min at 10.5 °C in the appropriate buffer. pFIX (2.1 μM) was then added, and the sample was mixed and returned to the fluorometer for time course anisotropy readings, which were taken every 30 s. Lower black points represent observations in control buffer, and upper black points represent observations in turnover buffer. Gray lines represent single exponential best fits (eq 7) to the data. The minimized parameters for k in the fitted models (trial 3) shown in this figure are as follows: control buffer, $k = 3.7 \times 10^{-4} \pm 0.2 \times 10^{-4}$ s^{-1}; turnover buffer, $k = 3.7 \times 10^{-5} \pm 0.3 \times 10^{-5}$ s^{-1}.

FpFIX to (1) confirm the modest K_d change identified using anisotropy and (2) identify any change of intensity of the fluorescein chromophore that may occur when substrates bind to the active site of saturating amounts of carboxylase. The conditions chosen in these experiments were identical to those of the anisotropy titrations. The difference in R between turnover and control conditions is 4.5 ± 0.3% (Figure 7, Table 1). This suggests that the environment surrounding the fluorescein chromophore at the propeptide binding site changes when substrates bind to the active site. In addition, an apparent 3.1-fold affinity increase for the FpFIX−Cbx interaction in turnover buffer ($K_d = 1.5 \pm 0.4$) compared to control conditions ($K_d = 4.7 \pm 0.7$, Figure 7, Table 1) was observed, confirming our observation made by anisotropy.

Off-Rates of FpFIX from Carboxylase during Turnover and Control Conditions. To test if carboxylase turnover of FLEEL and KH_2 affects the rate of FpFIX release from the propeptide−enzyme complex, the off-rate of FpFIX from carboxylase was determined in both turnover and control conditions at 10.5 °C as described in Materials and Methods. The time course of anisotropy loss due to FpFIX release from the propeptide−enzyme complex in control and turnover buffers was adequately fit by a single exponential decay model (Figure 8). The fitted first-order rate constant found for FpFIX release from carboxylase in turnover buffer ($k = 3.9 \times 10^{-5} \pm 0.4 \times 10^{-5}$ s^{-1}) is 9.2-fold less than that found in control buffer ($k = 3.6 \times 10^{-4} \pm 0.2 \times 10^{-4}$ s^{-1}, Figure 8, Table 3). The difference in off-rate we observe is not due to differences in the stability of the carboxylase in the conditions used. We carried out studies that indicated that the enzyme is stable through the entire time course of each experiment (in the presence of excess propeptide) and that carboxylation activity was linear through the time course.

Table 3: Fitted Parameters for Determination of Off-Rates of the FpFIX Propeptide from Recombinant Human Carboxylase in Control and Turnover Conditions[a]

buffer	trial[b]	$k \times 10^4$	f_{bi}	f_{bf}
control	1	3.9 ± 0.3	0.51 ± 0.003	0.021 ± 0.006
	2	3.4 ± 0.2	0.53 ± 0.003	0.029 ± 0.006
	3	3.7 ± 0.2	0.55 ± 0.002	0.026 ± 0.008
	av ± SD	3.6 ± 0.2	0.53 ± 0.02	0.025 ± 0.004
turnover	1	0.36 ± 0.02	0.69 ± 0.001	*c*
	2	0.43 ± 0.03	0.67 ± 0.001	*c*
	3	0.37 ± 0.03	0.70 ± 0.001	*c*
	av ± SD	0.39 ± 0.04	0.69 ± 0.02	*c*

[a] All experiments were carried out at 10.5 °C. f_{bi} and f_{bf} are the fitted values of the fraction of FpFIX bound to carboxylase at time 0 and infinite time, respectively. k is the first-order rate constant describing the release of FpFIX from carboxylase. [b] Uncertainties of the individual trials are standard error. [c] Parameter not minimized but set to 0.025 (minimized value in control conditions).

Also, the initial number of propeptide−enzyme complexes is significantly higher in the presence of substrates than in their absence (f_{bi}, Table 3). Both conditions have an equal number of propeptide binding sites. This difference is due to an improved affinity of enzyme for propeptide in these conditions, leading to increased formation of the complex (Figure 6).

To compare the rate of release of the FpFIX propeptide to the rate of carboxylation, we also measured the rate of turnover of FLEEL in turnover buffer at 10.5 °C. This rate was 0.12 ± 0.01 s^{-1} ($n = 3$), 3000-fold greater than the rate found for propeptide release under identical conditions.

