N-Bromoacetyl-Peptide Substrate Affinity Labeling of Vitamin K Dependent Carboxylase†

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ABSTRACT: Vitamin K dependent carboxylase (carboxylase) is a membrane-associated endoplasmic reticular enzyme that catalyzes the conversion of certain glutamate residues of essential blood coagulation proteins to γ-carboxyglutamyl (Gla) residues. A series of *N*-bromoacetyl-peptide substrate affinity labels based on the Gla domain of these blood-clotting proteins was synthesized, and the substrate and inactivator kinetic parameters were assessed. The most promising of these affinity peptides, *N*-bromoacetyl-FLEELY, was both substrate for carboxylase and an irreversible time-dependent inactivator of the enzyme, inactivating 80% of carboxylase under pseudo-first-order conditions. Addition of saturating amounts of a competing peptide substrate completely abolished the inhibitory properties of *N*-bromoacetyl-FLEELY, consistent with inactivation occurring at the active site. The partition ratio of inactivation/carboxylation was 1/30. The 94-kDa carboxylase was purified to 15–50% purity by a modification of a recent protocol [Wu, S.-M., Morris, D. P., & Stafford, D. W. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 2236–2240] and covalently labeled with *N*-bromoacetyl-FLEEL[^{125}I]Y. On silver-stained 10% sodium dodecyl sulfate–polyacrylamide gels, the predominant radiolabeled band was the 94 000 molecular weight species. This result independently validates that the 94-kDa protein is a carboxylase.

Blood coagulation proteins, such as the serine protease zymogens prothrombin, factor VII, factor IX, and factor X, form a class of secreted, calcium-binding proteins that contain γ-carboxyglutamyl residues (Furie & Furie, 1988). The zymogens are synthesized in the liver as pro-proteins that are subject to extensive intracellular posttranslational processing including pre- and prosequence proteolytic cleavage and the carboxylation of 9–12 glutamate residues clustered in their N-terminal region. The bidentate γ-carboxyglutamyl (Gla)[1] residues within these proteins serve as high-affinity binding sites for calcium ions (Furie et al., 1979). Once the proteins have chelated calcium, they can interact with membrane phospholipids, an event responsible for the propagation of the blood-clotting cascade (Mann et al., 1988).

The carboxylase that converts Glu residues to Gla residues requires stoichiometric amounts of O_2 and reduced vitamin K hydroquinone (KH_2), but has no requirement for either biotin or ATP (Suttie, 1985; Friedman & Shia, 1977). In the presence of CO_2, O_2, and KH_2 (K_m values of 0.3 mM, 70 μM, and 80 μM, respectively) and a Glu-containing peptide, the enzyme will yield a Gla residue, vitamin K epoxide, and H_2O as shown in Figure 1. The vitamin K epoxide (KO) formed is subsequently reduced back to KH_2 by either a thiol- or an NADH-requiring vitamin K epoxide reductase. This reductase is the site of action of warfarin and other vitamin K analogues (Whitlon et al., 1978).

In addition to the Glu-peptide-binding site, there is also a propeptide-binding site on carboxylase. The propeptide region (residues −18 to −1; Figure 2) of the precursor forms of the vitamin K dependent proteins confers 1000-fold tighter binding to Glu substrates when incorporated into the same synthetic peptide (Ulrich et al., 1988; Hubbard et al., 1989a). These propeptide residues are highly conserved (Pan & Price, 1985), and mutational analysis indicated that residues −18, −17, −16, −15, and −10 comprise the "γ-carboxylation recognition site" (γ-CRS) which directs the downstream and distant γ-carboxylation of the glutamates within the Gla domain (Ulrich et al., 1988; Huber et al., 1990). NMR studies showed that residues −13 to −3 of a synthetic prothrombin propeptide can form an amphipathic α-helix (Sanford et al., 1991) though the structure of the propeptide bound to carboxylase is unknown.

Recently, a 94-kDa carboxylase has been purified from bovine liver microsomes and the complete human and the majority of the bovine cDNA cloned (Wu et al., 1991a,b). The purification of the membrane-associated carboxylase required the construction of a recombinant 59^{mer} peptide affinity column (Wu et al., 1991b). In their one-step affinity purification, these workers achieved a 7000-fold purification of carboxylase, some 70-fold higher than had previously been reported by our groups. In our earlier work (Hubbard et al., 1989b), carboxylase was purified 100-fold from bovine liver microsomes but was highly enriched in a 77-kDa protein. On N-terminal sequence analysis, the 77-kDa protein was found to be the endoplasmic reticular GRP78 or BiP which we determined conclusively not to be carboxylase (unpublished data of B. R. Hubbard and T. Porter). The carboxylase

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[1] Abbreviations: carboxylase, vitamin K dependent γ-glutamyl carboxylase; Gla, γ-carboxyglutamate; GRP78, glucose-regulated protein of 78 kilodaltons; BiP, immunoglobulin heavy-chain-binding protein; FLEEL, Phe-Leu-Glu-Glu-Leu; KH_2, dihydrovitamin K_1; KO or vitamin K epoxide, *trans*-phytyl-2,3-epoxyvitamin K_1; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; RP-HPLC, reverse-phase high-performance liquid chromatography; TFA, trifluoroacetic acid; FAB-MS, fast atom bombardment mass spectrometry; AS carboxylase, ammonium sulfate precipitated microsomal preparation; KSI, ketosteroid isomerase; PBSBr, 20 mM sodium phosphate (pH 7.4)/150 mM NaBr buffer; MOPS, 3-(*N*-morpholino)propanesulfonic acid; γ-CRS, γ-carboxylation recognition site.

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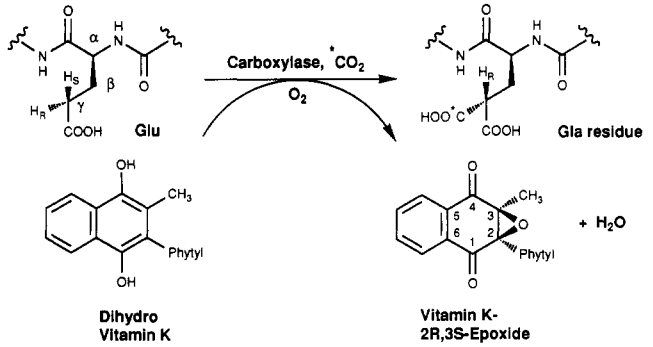

FIGURE 1: Carboxylase reaction of the conversion of CO_2, O_2, Glu-containing peptide, and dihydrovitamin K to a γ-carboxyglutamate (Gla)-containing peptide, vitamin K (2*R*,3*S*)-epoxide, and H_2O. The enzyme stereospecifically abstracts the *pro-S* γ-glutamyl proton and also carboxylates stereospecifically to form the (*R*)-[γ-$^{13}CO_2$]-glutamate residue with net inversion of configuration at the γ-carbon.

