Dioxygen Transfer during Vitamin K Dependent Carboxylase Catalysis†

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ABSTRACT: The vitamin K dependent carboxylase of liver microsomes is involved in the posttranslational modification of certain serine protease zymogens which are critical components of the blood clotting cascade. During coupled carboxylation/oxygenation this carboxylase converts glutamate residues, dihydrovitamin K, CO_2, and O_2 to a γ-carboxyglutamyl (Gla) residue, vitamin K (2*R*,3*S*)-epoxide, and H_2O with a stoichiometry of 1:1 for all substrates and products. In this paper we investigate the role of molecular oxygen in the reaction by following the course of the oxygen atoms using $^{18}O_2$. Two different mass spectroscopic techniques, electron ionization positive ion mass spectrometry and supercritical fluid chromatography–negative ion chemical ionization mass spectrometry, were used to quantitate the amount of ^{18}O incorporation into the various oxygens of the vitamin K epoxide product. We found that 0.95 mol atoms of oxygen were incorporated into the epoxide oxygen, 0.05 mol atoms of oxygen were incorporated into the quinone oxygen of vitamin K epoxide, and the remaining ca. 1.0 mol atoms of oxygen were incorporated into H_2O. No incorporation of oxygen into vitamin K epoxide from 50% $H_2{}^{18}O$ was observed. Thus, the carboxylase operates as a dioxygenase 5% of the time during carboxylation/oxygenation. The relevance of these findings with respect to the nonenzymic "basicity enhancement" model proposed by Ham and Dowd [(1990) *J. Am. Chem. Soc. 112,* 1660–1661] is discussed.

A number of proteins involved in blood coagulation such as the serine protease zymogens prothrombin and factors VII, IX, and X undergo posttranslational modification of 9–12 glutamyl (Glu) residues, located near their amino termini, to γ-carboxyglutamyl (Gla)[1] residues (Suttie, 1985). The bidentate Gla residues allow these proteins to chelate calcium and interact with membrane surfaces, an event that is essential for the initiation, progression, and regulation of the blood clotting cascade (Furie & Furie, 1988). The enzyme that catalyzes carboxylation of blood coagulation factors is the vitamin K dependent γ-glutamyl carboxylase. This integral membrane enzyme is an extremely unusual carboxylase since it requires stoichiometric amounts of dihydrovitamin K (KH_2), O_2, CO_2, and a glutamate-containing peptide to produce vitamin K epoxide (KO), Gla, and H_2O (Figure 1). The

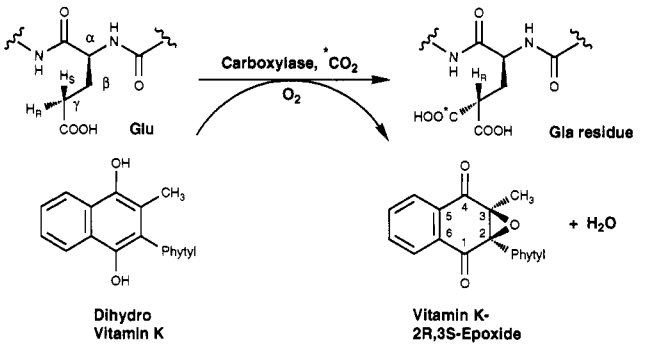

FIGURE 1: Carboxylase reaction of the conversion of CO_2, O_2, Glu-containing peptide, and dihydrovitamin K to a γ-carboxyglutamate-(Gla-) containing peptide, vitamin K (2*R*,3*S*)-epoxide, and H_2O. The enzyme stereospecifically abstracts the *pro-S* γ-glutamyl proton and also carboxylates stereospecifically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

enzyme is of crucial importance in hemostasis and thrombosis but is a completely new and as yet undetermined mechanistic type of carboxylase/oxygenase (Walsh, 1979).

Recently, the enzyme has been significantly purified from bovine liver microsomes (Wu et al., 1991b) and the human and bovine cDNAs have been cloned (Wu et al., 1991a). The enzyme is a single polypeptide of molecular weight 94 000 and when expressed in kidney 293 cells (Wu et al., 1991a) it retains the ability to carboxylate the FLEEL pentapeptide. The C-terminal portion of carboxylase shares approximately 19% sequence homology over a stretch of 198 amino acids with a well-studied dioxygenase, soybean lipoxygenase 1 (Vliegenthart & Veldink, 1982; Shibata et al., 1987). The soybean lipoxygenase is one of four isozymes that are part of a large family of lipoxygenases from plants and animals that share 40–90% sequence homologies in the C-terminal regions.