The previous results (3-fold and 9-fold changes in K_d and off-rate, respectively) suggest that the on-rates of the FpFIX peptide for carboxylase under turnover and control conditions

are not the same. Using very low concentrations of enzyme and propeptide and with the use of anisotropy or intensity, we observe that the time course of propeptide binding to carboxylase is approximately 2−3-fold slower for enzyme in turnover buffer compared to enzyme in control buffer.[2] This observation supports the results described above.

DISCUSSION

We developed an assay that allows the measurement of the number of propeptide binding sites, the equilibrium dissociation constant for a propeptide−carboxylase interaction, and the off-rate of a propeptide from the enzyme, without the use of steady-state kinetics. We employed these assays to make three significant observations. First, we showed that a tight-binding fluorescein-labeled consensus propeptide (FpCon) can be used to quantify the active fraction in a carboxylase preparation. This is important because our preparations of carboxylase (and presumably those of other laboratories) contain variable quantities of inactive enzyme. Second, we have determined the off-rate of a propeptide (FpFIX) from carboxylase and found that this rate is 3000-fold slower than the rate of turnover for FLEEL. This result could explain how carboxylase accomplishes multiple carboxylations of a macromolecular substrate during a single binding event. Third, our results provide evidence that substrates binding to the active site modify the propeptide binding site of the carboxylase. The difference in off-rate of FpFIX from carboxylase in turnover conditions (compared to control) suggests a mechanism for carboxylase's release of vitamin K-dependent substrates after they are completely modified. Additionally, our sedimentation data suggest that our carboxylase preparations are monodisperse in the buffer conditions we utilize, a property that will allow the enzyme to be studied by other bioanalytical techniques, such as surface plasmon resonance.

Most of our work utilized fluorescence anisotropy, although changes of intensity were also used (Figure 7). The large range of anisotropy, coupled with a low standard deviation of sample measurement, allows a more precise determination of binding parameters from these data (compare uncertainties for FpFIX data, Table 1). We demonstrated that the assay is sensitive and specific to the propeptide binding site on native forms of the carboxylase enzyme (Figures 1, 2, 3, and 5). The FpCon peptide, because it binds carboxylase with high affinity, is a sensitive titrant of propeptide binding sites (Figure 1) and can be used to determine the active fraction of a carboxylase preparation (Figure 5A). The FpFIX propeptide is useful for determining the K_d of the interaction (Figures 2, 6, and 7) because its affinity is ≈80-fold less than the consensus propeptide (*10*). Previous reports have relied on kinetic methods to determine affinity constants for propeptide−carboxylase interaction (*10*, *30*, *32*). Our binding assay (titration against FpFIX) should be useful for measuring structural−functional relationships in carboxylase studies. For example, it will be possible to compare propeptide binding of mutant and wild-type carboxylase in the absence of substrates. The affinity constant determined for pFIX in the present study for wild-type carboxylase in the competitive equilibrium assay (K_d = 7.8 ± 0.3 nM, Figure 3) differs 4−5-fold from that determined by a kinetic assay [K_i = 33 nM (*10*)]. Our earlier study, however, used a higher temperature (20 °C) to measure K_i values for propeptides. We have observed that the K_d for FpFIX−Cbx interaction is sensitive to temperature changes; for example, raising the temperature from 4.5 to 10.5 °C increased this value ≈3-fold in standard buffer.[2] This effect could account for the difference in affinity constants determined in our study and the earlier kinetic study.

Our carboxylase preparations contain a measurable fraction of an inactive form (or forms) of the enzyme. The inactive form of carboxylase appears not to affect the binding parameters determined for the propeptide's interaction with the active carboxylase, however (Figure 5). Moreover, the fact that our preparation was monodisperse in the buffer conditions utilized in this study indicates that the inactive form does not aggregate or induce aggregation of the active enzyme. All sedimentation data were well fitted by models describing a single homogeneous species (Figure 4), so the inactive form has hydrodynamic properties similar to those of the active form. Furthermore, the loss of activity is not due to proteolysis because, even for preparations with no activity, samples still run as a single species on PAGE.[2] Because the inactive carboxylase is well-behaved in sedimentation studies, the denaturation is subtle and could be due to oxidation. The important point is that there is a strict correlation between enzyme activity and propeptide binding (Figure 5A), and the active fraction of a carboxylase preparation (wild type or mutant) can be rapidly determined by titrating the preparation against the FpCon propeptide (Figure 1). This technique should allow comparison of kinetic results (e.g., k_{cat} of carboxylation) of the carboxylase prepared by different laboratories and between mutant and wild-type enzyme preparations.