	-18 propeptide -1	+5 Gla Domain +41
proFIX59	TVFLDHENANKILNRPKRYNSG	KLEEFVQGNLERECMEEKCSFEEAREVFENTEKTTEF
FIXGla	AVFLDHENANKILNRPKRYNSG	KLEEFVQGNLERECIEEKCSFEEAREVFENTEKTTEF
FIXQS	AVFLDHENANKILNQPKSYNSG	KLEEFVQGNLERECIEEKCSFEEAREVFENTEKTTEF
proPT54	HVFLAPQQARSLLQRVRRANT-	FLEEVRKGNLERECVEETCSYEEAFEALESSTA
proPT28	HVFLAPQQARSLLQRVRRANT-	FLEEVRK
TSYR28^{mer}	TVFLAPQQARSLLQRVRSYNT-	FLEEVRR

FIGURE 2: Peptide sequences of carboxylase substrates encompassing the propeptide and Gla domains of profactor IX and prothrombin. E designates a potential Gla (γ-carboxyglutamate) residue.

isolated by Wu et al. (1991a,b) is apparently a single polypeptide of predicted molecular weight 88 000 and when expressed in kidney 293 cells retains the ability to carboxylate the FLEEL pentapeptide (Wu et al., 1991a). Interestingly, the C-terminal portion of the amino acid sequence shares approximately 19% sequence homology with soybean lipoxygenase, a dioxygenase which utilizes a non-heme Fe to activate molecular oxygen for the peroxidation of long-chain fatty acids (Egmond et al., 1977).

It is not presently known whether carboxylase requires a redox-active transition-metal necessary for activation of molecular oxygen. Instead, vitamin KH_2 is thought to attack dioxygen to produce a highly basic vitamin K alkoxide which is sufficiently basic to effect proton abstraction from the γ-methylene of glutamate residues. This "basicity enhancement" mechanism proposed by Dowd and co-workers is based on a nonenzymic model reaction which mimics the overall chemical transformations occurring on the surface of the enzyme (Ham & Dowd, 1990; Dowd et al., 1991). We have recently tested Dowd's proposed carboxylase mechanism in ^{18}O-labeling experiments (Kuliopulos et al., 1992) and found that the carboxylase operates as a dioxygenase 5% of the time during coupled carboxylation/oxygenation consistent with Dowd's proposals.

In spite of these and other kinetic/mechanistic studies, the location and structure of the active site of carboxylase are unknown. The work presented here describes an initial foray into the delineation of the carboxylase active site. We have synthesized N-bromoacetylated peptides which are both substrates and time-dependent inactivators of carboxylase. In addition, we have repeated Wu's purification of carboxylase through the use of a recombinant 59^{mer} peptide affinity column (Wu et al., 1991b) to confirm labeling of the 94-kDa species. Covalent labeling of purified carboxylase with N-bromoacetylated FLEEL[^{125}I]Y has enabled us to begin to probe the active site of carboxylase and to independently demonstrate that the carboxylase has an apparent molecular weight of 94 000.

MATERIALS AND METHODS

Synthesis, Purification, and Characterization of N-Bromoacetylated Peptides. Peptides ranging from 10^{mers} to 28^{mers} were synthesized by the solid-phase procedure with Fmoc amino acids using a Milligen/Biosearch 9600 synthesizer or an Applied Biosystems Model 430A peptide synthesizer (Merrifield, 1965). The FLEEL pentapeptide was from Sigma and used without further purification. Peptides less than 10 amino acids in length were synthesized on a 0.25-mmol scale by manual stepwise solid-phase synthesis (Stewart & Young, 1984) using a wrist shaker and reaction vessels from Milligen. Solvents, solid support resin, and reagents were purchased from Applied Biosystems. Fmoc L-amino acid derivatives were from Peninsula Laboratories. The crude deprotected peptides were purified by reverse-phase high-performance liquid chromatography (RP-HPLC) using a 21.5 mm × 25 cm Bio-Rad Hi-Pore 318 C_{18} reverse-phase column and a Waters high-performance liquid chromatography system. A flow rate of 10 mL/min and a linear gradient (10% + 0.4%/min) of acetonitrile/0.1% trifluoroacetic acid (TFA) in distilled–deionized water/0.1% TFA was employed. Peptide peaks were detected by the UV absorbance at 214 nm. Fractions containing the desired peptides were combined and lyophilized. Reinjection of purified peptides showed them to be >95% homogeneous. The sequences of proPT18 and the 28^{mer} (TSYR28^{mer}) peptides were verified by automated N-terminal Edman degradation on an Applied Biosystems 470 protein sequencer.

The bromoacetyl moiety was attached to the N-terminal α-amino group of the purified peptide by the following modifications of the method of Hass and Neurath (1971). The peptide was dissolved in a minimal volume of 400 mM $NaHCO_3$/40% CH_3CN. While the solution was stirred on ice, the pH of the peptide solution was adjusted to pH 7.5 with small aliquots of 8.9 M hydrobromic acid. Bromoacetyl bromide (Aldrich) was diluted into cold CH_3CN just prior to use. A total of 6–10 equiv of the diluted bromoacetyl bromide was added to the vigorously stirred peptide solution in aliquots of 1–2 equiv every 1–2 min for 10 min. The pH of the solution was monitored with pH indicator strips (Merck) since it was imperative that the pH be maintained above pH 7 for the N-bromoacetylation to proceed. If the pH rose above 8.5, significant bromoacetylation of the tyrosine phenolic oxygen occurred in the tyrosine-containing peptides. After the last addition of bromoacetyl bromide, the peptide solution was allowed to stir for another 20 min on ice and then was acidified to pH 1.5 with several aliquots of 8.9 M hydrobromic acid. Depending on its solubility, the bromoacetylated peptide would often precipitate from solution under acidic conditions, so the reaction mixture was centrifuged at 10000*g* in 1.7-mL polypropylene microfuge tubes at 4 °C before HPLC purification. The supernatant contained mostly bromoacetic acid but often contained a minor amount of peptide starting material and bromoacetylated product. The peptide pellet was resolubilized in 40% CH_3CN/60% H_2O/0.1% TFA and purified by RP-HPLC as described above except that a semipreparative 10 mm × 25 cm, 5-μm, Vydac C_{18} reverse-phase column at a flow rate of 3 mL/min was used. The N-bromoacetylated peptide product (70–90%) was easily resolved from the peptide starting material (5–30%) and from dibromoacetylated peptide which had an additional bromoacetyl group at the tyrosine phenolic oxygen (<5%) in the tyrosine-containing peptides.

Lyophilized HPLC-purified peptide products of the bromoacetylation reaction were dissolved in 200 mM Na^2HCO_3/^2H_2O (99.95%), pD 7.5 (direct meter reading), and ^{1}H-NMR

spectra were measured on a Varian VXR500S spectrometer at 499.84 MHz at 298 K. The NMR spectra of the bromoacetylated peptides ($\leq 10^{mer}$) were recorded by using a 45° pulse of 10-μs duration, a sweep width of 8 kHz, 16 K data points, an acquisition time of 1.024 s, 96–256 transients, and low-power presaturation (field strength of 100 Hz for 1-s duration) of the residual HDO resonance. The spectra were processed with a line broadening of 0.3 Hz. All reported chemical shifts were relative to external sodium 2,2-dimethyl-2-silapentane-5-sulfonate. In addition, the constitution and sequence of the peptides were confirmed by FAB mass spectra analysis (Biemann, 1989) kindly provided by the MIT Mass Spectrometry Facility.

Tyrosine Iodination and Purification of N-Bromoacetyl-FLEEL[^{125}I]Y. FLEELY was iodinated on its tyrosine residue prior to N-bromoacetylation in order to avoid halogen exchange at the bromoacetyl group by I^-. The iodination of the tyrosine was based on the Chloramine-T method of Hunter and Greenwood (1962). Lyophilized FLEELY was dissolved in dimethyl sulfoxide to a final concentration of 1.7 mM. Five microliters of the peptide solution was added to 25 μL of 1.0 M potassium phosphate, pH 7.5, 25 μL of H_2O, and 10 μL (1 mCi) of Na^{125}I (Amersham) in a 1.7-mL Eppendorf tube. The iodination reaction was initiated by the addition of 10 μL of freshly prepared 1 mg/mL Chloramine-T (Aldrich) in H_2O and the solution gently mixed. The reaction was allowed to proceed at room temperature for 30 s and quenched with 20 μL of freshly prepared 10 mg/mL sodium metabisulfite (Aldrich) in H_2O. The quenched reaction was diluted with 400 μL of 10 mM TFA (in water) and 10 μg of bovine serum albumin which served as a carrier protein.