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[1] Abbreviations: carboxylase, vitamin K dependent γ-glutamyl carboxylase; Gla, γ-carboxyglutamate; FLEEL, Phe-Leu-Glu-Glu-Leu; KH_2, dihydrovitamin K_1; KO or vitamin K epoxide, *trans*-phytyl-2,3-epoxyvitamin K_1; hsp70, 70-kDa heat shock protein; BiP, immunoglobulin heavy chain binding protein; DE52, (diethylaminoethyl)cellulose; ATP, adenosine triphosphate; CGG-proPT18, Cys-Gly-Gly-His-Val-Phe-Leu-Ala-Pro-Gln-Gln-Ala-Arg-Ser-Leu-Leu-Gln-Arg-Val-Arg-Arg; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate; EDTA, ethylenediaminetetraacetic acid; RP-HPLC, reverse-phase high-performance liquid chromatography; EI-MS or EI-PI-MS, electron ionization (positive ion) mass spectrometry; SFC-NICI-MS, supercritical fluid chromatography–negative ion chemical ionization mass spectrometry; M, molecular ion.

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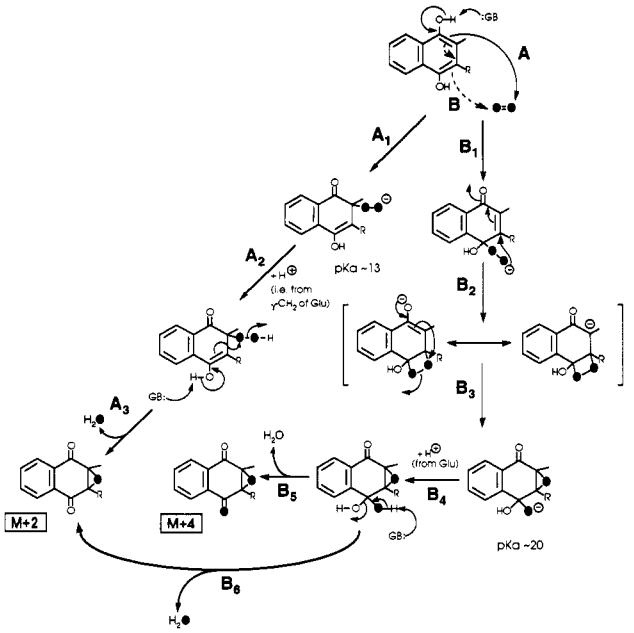

FIGURE 2: Basicity enhancement mechanism showing possible routes of ^{18}O incorporation into the vitamin K epoxide (KO) products of the carboxylase reaction.

The animal enzymes include those involved in the arachidonic acid biosynthetic pathway (Dixon et al., 1988; Funk et al., 1990). These monomeric lipoxygenases each contain 1 mol of tightly bound Fe which presumably forms an Enz–Fe–O_2 complex that is the basis for the extraordinary reactivity of the dioxygen to their respective aliphatic substrates. It is not known whether carboxylase contains a metal required for the activation of O_2.

In a previously proposed mechanistic scheme (Suttie et al., 1978), the activation of molecular oxygen was thought to occur by the formation of a 3-hydroperoxy adduct with dihydrovitamin K which then acts as a base ($pK_a \sim 13$). The base could then abstract the γ-methylene *pro-S* proton from the glutamate (see path A_1–A_3 in Figure 2) forming a glutamate carbanion intermediate (Suttie et al., 1978; Dubois et al., 1983). Oxygen and vitamin KH_2 dependent exchange of tritium from water into the glutamyl γ-methylene of the *N-tert*-butoxycarbonyl-Glu-Glu-Leu-OMe substrate is interpretable as evidence for a carbanion mechanism (McTigue & Suttie, 1983). A nucleophilic attack on CO_2 by the glutamate carbanion proceeds with net inversion of configuration at the γ-methylene carbon (Dubois et al., 1991). Dowd and co-workers (Ham & Dowd, 1990; Dowd et al., 1991) have proposed an elegant "basicity enhancement" mechanism for carboxylase based on the analogous nonenzymic oxidation of 2,4-dimethyl-1-naphthoxide anion to a tertiary alkoxide base. This strong base can then abstract a proton on another molecule and thereby effect ring closure of that molecule. Since this nonenzymic chemical model closely simulates what happens on the surface of the enzyme, it has been speculated (Maddox, 1991) that nature has retained many of these energetically favorable features over the course of evolution. Other catalytic aspects such as substrate specificity and the reduction of unfavorable entropic barriers that occur when there are four separable substrates would require substantial improvements of the chemical template.