The off-rate for FpFIX from carboxylase as the enzyme undergoes turnover at 10.5 °C was found to be ≈0.00004 s^{-1} (Figure 8). To compare this rate to the rate of vitamin K-dependent carboxylation, we determined the rate of FLEEL turnover in identical conditions and found it to be 0.12 ± 0.01 s^{-1}. We assume that this rate is comparable to the rate of vitamin K-dependent carboxylation, because addition of CO_2 to the first of the adjacent Glu residues of FLEEL [the second is not carboxylated (*33*)] is likely to be the rate-limiting step in the turnover of this weakly binding (K_m ≈ 1.5 mM) substrate. The approximately 3000-fold difference between these two rates indicates that propeptide release is probably the rate-limiting step in the turnover of factor IX from carboxylase in vitro; this hypothesis is supported by previous in vitro work from our laboratory (*34*, *35*). The large difference between the rate of carboxylation and the off-rate of the propeptide provides compelling evidence that carboxylase can convert the 12 amino-terminal glutamates of factor IX to Gla during a single binding event in these conditions. A similar rate difference in vivo may ensure that all vitamin K-dependent substrates are fully carboxylated in the normal physiological state. The pathological consequences of diminishing this rate difference in vivo may be demonstrated by a patient with a factor IX propeptide mutation (A −10 T) (*36*). When the patient was placed on warfarin therapy, the factor IX activity levels dropped to 1% of normal, while the activity of the other vitamin K-dependent coagulation factors were reduced to the

[2] S. R. Presnell and D. W. Stafford, unpublished observations.

expected 30−40% of normal. The mutant propeptide binds with 600-fold weaker affinity in vitro compared to the wild-type propeptide (*28*); this would substantially increase the off-rate of factor IX from carboxylase. Warfarin therapy reduces vitamin KH_2 levels, consequently reducing the rate of carboxylation. As a result, the rate of carboxylation in the patient may no longer be sufficiently different from the off-rate of factor IX to allow full carboxylation of this substrate. This could lead to the production of an incompletely carboxylated factor IX product, rendering the very low levels of factor IX activity.

Our studies provide evidence that substrates binding to the active site modify the propeptide binding site of carboxylase. The strongest evidence for this is a 9-fold slower off-rate for the release of FpFIX from carboxylase under turnover conditions compared to control (no FLEEL or KH_2, Figure 8). The 3-fold higher affinity of FpFIX for carboxylase (measured using either anisotropy or intensity as observables in independent experiments, Figures 6 and 7) and the ≈5% change of fluorescence intensity of carboxylase-bound FpFIX (Figure 7) under turnover conditions compared to control also support this conclusion. The fact that the off-rate of the FpFIX propeptide from carboxylase is dependent on the occupancy of the active site suggests a mechanism that carboxylase uses to regulate the release of factor IX (and other vitamin K-dependent substrates) in vivo. The off-rate of FpFIX from carboxylase in the absence of substrates is significantly (9-fold) faster than this rate measured in the presence of substrates in vitro. This suggests that the off-rate of the entire factor IX substrate from carboxylase with no Glu or vitamin KH_2 present in the active site may be significantly faster compared to when the enzyme is carrying out posttranslational processing in vivo. Hence, after all 12 Glu's on factor IX are converted to Gla, the off-rate of the substrate could be substantially increased. Additional work will be necessary to understand the regulation of carboxylase turnover. It is not clear whether the off-rate difference we observe in vitro can act as a significant release mechanism of factor IX from carboxylase in vivo. However, the difference may be more pronounced at a physiological temperature or in a physiological setting (such as when integrated into the lipid bilayer of the endoplasmic reticulum). Nevertheless, this work provides the first evidence for the regulation of the propeptide binding site by the active site of γ-glutamyl carboxylase.

In sum, the fluorescence and sedimentation techniques described in this report should allow additional structural and functional characterization of wild-type and mutant forms of carboxylase. Our work shows that titration of preparations against FpCon will be useful to quantify the active fraction of the enzyme. Additionally, the off-rate for a fluorescein-labeled factor IX propeptide was found to be 3000-fold slower than the rate of carboxylation, a difference that many explain how carboxylase can carry out multiple carboxylations of a substrate during the same binding event. Finally, we show evidence that substrate binding to the active site modifies the propeptide binding site of carboxylase. It will be important to measure off-rates of vitamin K-dependent substrates in vivo using cell culture techniques to better understand how complete carboxylation of macromolecular substrates occurs in humans.

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SUPPORTING INFORMATION AVAILABLE

Additional information about (1) the purity of the carboxylase used in this work and (2) studies that indicate that the enzyme is stable through the time course of the experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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