Free unreacted Na^{125}I was separated from the iodinated peptide by C_{18} Sep-Pak (Waters) chromatography. The Sep-Pak was preequilibrated with H_2O and ethanol as indicated by the manufacturer. The reaction mixture was loaded onto the Sep-Pak and washed with 1 mL of 10 mM TFA. A 10 mM TFA/ethanol gradient consisting of sequential 0.5-mL injections of 10%, 20%, 40%, 60%, 80%, and 100% ethanol was used to elute the iodinated peptide. The unreacted Na^{125}I (4%) eluted in the initial 10 mM TFA wash, and the iodinated peptide (93%) eluted at 60% and 80% ethanol. The fractions containing the iodinated peptide were combined (1 mL) and reduced to 50 μL with a heat gun and by blowing a stream of nitrogen gas over the surface of the liquid. The crude iodinated peptide mixture was reconstituted with 500 μL of 10 mM TFA and purified by RP-HPLC on a Vydac analytical C_{18} column as described above except that an in-line γ-counter was used to detect radiolabeled peaks. The FLEEL[^{125}I]Y (700 μCi) was separated from both the unlabeled FLEELY and the FLEEL[^{125}I]$_2$Y (200 μCi). Fractions containing FLEEL[^{125}I]Y were pooled, evaporated down to 700 μL with a heat gun and a N_2 stream, and stored at −20 °C.

The microscale N-bromoacetylation of FLEEL[^{125}I]Y was carried out as described above with a few modifications. FLEELY (0.5 mg; 0.62 μmol) was dissolved in 450 μL of CH_3CN, 325 μL of 1 M $NaHCO_3$, pH 8.5, and 675 μL of the partially evaporated FLEEL[^{125}I]Y solution in 10 mM TFA. The pH of the reaction mixture was 8.0. The solution was vigorously stirred with a magnetic micro-stir bar on ice. A total of 8 equiv of bromoacetyl bromide (5 μmol), diluted 50-fold into cold CH_3CN, was added as 10 2-μL aliquots every 1 min to the stirring peptide solution. The pH remained at 8.0. After being stirred on ice for a total of 30 min, the reaction was stopped with 30 μL of 8.9 M HBr. The final pH was 1.5. The quenched bromoacetylated peptide reaction mixture was directly injected onto the RP-HPLC system and purified as before. The *N*-bromoacetyl-FLEEL[^{125}I]Y (69%) was separated from FLEEL[^{125}I]Y (23%) and *N*-bromoacetyl-FLEEL[^{125}I]-*O*-bromoacetyl-Y ($\leq$5.4%). The fractions containing the *N*-bromoacetyl-FLEEL[^{125}I]Y were combined, aliquoted into Eppendorf tubes, evaporated to dryness with a Speed-Vac concentrator (Savant), and stored at −20 °C. The final yield of *N*-bromoacetyl-FLEEL[^{125}I]Y was 209 μCi at a specific activity of 2.2 Ci/μmol.

Determination of K_m and V_m Values for Peptide Substrates. The initial rate of $^{14}CO_2$ incorporation into peptide substrates was measured in reaction mixtures of 125 μL containing peptide at five to eight different concentrations, 2.7 mg (25 μL) of partially purified ammonium sulfate precipitated carboxylase (Ulrich et al., 1988; Hubbard et al., 1989b), 0.88 mM vitamin K hydroquinone [prepared by the in situ reduction of AquaMEPHYTON (Merck) with $NaBH_4$], and 1.5 mM (10 μCi) $NaH^{14}CO_3$ (Amersham, 51.7 mCi/mmol) in 20 mM sodium phosphate/150 mM NaBr (PBSBr) buffer, pH 7.4. For each peptide kinetic run, a master cocktail (excluding peptide) for the individual kinetic reactions was freshly prepared and stored on ice. Aliquots of this reaction mixture were pipetted into 5-mL polycarbonate tubes containing peptide in 20 mM sodium phosphate/150 mM sodium bromide buffer, pH 7.4. The tubes were sealed and incubated at 25 °C. Reactions were quenched after 6 min by the addition of 2 mL of 10% trichloroacetic acid solution. The samples were transferred to 7-mL glass scintillation vials, and traces of remaining volatile $^{14}CO_2$ were removed by boiling the samples down to approximately 200 μL. After being cooled, the samples were dispersed in 5 mL of Aquasol scintillation fluid (DuPont–New England Nuclear) and analyzed by scintillation counting on a Beckman LS1801 liquid scintillation counter. The concentration of tyrosine-containing peptides was determined directly by the tyrosinate absorbance at 295 nm (ϵ = 2390 M^{-1} cm^{-1}), pH 13, by the method of Goodwin and Morton (1946). Concentrations of non-tyrosine-containing peptides were estimated from the weight of the lyophilized peptide–TFA salt. The K_m and V_m values were obtained from double-reciprocal plots and the data fit by Wilkinson (1961) hyperbolic weighted least-squares analysis.

Inactivation of Carboxylase with N-Bromoacetyl Affinity Peptides. Peptides were dissolved in 40% CH_3CN/60% PBSBr, and the concentration was determined by tyrosinate absorption. The carboxylase used in the inactivations was the ammonium sulfate precipitated microsomal preparation as described above. Inactivation of carboxylase by *N*-bromoacetyl-peptides was performed at 25 °C in 150-μL volumes containing 75 μL of ammonium sulfate precipitated microsomes (AS carboxylase), 10 μM proPT18 (unless the peptide already contained the propeptide sequence), and *N*-bromoacetyl-peptide or control non-bromoacetylated peptide in PBSBr. The inactivation reactions were carried out in sealed 5-mL polystyrene tubes and were initiated by the addition of the peptide followed by brief vortexing and were incubated at 25 °C in a circulating water bath. In order to assay for remaining carboxylase activity, 14-μL aliquots were removed at various times and diluted into 111 μL of a master cocktail mixture. The master cocktail mixture, when diluted with an additional 14 μL per assay reaction, contained 10 mM FLEEL, 0.88 mM KH_2, 1.5 mM $NaH^{14}CO_3$ (10 μCi), and 10 μM proPT18 in PBSBr. The individual assay reactions were incubated at 25 °C for 30 min and quenched with 2 mL of 10% TFA, and the amount of [γ-$^{14}CO_2$]Gla was quantitated as described above.

The kinetic analysis of the time-dependent inactivation of carboxylase by bromoacetyl-peptides followed that of Kitz and Wilson (1962). Plots of the log (percent activity remaining) as a function of time gave straight lines with slopes that were the observed pseudo-first-order rate of inactivation, k_{obs}, at each peptide concentration. The k_{obs} rates were corrected for the rate of inactivation, $k_{control}$, which occurred in the presence of an identical concentration of the parent nonbromoacetylated peptide. Replots of the reciprocal of the corrected k^c_{obs} ($=k_{obs} - k_{control}$) as a function of reciprocal peptide concentration had intercepts of k_{inact}, which is the rate of maximal inactivation when all enzyme is complexed with the bromoacetyl-peptide, and K_i, which represents the peptide concentration giving half-maximal inactivation.