Dowd's model would predict that the enzymatic base is not a hydroperoxy adduct of vitamin K; instead the peroxyanion adduct would rearrange to form a vitamin K alkoxide intermediate with a greatly enhanced basicity of $pK_a \sim 20$ (path B_1–B_3 in Figure 2). If the alkoxide is an intermediate in the carboxylation reaction, then one could detect ^{18}O incorporation from $^{18}O_2$ into both the epoxide oxygen position, as previously shown (Sadowski et al., 1977), and into the 1-oxo position, which has not yet been demonstrated. If the enzyme reaction follows this path (path B_1–B_5) one would expect an elevated abundance of a molecular ion in which the molecular weight is increased by 4 mass units [(M + 4) or two ^{18}O atoms as opposed to two ^{16}O atoms] appearing in the vitamin K epoxide product. If the enzyme operates through a peroxyanion base mechanism (path A_1–A_3) one would expect the appearance of (M + 2) vitamin K epoxide arising from ^{18}O incorporated exclusively at the epoxide oxygen. In this paper we describe the results of two different mass spectroscopic techniques used to quantitate the amount of ^{18}O incorporated into the epoxide and quinone (1-oxo) positions of the vitamin K epoxide produced during coupled carboxylation/oxygenation. By following the course of ^{18}O incorporation into vitamin K epoxide we can place constraints on the role of dioxygen in the reaction mechanism.

MATERIALS AND METHODS

Silica gel chromatography was used to purify the biologically active *trans*-phytylvitamin K_1 from a mixture of *cis*- and *trans*-phytylvitamin K_1 (Sigma) as described by Fasco et al. (1983). *trans*-Phytyl-2,3-epoxyvitamin K_1 (KO) was synthesized (Tishler et al., 1940) and purified by reverse-phase HPLC in methanol–water (95:5). The specific activity of the $NaH^{14}CO_3$ (Amersham) used in the carboxylation reactions was 53.1 mCi/mmol. Cambridge Isotope Laboratories supplied 98% $^{18}O_2$, 50% $^{18}O_2$, and 50% $H_2{}^{18}O$. 100% $^{16}O_2$ was from Med Tech Gases. FLEEL was from Sigma and was used without further purification.

Carboxylase was partially purified 90-fold from bovine liver microsomes by the following modification of the method of Hubbard et al. (1989b). After removal of intact organelles by low-speed centrifugation (10000*g*), crude microsomes were precipitated by the addition of solid $LaCl_3$ to a final concentration of 8 mM. This initial microsomal-enriched fraction was pelleted at 10000*g*, thereby avoiding a volume-limiting ultracentrifugation step. Contaminating hsp70, immunoglobulin heavy chain binding protein (BiP), was removed by successive batch absorptions to DE52 (Whatman) and ATP–agarose (Sigma) prior to CGG-proPT18 affinity chromotography. Carboxylase was eluted from the affinity column as before and concentrated from 0.25% CHAPS, 1 mg/mL phospholipids [for exact composition see Hubbard et al. (1989b)], 15% glycerol, 35 mM dithiothreitol, 1 mM EDTA, 20 mM sodium phosphate, and 150 mM NaCl, pH 7.4, using Centriprep-30 ultrafiltration units (Amicon). All steps were performed at 4 °C. The specific activity of carboxylase used in the carboxylation/oxygenation reactions was 2.5×10^7 dpm h^{-1} (mg of protein)$^{-1}$.