Construction and Expression of Recombinant Steroid Isomerase–Factor IX Fusion Protein in Escherichia coli and Purification of FIX59^{mer} Peptides. The propeptide and Gla domain of factor IX encoding residues −18 through 41 were fused to the C-terminus of the 125 amino acid bacterial steroid isomerase gene (Kuliopulos et al., 1987). After expression of the fusion protein, the C-terminal FIX region was to be cleaved with CNBr at a junctional methionine residue. The polymerase chain reaction was used to insert a *Nco*I site between the two genes at codon −19 of FIX to create an in-frame methionine. The adjacent threonine at −18 was changed to alanine in order to promote efficient cyanogen bromide (CNBr) cleavage at methionine. The (+)PCR deoxyoligonucleotide used to create the Met-19/Ala-18 codons in the FIX gene was 5′-GCCAAGCTTCCATGGCAGTTTTTCTTGATCATG-3′, and the (−)PCR oligonucleotide was 5′-CAAAGCATGCGGATCCTCAAAATTCAGTTGTCTTA-3′, which placed a TGA stop codon and a *Bam*HI site after the Phe-41 codon in the FIX gene. The PCR-amplified product was digested with *Nco*I and *Bam*HI to create a 186 bp fragment which was ligated into *Nco*I/*Bam*HI sites placed at the stop codon of the KSI gene within pAK1370Y14F (Kuliopulos et al., 1987, 1989) to create the pAK808KSIFIX fusion construct which was the basis for all subsequent mutations. The M19I and R-4Q/R-1S mutations were introduced as previously described by Wu et al. (1990). The two fusion genes, the KSI-FIX59/T-18A/M19I double mutant (FIXGla), and the KSI-FIX59/T-18A/R-4Q/R-1S/M19I quadruple mutant (FIXQS) were sequenced completely on both strands to rule out adventitious mutations.

The fusion proteins KSIFIXGla and KSIFIXQS were expressed directly from the pAK808 construct which placed the KSIFIX gene under the control of the lac promoter in the pUC 19 derived pAK1370Y14F vector. The steroid isomerase–factor IX fusion proteins were expressed in the protease-deficient *E. coli* strain BL21(DE3) and processed into inclusion bodies. Isolation of inclusion bodies and CNBr cleavage at the junctional methionine between the N-terminal KSI fragment and the C-terminal FIX59^{mer} followed the procedure of Wu et al. (1990) except that the CNBr/protein ratio was 1/2 (w/w). The 59^{mer} was purified to homogeneity on a 1 × 10 cm HR10/10 Mono Q anion-exchange column (Pharmacia) using the same chromatographic conditions described by Wu et al. (1990).

FIXQS Affinity Purification of Carboxylase. The preparation of solubilized, ammonium sulfate precipitated carboxylase was carried out at 4 °C and is largely based on the method of Hubbard et al. (1989b). One liter of filtered bovine liver homogenate was centrifuged at 10000*g* for 10 min. The supernatant was diluted to 850 mL with buffer I (50 mM Tris-HCl, pH 7.4, and 0.1 M NaCl) and centrifuged at 150000*g* for 1 h. The dark brown supernatant was discarded, and the pellet was homogenized with a Brinkmann Polytron PT 3000 in buffer II (50 mM Tris-HCl, pH 7.4, and 1.0 M NaCl). The homogenate was pelleted and resuspended in buffer III (25 mM Tris-HCl, pH 7.4, and 0.5 M NaCl). Solid 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS, Sigma) was added to a final concentration of 1% (w/v) and allowed to stir on ice for 15 min. Powdered ammonium sulfate was then added to 30% saturation, and the mixture was stirred on ice for an additional 15 min. The first ammonium sulfate cut was pelleted at 150000*g* for 1 h, and the supernatant was brought to 57.5% saturation with powdered ammonium sulfate. After being stirred for 15 min on ice, the mixture was centrifuged at 30000*g*. The top pellets were pooled and resuspended in 150 mL of buffer A1 [25 mM MOPS, pH 7.0, 500 mM NaCl, 20% (v/v) glycerol, 0.1% (v/v) phosphatidylcholine, 0.1% (w/v) CHAPS, and 1X protease inhibitor cocktail (Wu et al., 1991b)]. The determination of specific activities during the entire carboxylase purifications was done under nearly identical assay conditions described by Wu et al. (1991b) in order to allow direct comparisons. These assays were carried out in 125-μL systems in buffer A1, 3.6 mM FLEEL, 880 μM KH_2, 1.5 mM $NaH^{14}CO_3$, 0.8 M ammonium sulfate, and 16 μM proPT18 instead of proFIX19.

An FIXQS–Affigel-10 affinity matrix was prepared by coupling 108 mg of FIXQS to 25 mL of Affigel-10 resin according to the manufacturer's protocol. The final concentration of covalently bound peptide was 406 μM as determined by the Bradford method using FIXQS as a standard (Bradford, 1976). The 150 mL of ammonium sulfate precipitated microsomes in buffer A1 contained 8 g of total protein as determined by the Bradford method using IgG as a standard. The microsomes were sonicated and applied to the FIXQS column, and bound carboxylase was washed with buffer A1 according to the method of Wu et al. (1991b). We departed slightly from Wu's elution II protocol by washing the bound carboxylase with linear 0.05–0.5% (50 mL)/0.5% (50 mL)/0.5–1.0% (50 mL)/1.0–0.05% (50 mL) Triton X-100 gradients and eluting with a linear 0.1–1.0% (200 mL)/1.0% (50 mL) CHAPS gradient, thus eliminating an intermediary 0.1–1.0% CHAPS (w/v)/buffer A1 (50 mM NaCl) gradient. The compositions of the Triton X-100- and CHAPS-containing buffers were identical to those described by Wu et al. (1991b). The purity of carboxylase was monitored by 10% SDS–PAGE analysis of silver-stained material and assayed as described in this section.

RESULTS AND DISCUSSION

Time-Dependent Inactivation of Carboxylase with N-Bromoacetyl-Peptide Affinity Labels. Carboxylase specifically interacts with peptides at two sites: the propeptide-binding site (γ-CRS) and the active site (Jorgensen et al., 1987). The γ-CRS directs and enhances active-site γ-carboxylation of certain Glu residues located within the Gla domain (see Figure 2) of the same peptide substrate with 1000-fold higher affinities relative to γ-carboxylation of the free Glu-containing peptide [i.e., compare the K_m values of proPT28 and FLEEL, Table I; Ulrich et al., 1988)]. We made *N*-bromoacetyl-peptide affinity labels of varying length and composition which could potentially covalently label carboxylase at one or both of these sites. The bromoacetyl moiety was an attractive choice for the electrophile because of its relative stability in aqueous solution and for the ease of introduction of the group at the α-amino group of many peptides (Wilcheck & Givol, 1977).