Determination of Coupling Ratios between Carboxylation (Gla Formation) and Oxygenation (KO Formation). Carboxylation/oxygenation reactions were performed in sealed 2-mL Wheaton crimp vials. Equilibration with various isotopic mixtures of molecular oxygen was achieved with 1 L of gas at 1 L/h at 25 °C except in the case of 98% $^{18}O_2$, where the flow rate was 0.1 L/h for a total volume of 100 mL. Assay mixtures of 1 mL total volume included 200 μL of partially purified carboxylase (200 μg of total protein), 200 mM dithiothreitol, 10 mM FLEEL, 3 mM $NaH^{14}CO_3$, 0.7 mM dihydrovitamin K (KH_2) in 100 mM sodium phosphate, and 150 mM NaCl, pH 7.4. The dithiothreitol, FLEEL, KH_2, and $NaH^{14}CO_3$ in a volume of 270 μL were added together by

syringe to the buffered enzyme solution equilibrated with O_2. After incubation for 1 h at 25 °C, 50 μL was removed by syringe, quenched with 10% trichloroacetic acid, and boiled, and $^{14}CO_2$-Gla was quantitated by scintillation counting (Hubbard et al., 1989a). The remaining 950 μL of the reaction mixture was transferred to another sealed vial and vortexed with 5 mL of 2-propanol–hexane (3:2). The hexane layer was removed by syringe and then rotary evaporated to dryness. The dried residue was resuspended in 150 μL of methanol and injected in 3 equal volumes onto an analytical C_{18}, 4.6 mm × 25 cm, 5-μm Vydac RP-HPLC column at a flow rate of 1 mL/min in 100% methanol. KO was quantitated at 254 nm using a standard solution of KO in methanol (ϵ_{225} 30 800 M^{-1} cm^{-1}) by the method of Fasco et al. (1983). The KO peaks which eluted at ca. 6.9 min were pooled and dried with a Speed-Vac concentrator (Savant), and duplicates of each sample were analyzed by both EI and SFC–mass spectrometry.

Electron Ionization (EI) Positive Ion Mass Spectrometry. In-beam electron ionization mass spectrometry was performed on a VG ZAB2-SE-FPD mass spectrometer (VG Instruments, Manchester, UK) operated at 8 kV in the positive ion mode. Current-controlled scans were acquired at a rate of 5 s/decade from 300 to 500 amu. The resolution was 1:1000 and perfluorokerosene was used for calibration. The source temperature was maintained at 250 °C and the direct inlet probe was heated from ambient to 250 °C at a rate of 50 °C/s.

Supercritical Fluid Chromatography–Negative Ion Chemical Ionization Mass Spectrometry (SFC-NICI-MS). The chromatographic system was a Series 601 supercritical fluid chromatograph (Lee Scientific, Division of Dionex Corp., Salt Lake City, UT). The samples were introduced into the column by a solid-phase injector (Koski et al., 1992). The mobile phase CO_2 (Scott Specialty Gases, Plumsteadville, PA), was density programmed from 0.20 to 0.76 g/mL at 0.02 (g/mL)/min. The oven was maintained at 100 °C. The analytical fused silica column (10 m) was coated with a 50% (cyanopropyl)methylpolysiloxane stationary phase and had an internal diameter of 50 μm. Mobile-phase density and flow were regulated with an integral restrictor located at the distal end of the column.

Negative ion chemical ionization mass spectrometry (NICI-MS) was performed on a VG ZAB2-SE-FPD mass spectrometer operating at 8 kV. Carbon dioxide was used as the reagent gas. The end of the SFC column was introduced into the source and maintained at 350 °C. The interface has been previously described (Reinhold et al., 1988).

RESULTS

Fate of Molecular Oxygen in the Vitamin K Dependent Carboxylation Reaction. The biologically active substrate, *trans*-phytylvitamin K_1, was separated from the inactive *cis*-phytylvitamin K_1 in order to simplify interpretation of the data. We were careful to run the reactions under saturating amounts of all substrates (especially O_2) in order to limit uncoupling of carboxylation and oxygenation activities where KO is produced in excess relative to the Gla coproduct (Wood & Suttie, 1988). In preliminary ^{18}O labeling experiments we had already determined that the presence of non-glutamate peptide substrates such as FLDDL or FLAAL did not further uncouple Gla formation from KO formation as compared to the same reactions run in the absence of exogenous peptide. We chose the small FLEEL pentapeptide for these experiments because it is the best characterized substrate for carboxylase and is a very high V_m peptide and produces sufficient quantities of vitamin K epoxide product (30 nmol) to perform mass spectrometric analyses. Other low K_m, low V_m Glu substrates such as 28mers (i.e., proPT28) which are based on the sequences of the physiological substrates of carboxylase do not turn over enough vitamin K and therefore preclude mass spectral analysis of the vitamin K epoxide products. Under the experimental conditions employed here, the relative amounts of vitamin K epoxide versus Gla produced by carboxylase ranged from 1.1:1 to 1.6:1 indicating close coupling between oxygenase and carboxylase activities on the same enzyme. Thus having established that we were analyzing the products of the predominantly coupled reaction, we proceeded to follow the course of the labeled oxygen atoms by mass spectrometry.

Two different mass spectrometric techniques were employed since our goal was to quantitate potentially small increases in the abundance of mass isotopes which were already of low natural abundance. The first method was positive ion electron ionization mass spectrometry which has the advantage of being able to generate numerous fragment ions of high intensity (McLafferty, 1973). The fragments are of considerable utility in the diagnosis of structure and the location of individual atoms. This method, however, under conditions of high sample concentration, is liable to exaggerate the isotope ratios [i.e., increased (M + 1)/M, (M + 2)/M, etc.] as a result of radical recombination:

$$R{=}O^{\bullet +} + H^{\bullet} \rightarrow (R{=}OH)^{+} \quad (1)$$

$$M^{\bullet +} + 1 \rightarrow (M + 1)^{+} \quad (2)$$

$$(M + 1)^{\bullet +} + 1 \rightarrow (M + 2)^{+}, \text{ etc.} \quad (3)$$

The most common of these radical reactions is addition of a hydrogen to a heteroatom such as a carbonyl oxygen (eq 1). The second mass spectrometric technique used was supercritical fluid chromatography–negative ion chemical ionization MS. In SFC-NICI-MS the analytes are eluted from the analytical column with supercritical carbon dioxide. The end of the column is inserted directly into the ion source of the mass spectrometer. Hence, the solutes are immediately ionized after elution from the column. When mild reagent gases such as CO_2 or ammonia are used, the amount of internal energy transferred from the reagent gas to the product is low, thereby providing abundant molecular ions (Lee & Markides, 1990; Caesar et al., 1990). The two methods, EI-MS and SFC-NICI-MS, when used in conjunction, are complementary for the quantitation of potentially subtle changes in the relative abundancies of isotopic species.

The EI-MS data will be considered first. The vitamin K epoxide molecular ion (M = 466) readily fragmented into three major species, m/z 306, 423, and 450, consistent with structures shown in Figure 3. The epoxide oxygen is present in the 306 and 466 species whereas the quinone oxygen(s) is(are) present in the 423, 450, and 466 species. If ^{18}O is incorporated into the epoxide oxygen there should be an increase in the intensity of the relative abundance of the (M + 2) species relative to the natural isotopes of 306 and 466. If ^{18}O is incorporated into the quinone oxygen then an increase in the (M + 2) species of 423, 450, and 466 will likewise occur. The 466 species contains both epoxide and quinone oxygens and could give rise to a double labeled (M + 4) ion if two atoms of ^{18}O were incorporated as illustrated by path B_1–B_5 in Figure 2. The relative abundancies of the 306 (M) and 308 (M + 2) species were plotted as a function of percent ^{18}O in the reaction mixture as shown in Figure 4A. The slopes