Table I: Physical and Kinetic Parameters of Carboxylase Substrates and N-Bromoacetylated Peptide Inactivators[a]

peptide	molecular ion[b] (m/z)	chemical shift of *N*-(haloacetyl)methylene singlet[c] (ppm)	K_m[d] (μM)	V_m[d] (dpm/min)	V_m/K_m[d] [M^{-1} (dpm/min)]	% inactivation[e]
FIXgla[f]	7053[g]		0.3 ± 0.1	460 ± 20	1.5×10^9	
FIXQS[f]			0.3 ± 0.1	580 ± 20	2.1×10^9	
proPT28			1.5 ± 0.7	690 ± 80	4.6×10^8	
TSYR28^{mer}	3379		6.9 ± 1.5	1400 ± 200	2.0×10^8	
BrAcTSYR28^{mer}	3500		7.0 ± 5.0	900 ± 200	1.3×10^8	20 (41 μM)
SYNSGFLEEV[f]			300 ± 50	10000 ± 1000	3.3×10^7	
BrAcSYNSGFLEEV		3.87	1500 ± 200	26000 ± 5000	1.7×10^7	<10 (1.8 mM)
FLEEYNA			400 ± 100	8200 ± 700	2.1×10^7	
BrAcFLEEYNA		3.82	500 ± 100	11000 ● 1000	2.2×10^7	<10 (5 mM)
ClAcFLEEYNA	961	4.15				<10 (1.3 mM)
FLEEYRK			500 ± 200	3700 ± 200	7.4×10^6	
GEEEYQE			340 ± 100	4600 ± 1000	1.3×10^7	
YLEEL			1000 ± 100	41000 ± 4000	4.1×10^7	
BrAcYLEEL		3.82				<10 (9.5 mM)
FLEELY	813		900 ± 200	24000 ± 1300	2.7×10^7	
BrAcFLEELY	933	3.85	800 ± 100[h]	33600 ± 1300[h]	4.2×10^7	80 (3 mM)
FLEEL			**2400 ± 100**	**32000 ± 300**	$\mathbf{1.3 \times 10^7}$	
BrAcFLEEL	770	3.87	1000 ±100[h]	20000 ± 2000[h]	2.0×10^7	83 (10 mM)

[a] Incorporation of $^{14}CO_2$ into peptide was linear up to 6 min when the reactions were done at 25 °C in 125-μL systems containing 25 μL of ammonium sulfate precipitated bovine liver microsomes [~380 ng of carboxylase in 2.7 mg of total protein with a specific activity of 3.2×10^5 dpm h^{-1} (mg of total protein)$^{-1}$], 880 μM dihydrovitamin K_1, 1.5 mM $NaH^{14}CO_3$ (10 μCi) in 150 mM NaBr/20 mM sodium phosphate, pH 7.4, and 10 μM proPT18. The proPT18 was omitted when the peptide substrate/inactivator already contained the proPT18 sequence such as in proPT28, FIXgla, FIXQS, TSYR28^{mer}, and BrAcTSYR28^{mer}. Note that DTT and Cl^- (both present in the standard assay) were excluded from the assays in order to prevent nucleophilic attack on the *N*-bromoacetyl groups. [b] Determined by FAB-MS. The molecular ion peak (MH^+) of the bromoacetylated peptides is actually a doublet arising from the two isotopes of bromine, M = 79 (50.54%), 81 (49.46%); the reported molecular ion is the lower mass isotope. [c] The *N*-(haloacetyl)methylene singlet resonance was easily assigned in all N-haloacetylated peptides except in the case of the *N*-bromoacetyl-TSYR28^{mer}. The chemical shifts of the nonexchangeable protons of the various amino acid side chains (A, E, F, G, L, N, S, V, Y) were the same, within error, as the "random-coil" chemical shifts of the side-chain protons of X in GGXA tetrapeptides in aqueous solutions reported by Wuthrich (1986). The chemical shifts of the side chains did not change upon N-bromoacetylation. The Cα proton resonances were not unequivocally assigned except with *N*-ClAcFLEEYNA where 2D-NMR (TOCSY) experiments were employed. The tyrosine ring proton resonances were shifted upfield from 6.82 to 6.81 ppm (3,5 H) and from 7.12 to 7.11 ppm (2,6 H) if bromoacetylation of the tyrosine phenolic oxygen occurred. [d] Kinetic parameters, K_m and V_m, were determined from double-reciprocal plots of velocity with respect to substrate concentration by the hyperbolic weighted least-squares method of Wilkinson (1961). The V_m for each peptide is the value relative to the normalized V_m value (=32 000 dpm/min) obtained for the standard FLEEL peptide run simultaneously under the same conditions. This normalized V_m value was the average value determined from 12 separate experiments. [e] The extent of time-dependent inactivation of carboxylase by bromoacetylated peptides over a 90-min time period was determined from plots of log (percent activity remaining) with respect to time, corrected for the first-order loss of activity in the control reactions. Values in parentheses are the concentrations of *N*-bromoacetyl-peptide used during the inactivations. [f] Produced by recombinant methods (see text for details). [g] Determined by Wu et al. (1990). [h] Kinetic parameters, K_m and V_m, were determined from the slopes of the double-reciprocal data extrapolated to zero substrate concentration when >20% of the enzyme was inactivated at high substrate concentrations (see Figure 4 for example). [i] Amino acid composition determined by total amino acid hydrolysis.

In spite of its stability to H_2O, the bromoacetyl group will be readily attacked by a wide range of amino acid side-chain nucleophiles. The first *N*-bromoacetyl-peptide synthesized was *N*-bromoacetyl-FLEEL, chosen because of the commercial availability of FLEEL, its high solubility, and its well-characterized kinetic properties as a carboxylase substrate and the lack of any reactive nucleophiles such as Cys-SH or Lys-NH_2. The *N*-bromoacetyl-FLEEL was synthesized, purified by HPLC, and characterized by high-field ^{1}H-NMR and FAB-MS (Table I). In the following kinetic studies, we used the concentrated and stable ammonium sulfate precipitated microsomal carboxylase preparation (AS carboxylase) rather than the dilute and relatively unstable (at 4–25 °C) affinity-purified carboxylase (vide infra). This choice of carboxylase preparation was made because the dynamic range of the measurement of [γ-$^{14}CO_2$]Gla incorporation for both substrate and inactivation studies was 10–20-fold higher in the AS carboxylase as compared to the affinity-purified carboxylase, thus giving a much better signal to noise ratio.

The *N*-bromoacetyl-FLEEL was a high-V_m substrate with K_m and V_m values similar to those of the parent FLEEL peptide (Table I). In addition to being turned over as a substrate, the *N*-bromoacetyl-FLEEL was a time-dependent inactivator of carboxylase with 83% of carboxylase activity lost over a 90-min period as compared to a control incubation with FLEEL. The amount of inhibition was not affected by addition of the other cosubstrates including 1.5 mM $NaHCO_3$ or 880 μM KH_2. However, some residual CO_2 and O_2 were still present in the inactivation reaction mixtures since we did not degas any of the reaction components. Addition of 10 μM proPT18 was found to enhance inactivation by ≥2.1-fold as was previously observed for substrate turnover (V_m) with FLEEL (Knobloch & Suttie, 1987; Ulrich et al., 1988). This enhancement effect on inactivation by proPT18 is further evidence that *N*-bromoacetyl-FLEEL interacts with the carboxylase active site. All subsequent inactivations included 10 μM proPT18 except in the case of BrAcTSYR28^{mer} (vide infra) which already contained the ProPT18 peptide sequence (Figure 2). All of these inactivations obeyed pseudo-first-order kinetics over the first 80–90 min of the reaction, consistent with the kinetic condition that presupposes a single inactivation site per carboxylase monomer. Furthermore, inactivation by 10 mM *N*-bromoacetyl-FLEEL could be completely prevented by addition of 10 mM FLEEL, which demonstrated overlap between the site of carboxylation and the site of inactivation.

Next, we wished to introduce a tyrosine residue into the *N*-bromoacetyl-peptide for the convenient introduction of high specific activity ^{125}I into the ortho positions of the tyrosine ring. This *N*-bromoacetyl[^{125}I]Tyr-peptide would be useful in the identification of a radiolabeled carboxylase active-site peptide. Several locations for the tyrosine residue were selected with the goal to place the tyrosine at isosteric/aliphatic positions using the wild-type substrate as a template (Figure

2) and thus retain favorable active-site–*N*-bromoacetyl-Tyr-peptide interactions. These peptides and their kinetic parameters are listed in Table I. Since the introduction of a Tyr into these small peptides drastically decreased their solubility in water, some peptides were made which contained ionizable or hydrophilic side chains which would counteract the adverse solubility effects of the Tyr. Two of these peptides, FLEEYRK and GEEEYQE, were not good substrates and were not tested further. *N*-Bromoacetyl-FLEEYNA, *N*-chloroacetyl-FLEEYNA, and *N*-bromoacetyl-YLEEL were carboxylase substrates but failed to demonstrate appreciable inactivation.

Apparently, the enzyme nucleophile which attacks the *N*-bromoacetyl of *N*-bromoacetyl-FLEEL is not properly disposed to attack other closely related *N*-bromoacetyl-peptides which differ in the composition and length of more distally located amino acids. Thus, minor changes in sequence content and length can have adverse effects on K_{inact} yet have no effect on K_m. This directs attention to the real mystery of how carboxylase can discriminate among millions of different Glu-containing peptides (20^5 for just a -XXEEXXX- heptapeptide sequence), some of which will undoubtedly differ only very slightly in their amino acid composition. The limited number of these affinity probes which can bind in the requisite geometry for covalent modification of a particular nucleophile must necessarily reflect the specific ground-state interactions that occur between the carboxylase Glu-peptide site and its substrates. Therefore, it is not surprising that the location of a hydrophobic and bulky side chain like Tyr could effectively modulate the k_{inact} and K_m values of these peptide probes of the carboxylase active site. The possibility remains, however, that these *N*-bromoacetyl-peptide substrates are also poor inactivators (<20%), but the degree of inactivation could not be reliably quantitated due to the narrow range of signal to noise ratio at low levels of activity loss.

N-Bromoacetyl-SYSNGFLEEV was synthesized in hopes of labeling a somewhat more distant active-site nucleophile as compared to that labeled by *N*-bromoacetyl-FLEEL. This peptide was also a substrate but not an inactivator. Finally, the *N*-bromoacetyl-FLEELY peptide was synthesized and was found to be both a high-V_m substrate and an excellent time-dependent inactivator (Table I, Figure 3). In the presence of 3 mM *N*-bromoacetyl-FLEELY, 80% of carboxylase activity was lost after 80-min incubation at 25 °C. This inactivation could be either completely protected against with saturating amounts of recombinant FIXQS (Figure 3) or partially protected against (20%) with 10 mM FLEEL, and consistent with the inactivation occurring at the active site or at least that the site of inactivation had substantial overlap with the active site. Addition of 0.5 mM DTT, a potent nucleophile, to the inactivation reaction mixture completely abolished the inhibitory properties of the *N*-bromoacetyl-FLEELY peptide.

The dependence of the first-order rates of inactivation on *N*-bromoacetyl-FLEELY concentration was determined and is shown in the insert to Figure 3. The intercepts of this double-reciprocal plot gave the maximal first-order rate of inactivation, k_{inact}, where all enzyme is complexed with *N*-bromoacetyl-FLEELY, and the K_i or concentration of *N*-bromoacetyl-peptide representing half-maximal inactivation. These values were 0.043 ± 0.021 s^{-1} and 9 ± 1 mM, respectively. The partition ratio, k_{inact}/k_{cat}, which compares the rate of inactivation to substrate turnover (Walsh et al., 1978) was also determined for *N*-bromoacetyl-FLEELY. In order to calculate k_{cat}, one must know the concentration of enzyme in the assay mixture and the V_m. The V_m was obtained from the slope of the double-reciprocal data extrapolated to *zero*

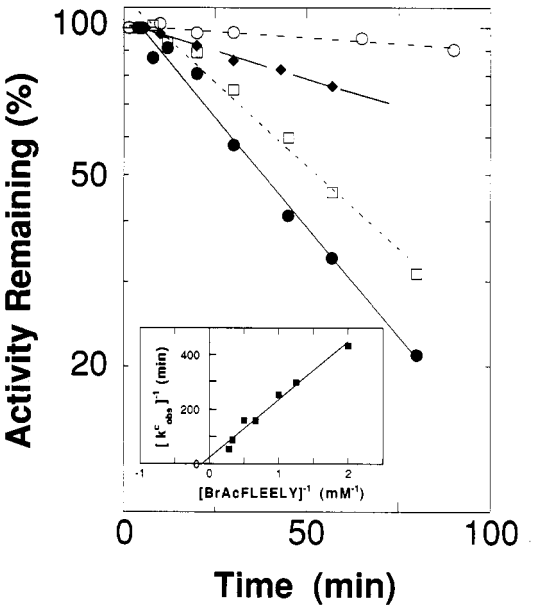

FIGURE 3: Time-dependent inactivation at the carboxylase active site by *N*-bromoacetyl-FLEELY. For each inactivation experiment, the percentage of carboxylase activity remaining was plotted as a function of time. The least-squares slopes of these lines were equal to the pseudo-first-order rate constant of inactivation, K_{obs}, at each peptide concentration. The peptide concentrations were 3.0 mM *N*-bromoacetyl-FLEELY (●), 3.0 mM *N*-bromoacetyl-FLEELY + 13 μM FIXQS (○), 0.8 mM *N*-bromoacetyl-FLEELY (□), and 3.5 mM FLEELY (♦). The insert to the figure is a double-reciprocal replot of the k^c_{obs} values corrected for the control rates of inactivation ($k^c_{obs} = k_{obs} - k_{control}$).

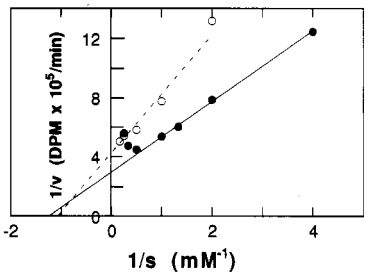

FIGURE 4: Substrate turnover of *N*-bromoacetyl-FLEELY and FLEELY. Double-reciprocal plots of the initial velocity of carboxylation at various FLEELY (○) and *N*-bromoacetyl-FLEELY (●) concentrations. Kinetic parameters, K_m and V_m, for *N*-bromoacetyl-FLEELY were determined from the slopes of the double-reciprocal data extrapolated to zero peptide concentration. K_m and V_m values for FLEELY were determined by Wilkinson hyperbolic weighted least-squares analysis.

substrate concentration under initial velocity conditions since appreciable inactivation will occur at concentrations approaching K_i (Figure 4, Table I). The amount of carboxylase in the assay mixtures was 3.5 pmol on the basis of the values of Wu et al. (1991b), who reported a 7000-fold purification from the AS carboxylase preparation. We achieved ~4000-fold purification from AS carboxylase (vide infra). The total protein concentration of our AS carboxylase preparation (107 ± 64 mg/mL) was in turn based on the average of three separate measurements, including the Bradford method using both IgG and BSA as standards, and by densitometry of silver-stained material run on 10% SDS–PAGE using molecular weight markers and IgG as standards. Thus, on the basis of the V_m and the estimated carboxylase concentration, the k_{cat} for *N*-bromoacetyl-FLEELY was 1.3 ± 0.6 s^{-1}. The k_{inact}/k_{cat} ratio was 0.033 ± 0.016, defining a highly favorable partition ratio of 1 inactivation for every 30 turnovers (Figure 5).