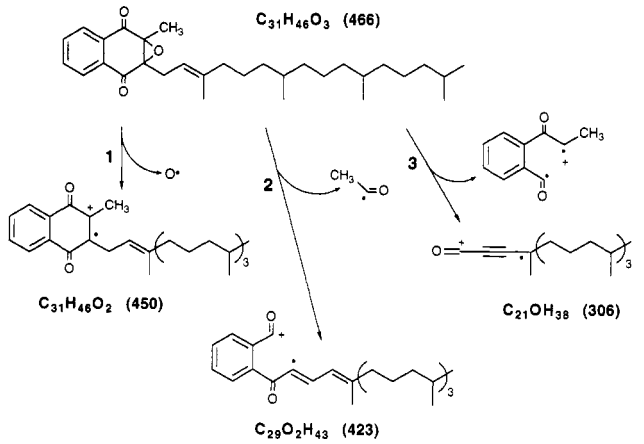

FIGURE 3: Fragmentation of vitamin K epoxide produced during electron ionization mass spectrometry. The parent molecule, vitamin K epoxide (M = 466), readily fragmented into three highly abundant species with masses 306, 423, and 450. All three fragments resulted from breakage of the bonds of the relatively unstable epoxide ring.

of the lines, which are equal to the mol atoms of ^{18}O incorporated, indicated that 0.837–0.954 atoms of oxygen from $^{18}O_2$ are incorporated into the epoxide oxygen, in agreement with preliminary results from early EI-MS studies (Sadowski et al., 1977). There was no incorporation of ^{18}O from $H_2{}^{18}O$ into the epoxide oxygen when the reaction was run in the presence of 50% $H_2{}^{18}O$ (Figure 4A). The same analysis of the 466 (M) and 468 (M + 2) species gave 0.832–0.985 mol atoms of ^{18}O incorporation from $^{18}O_2$ into the epoxide oxygen (Table I). Again, there was no incorporation of oxygen (≤0.011 mol atoms) from 50% $H_2{}^{18}O$ into the 466 species. These results clearly confirm that the epoxide oxygen of the KO product of the coupled carboxylation/oxygenation reaction derives from molecular oxygen as would occur by either pathway A_1–A_3 or B_1–B_5,B_6 (Figure 2).

The crucial and remaining mechanistic issue is whether the quinone oxygen of the KO product also derives from $^{18}O_2$. Three of the KO species, 423, 450, and 466, contain the quinone oxygen, and the relative abundance of each isotope (M + 2) or (M + 4) plotted as a function of percent ^{18}O is shown in Figure 4B. The mol atoms of ^{18}O incorporated into the quinone oxygen position ranged from 0.023 to 0.089 (average of 0.056) as determined by EI-MS. No exchange (≤0.007 mol atoms) of the quinone oxygen with 50% $H_2{}^{18}O$ was observed (Table I). In addition, less than 0.18% KO product was produced in the absence of enzyme, establishing that all pathways of oxygen incorporation into vitamin K were enzyme dependent. As another control, the (M + 2) peak of the 306/308 fragment, which contains no quinone oxygen, was analyzed as above (Figure 4B) and showed no increase in abundance as expected. Thus, one in 20 oxygenase reactions results in incorporation of ^{18}O into the 1-oxo position by path B_1–B_5.

As was discussed above, EI-MS can suffer from exaggerated levels of isotope contribution due to a concentration-dependent addition of hydrogen radicals to $M^{\bullet +}$ (eqs 1–3). The magnitude of this spurious increase in isotopic contribution can be quantitated by monitoring the (M + 1)/M ratio as a function of the intensity of M, which is directly proportional to the concentration of the sample in the ion beam. Furthermore, for any particular sample the percent increase in the intensity of all mass isotopes will be identical regardless of the mass since the isotope effect on radical recombination will be insignificant at low concentrations. The (M + 1)/M isotopic ratio for the EI-MS data remained relatively constant at 39.3 ± 1.4 (n = 23) at M intensities ≤1.8 × 10^7 ion current

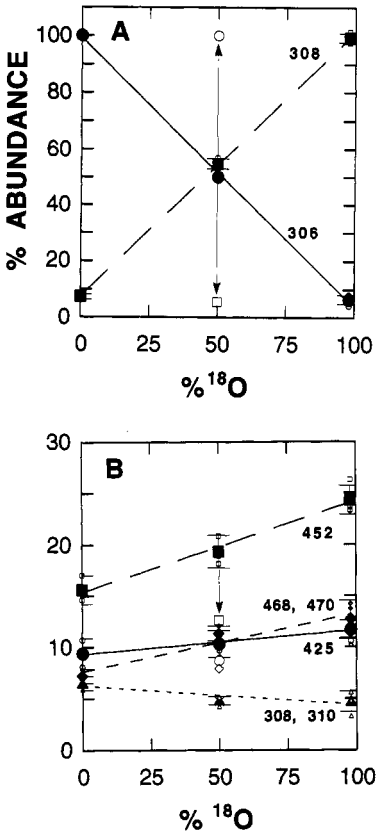

FIGURE 4: Incorporation of ^{18}O by carboxylase into vitamin K epoxide as determined by electron ionization positive ion mass spectrometry and supercritical fluid chromatography–negative ion chemical ionization mass spectrometry. The abundance of each vitamin K epoxide molecular species or fragment as a percentage of the amount of the reference peak (most abundant peak) is plotted as a function of the percent $^{18}O_2$ included in the carboxylation reaction mixture. In certain cases, the mass of the reference peak of a particular species and its isotopes changed depending on the percent ^{18}O included in the reaction mixture. (A) Average percent abundance of the 306 peak, (●) and its 308 (M + 2) isomer (■) relative to the reference peak at three different percent ^{18}O amounts in the reaction mixtures. The reference peak used at 0% $^{18}O_2$ was the 306 peak; otherwise the reference peak was the 308 peak. The lines represent the least-squares fit of the data; the error bars encompass 2 standard errors (95.5% confidence interval). The slope of these lines is equal to the mol atoms of ^{18}O incorporated for each fragment. The small ○ and □ symbols are the individual data points (n = 3, 4 for each percent ^{18}O). The large ○ and □ symbols are the percent abundance of the 306 and 308 peaks, respectively, at 50% $H_2{}^{18}O$ abundance. (B) The (M + 2) peaks, 308/310 (▲), 425 (●), and 452 (■), and the (M + 4) peak, 468/470 (◆), are normalized to their respective (M + 1) peak intensities and analyzed as in (A). The 425, 452, and 468/470 species contain the quinone oxygens of vitamin K epoxide; the 308/310 species does not and thus serves as a control. The large □, ○, ◇, and × symbols are the average values (n = 4) for the 452, 425, 468/470, and 308/310 data at 50% $H_2{}^{18}O$, respectively. The reference peaks used for the 306/308 data are as indicated above for panel A. The reference peaks used for the 425 and 452 data were the 423 and 450 peaks, respectively. The reference peak used for the 468/470 data obtained at 0% $^{18}O_2$ was the 466 peak; otherwise, at higher percent ^{18}O values, the 468 peak was used.

units (Figure 5). The theoretical natural abundance of (M + 1)/M for this data (M = 450, 466) is 35.3. At values >1.8 × 10^7 ion current units the (M + 1)/M ratio increased proportionately with M intensity. Thus, data normalized to M included only those data with intensities ≤1.8 × 10^7 ion current units where the (M + 1)/M ratio was constant. For purposes of comparison, all of the EI-MS intensities for each species and its isotopes were normalized to the respective (M + 1) peak. Assuming that the percent increase in intensity for each isomer was identical for all isotopes of that molecular

Table I: Incorporation of ^{18}O into Vitamin K Epoxide by Partially Purified Carboxylase As Analyzed by Electron Ionization Positive Ion Mass Spectrometry and Supercritical Fluid Chromatography–Negative Ion Chemical Ionization Mass Spectrometry

molecular species	fragment (m/z)	type of oxygen[a]	data normalized to[b]	mol atoms of ^{18}O incorp from $^{18}O_2$[c]	mol atoms of ^{18}O incorp from $H_2{}^{18}O$[c]
			Electron Ionization MS		
M	306	E	M	−0.954 ± 0.020	0.000
			M + 1	−0.837 ± 0.039	−0.051 ± 0.027
M	466	E	M	−0.985 ± 0.005	0.000
			M + 1	−0.832 ± 0.123	−0.050 ± 0.229
M + 2	308	E	M	0.938 ± 0.027	−0.019 ± 0.010
			M + 1	0.885 ± 0.083	−0.019 ± 0.008
M + 2	468	E	M	0.985 ± 0.023	0.011 ± 0.022
			M + 1	0.954 ± 0.070	0.007 ± 0.009
M + 2	310[d]	E	M	−0.024 ± 0.023	−0.018 ± 0.010
			M + 1	−0.018 ± 0.017	−0.018 ± 0.008
M + 2	425	Q	M	0.025 ± 0.028	−0.003 ± 0.030
			M + 1	0.023 ± 0.022	−0.007 ± 0.015
M + 2	452	Q	M		
			M + 1	0.089 ± 0.028	−0.030 ± 0.014
M + 4	470[d]	Q	M	0.051 ± 0.016	0.001
			M + 1	0.056 ± 0.026	0.007 ± 0.009
			Supercritical Fluid Chromatography–MS		
M	466	E	M[e]	−0.929 ± 0.021	0.000
M + 2	468	E	M[e]	0.923 ± 0.003	−0.014 ± 0.058
M + 4	470[d]	Q	M[e]	0.036 ± 0.024	−0.014 ± 0.058