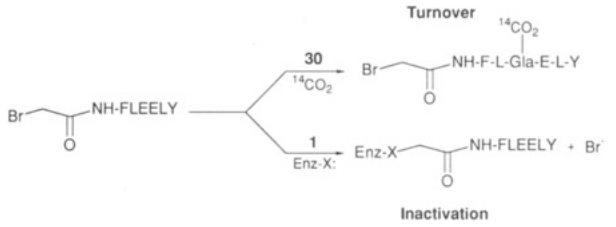

FIGURE 5: Alternative reaction pathways of *N*-bromoacetyl-FLEELY substrate turnover or irreversible inactivation of carboxylase. In the upper pathway, the *N*-bromoacetyl-FLEELY is γ-carboxylated to form a Gla-peptide product. In the lower pathway, an enzyme nucleophile, Enz-X:, located either within or nearby the active site, attacks the *N*-bromoacetyl methylene, displacing Br^- and forming the inactive carboxylase–Ac-FLEELY complex.

An initial attempt was made to label the γ-carboxylation recognition site of carboxylase. We synthesized an *N*-bromoacetyl-28^{mer} (*N*-BrAcTSYR28^{mer}) which incorporated residues −18 to −1 of the propeptide region and residues 1–10 of the Gla domain of prothrombin (Figure 2). Potential nucleophiles such as His-18 and Lys-10 were substituted by Thr and Arg, respectively, to avoid self-reactivity with the *N*-bromoacetyl electrophile. A Tyr was placed at position 1 (as in factor IX) to allow for radioiodination of tyrosine, and the Arg at −1 was changed to Ser to avoid propeptidase cleavage at this site. All these changes were conservative and based on sequence homologies with known carboxylase substrates (Figure 2). The *N*-bromoacetyl-TSYR28^{mer} was synthesized and purified, and its composition was verified by FAB-MS. Like its predecessor *N*-bromoacetyl-peptides, the *N*-bromoacetyl-TSYR28^{mer} was an excellent substrate (Table I). However, it did not inactivate carboxylase to any appreciable extent (20%).

Given the observation of time-dependent inactivation of crude carboxylase by *N*-bromoacetyl-FLEELY, we needed to purify carboxylase to sufficient purity to test for labeling of the enzyme species by ^{125}I-labeled inactivator. To this end, we turned to the recent purification strategy of Wu et al. (1991b) which required an affinity resin containing a profactorIX 59^{mer}.

Production of Recombinant ProFIX59^{mer} Peptides. Since it was necessary to use a 59^{mer} peptide affinity column in large amounts to purify carboxylase from bovine liver microsomes, we had to develop a method to produce >100-mg quantities of the peptide. We decided to adopt the general approach of Wu et al. (1990) and produce the 59^{mer} as a recombinant fusion protein.

Our fusion construct consisted of the full-length bacterial steroid isomerase (KSI) Y14F mutant, residues 1–125, as the N-terminal portion, attached downstream by a junctional methionine to residues −18 to 41 of proFIX59. Several mutations were incorporated into the amino acid sequence of proFIX59 as previously done by Wu et al. (1990). These included the T-18A/M19I double mutant (FIXGla), which allowed for efficient and specific CNBr cleavage at the junctional methionine, and the additional mutations, R-4Q/R-1S (FIXQS), which prevented cleavage of the propeptide from the Gla domain by microsomal propeptidases. The fusion gene, KSIFIX, was placed under the control of the lac promoter in a pUC-derived vector, pAK808, and the protein was expressed in *E. coli* BL21(DE3) to levels of 8% of the total cellular protein (Figure 6). The fusion protein was processed into inclusion bodies, partially purified with detergents, solubilized in 70% formic acid, and cleaved with CNBr. The desired 59^{mer} peptide product of the CNBr cleavage was purified to homogeneity by Mono Q anion-exchange chromatography (Figure 6). Average yields were 47 mg of pure FIX59^{mer} starting from 9 L of culture. Wu et al. (1990) had reported that a significant portion of the FIX59^{mer} peptides existed as nonreducible dimers of MW ~14 000. We believed that the covalent bond linking the monomers together was due to intermolecular amine nucleophilic attack onto the methylene adjacent to the cysteine sulfonium, Cys-S^+-$(CN)_2$, generated by treatment with excess CNBr. We also observed dimer formation but were able to limit production of this species by lowering the amount of CNBr used in the cleavage reaction. The recombinant monomeric FIXGla and FIXQS peptides were excellent carboxylase substrates (Table I) and had similar kinetic parameters as reported by Wu et al. (1990). The isolated $(FIXGla)_2$ and $(FIXQS)_2$ dimeric species had identical kinetic parameters as the monomeric species.

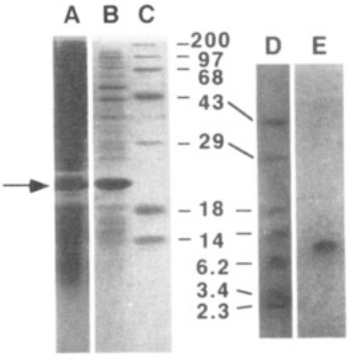

FIGURE 6: Expression of the KSIFIXQS fusion protein in *E. Coli* (BL21/DE3) and purification of FIXQS as analyzed by 15% SDS–polyacrylamide gels stained with Coomassie Blue R-250. (A) Total cellular extract of IPTG-induced *E. coli* BL21 (DE3) containing the pUC19/KSIFIXQS fusion gene. The KSIFIXQS fusion protein (MW 21K) is indicated by the arrow. (B) Inclusion bodies from material in lane A extracted with successive deoxycholate/Nonidet P-40 and Triton X-100 washes. (C) Mono Q purified FIXQS after CNBr/70% formic acid cleavage of material in lane B.

FIXQS Affinity Purification of Carboxylase. Now that we had successfully made large quantities of FIXQS suitable for the preparation of an affinity column, we repeated the carboxylase purification procedure of Wu et al. (1991b). A 25-mL FIXQS affinity column was prepared, and three separate affinity purifications were carried out. Typically, 8.0–9.5 g of sonicated ammonium sulfate precipitated microsomes from bovine liver was applied to the affinity column. The specific activity of the carboxylase preparation loaded onto the column ranged from 3.2×10^5 to 3.7×10^5 dpm h^{-1} (mg of protein)$^{-1}$. We followed Wu's elution II protocol with a few minor modifications of the detergent wash and elution gradients. Carboxylase was eluted with a 0.1–1.0% CHAPS gradient in the presence of 2 μM proPT18 as a stabilizer of carboxylase activity. The elution profile of the 94-kDa band, as analyzed by silver-stained 10% SDS–PAGE, was similar to that obtained by Wu and co-workers. The peak of the 94-kDa band eluted at the top of the 1.0% CHAPS gradient but was very broad (150 mL). The activity profile did not completely match that of the 94-kDa band. The activity correlated well with the initial appearance and rise of the 94-kDa band but peaked at ~0.7% CHAPS and slowly decreased during the remaining elution of the 94-kDa band. This diminution of carboxylase activity may be attributed to the progressively higher CHAPS concentration of the latter fractions. In separate experiments, high CHAPS concentration was shown to effectively inhibit dilute carboxylase. The final specific activity of the most active fractions (see Figure 8, lane B, for example) ranged from 1.3×10^9 to 1.9×10^9 dpm h^{-1} mg^{-1} which reflected a 3500–5200-fold

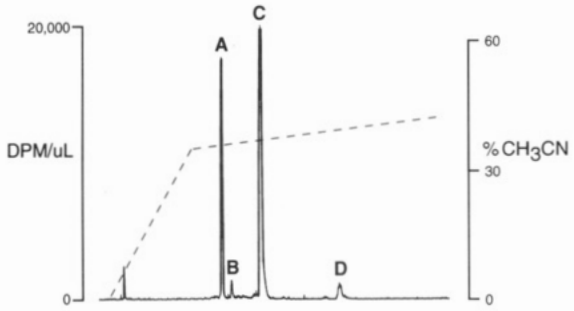

FIGURE 7: Reverse-phase high-performance liquid chromatography of the reaction products of the N-bromoacetylation of FLEEL[^{125}I]Y. The microscale N-bromoacetylation of FLEEL[^{125}I]Y was carried out as described under Materials and Methods. The reaction mixture was quenched with HBr and injected directly onto a 4 × 250 mm Vydac RP-HPLC column, and a CH_3CN/H_2O/0.1% TFA gradient was applied. Radioactivity was monitored by an in-line γ-counter. Peaks A and B are unreacted FLEEL[^{125}I]Y and FLEEL[^{125}I]$_2$Y, respectively. Peaks C and D are *N*-bromoacetyl-FLEEL[^{125}I]Y and *N*-bromoacetyl-FLEEL[^{125}I]$_2$Y, respectively.

purification from the AS carboxylase starting material. The yield was only 4% of the activity loaded onto the column, but 90% of the activity that remained bound to the column after extensive washing was successfully recovered as ascertained by directly measuring the activity of a portion of the washed affinity matrix. Depending on the column fraction, the purity of carboxylase ranged from 15% to 50% as assessed by silver staining of material run on 10% SDS–polyacrylamide gels. The average amount of the 94-kDa protein recovered was ~100 μg.

Specific and Irreversible Labeling of Affinity-Purified Carboxylase with N-Bromoacetyl-FLEEL[^{125}I]Y. Using affinity-purified carboxylase, we tested for the specific labeling of the 94-kDa species using an *N*-bromoacetyl-FLEEL[^{125}I]Y inactivating substrate. The *N*-bromoacetyl-FLEEL[^{125}I]Y peptide was synthesized and purified by RP-HPLC (Figure 7). The final specific activity was 2.2 Ci/μmol, a sufficiently high activity to measurably label femtomole amounts of carboxylase. FIXQS affinity column fractions containing the highest amount of carboxylase activity were incubated with the radiolabeled peptide. The inactivation mixtures were boiled in a β-mercaptoethanol/SDS-containing sample buffer and analyzed by 10% SDS–PAGE. The gels were stained with silver, and radioactivity was assayed by autoradiography. As shown in Figure 8, the 94-kDa band was the predominant labeled protein, thus independently demonstrating that this species was carboxylase. Since the carboxylase–FLEEL[^{125}I]Y complex survived boiling in β-mercaptoethanol/SDS and subsequent electrophoresis, one can conclude that the interaction was covalent and therefore that the time-dependent inactivation observed was irreversible. Since the covalent bond was not susceptible to nucleophilic displacement by β-mercaptoethanol, one can also conclude that the enzyme nucleophile is not likely to be Asp, Glu, or His.

Coincubation with FIXQS peptide substrate partially displaced the radiolabeled peptide, consistent with the inactivation occurring at the active site as was discussed earlier (Figure 8, lanes D–F). Addition of DTT to the inactivation reaction mixture completely blocked covalent labeling of carboxylase, confirming that an intact electrophilic *N*-bromoacetyl moiety was essential for inactivation to occur (Figure 8, lane C). Interestingly, a distinct higher molecular mass species (>200 kDa) was also covalently labeled by *N*-bromoacetyl-FLEEL[^{125}I]Y and showed identical displacement by FIXQS as occurred with the 94-kDa species. Attempts to

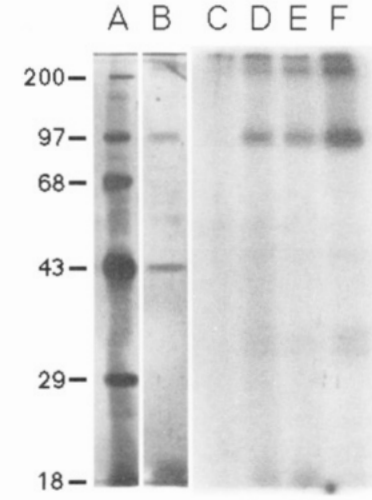

FIGURE 8: Irreversible and specific labeling of affinity-purified carboxylase with *N*-bromoacetyl-FLEEL[^{125}I]Y as analyzed by 10% SDS-PAGE and autoradiography. Fifty-microliter aliquots of HPLC-purified *N*-bromoacetyl-FLEEL[^{125}I]Y were dried in 1.7-mL Eppendorf tubes. The dried peptide residue was partially solubilized with 3 μL of 0.5 M potassium phosphate, pH 7.5, and varying amounts of FIXQS peptide substrate in PBSBr were added to each tube. Inactivation reactions were initiated by addition of 40 μL of affinity-purified carboxylase in 0.7% (w/v) CHAPS, 0.2% (v/v) phosphatidylcholine, 25 mM MOPS, pH 7.0, 500 mM NaCl, 20% glycerol (v/v), and 2 μM proPT18. The final volume of each inactivation mixture was 50 μL. The inactivation reactions were incubated at 25 °C for 2 h and then quenched with 10 μL of β-mercaptoethanol (βME)/SDS sample buffer and boiled for 5 min before electrophoresis. The βMe/SDS sample buffer contained 316 mM Tris-HCl, pH 6.8, 10% SDS (w/v), 3.6 M βME, 40% glycerol (v/v), and 0.005% bromophenol blue (w/v). After electrophoresis, the gel was stained with silver and dried, and autoradiography was performed. (A) Protein molecular weight standards; (B) silver-strained material shown in lane D; (C) autoradiography of inactivation reaction mixture preincubated with 30 mM DTT for 15 min at 25 °C prior to addition of carboxylase; (D) autoradiography of inactivation reaction mixture with 13 μM FIXQS; (E) same as lane D except that 5.6 μM FIXQS was present; (F) same as lane D except that FIXQS was not present.

quantitate the ratio of label to 94-kDa and >200-kDa species proved difficult since the silver staining of the radiolabeled material was highly irreproducible and the apparent relative proportion of the 94-kDa band to the >200-kDa band also varied upon silver staining. One can say, however, that upon visual inspection of the gel shown in Figure 8 and other similar gels (unpublished data), the ratios of label to 94- and >200-kDa proteins are nearly the same. It may be that this higher molecular weight species either was an aggregated form of carboxylase or may possibly be a higher order species (i.e., nonreducible dimer) though this remains to be confirmed by further experiments. Currently, studies are underway to isolate and identify the labeled active-site peptide and to determine which amino acid side chain is the critical nucleophile which attacks the *N*-bromoacetyl group.

Conclusions. We have synthesized a series of *N*-bromoacetyl-peptides ranging from 5^{mers} to 28^{mers} which vary in their efficacies as substrates and irreversible inhibitors of carboxylase. *N*-Bromoacetyl-FLEELY is the most useful of these peptides and has an efficient partition ratio of 30 turnovers as substrate for every 1 inactivation event. In addition, competing carboxylase substrates partially or completely abolish the inhibitory activity of *N*-bromoacetyl-FLEELY, establishing that the site of inactivation either overlaps or is identical with the site of carboxylation. The subsequent covalent labeling of affinity-purified 94-kDa carboxylase with *N*-bromoacetyl-FLEEL[^{125}I]Y was also

shown to be partially competed against by recombinant FIXQS. These observations independently establish that the 94-kDa protein is an authentic carboxylase and uses *N*-bromoacetyl-FLEEL and *N*-bromoacetyl-FLEELY as both substrates and inactivators. Although the *N*-bromoacetyl moiety of the *N*-bromoacetyl-FLEELY peptide is approximately three amino acids away from the first carboxylatable glutamate, the identification and location of the nucleophile which attacks the N-bromoacetyl group should provide the initial information necessary to identify the carboxylase active site.

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