[a] E = epoxide; Q = quinone oxygens of vitamin K epoxide. [b] Data normalized to M included only those data with M intensity $<1.8 \times 10^7$ ion current count, conditions under which (M + 1)/M remained constant as discussed in the text. When the data were normalized to (M + 1) all experimentally-derived data were included. [c] The number of mol atoms of ^{18}O incorporated into the various molecular species of KO and its fragments was calculated from the slope of the least-squares fitted line of relative abundance of each molecular species plotted against percent ^{18}O (for example, see Figure 4A). Negative values arise from negative slopes such as occurs when there is a loss of an isotopic peak *relative* to its abundance at 0% ^{18}O. Errors were derived from 2 standard errors of the mean (95.5% confidence interval) of the slopes of these lines. [d] Data obtained at 0% $^{18}O_2$ was for the (M + 2) peaks since the (M + 4) peaks at higher ^{18}O amounts are actually 2 mass units higher than the most abundant species. [e] Since (M + 1)/M remained nearly constant in all experiments (35.7 ± 1.2), it was unnecessary to make the additional normalization to (M + 1).

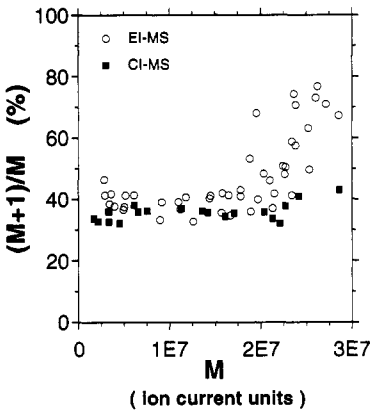

FIGURE 5: Relative abundance of the (M + 1) peak to the M peak as a function of the intensity (ion current units) of the M peak for both the EI-MS and SFC-NICI-MS (CI-MS) data. The ion current units are relative values based on the particular operating parameters of the mass spectrometer and are directly proportional to the amount of the sample measured. The EI data (○) includes all 450 and 466 data points (n = 45). The CI data (■) are of the parent species (M = 466). The theoretical natural abundance of (M + 1)/M was 35.3 for these molecules. All EI and CI mass spectra were collected on a VG ZAB2-SE-FPD instrument operated at 8 kV.

species, one would expect that the data normalized to (M + 1) (all values) would agree with the data normalized to M ($\leq 1.8 \times 10^7$ ion current units). This was indeed the case. Data normalized to either M or (M + 1) are compared in Table I and the values determined by each method agreed within 3–15% of each other.

The second mass spectral method, SFC-NICI-MS, was then employed in order to independently test the validity of the above analysis. In SFC-NICI-MS the signal is also concentration dependent, but the (M + 1) signal was not artifically increased due to the concentration of the sample (Figure 5). The (M + 1)/M ratio remained nearly constant over the entire range of intensities at 35.7 ± 1.2 (n = 20), which is the expected natural abundance within error for the 466 species. The mol atoms of ^{18}O incorporated into both the epoxide and quinone oxygens was the same within error as determined by EI-MS (Table I). Given the consistency of the ^{18}O incorporation results derived by the two methods, we are highly confident that we measured a real, albeit small, amount of ^{18}O exchange at the quinone oxygen. Thus, for every mole of molecular oxygen consumed in one vitamin K dependent turnover by carboxylase, approximately 0.95 mol of oxygen is incorporated into the epoxide oxygen position concomitant with approximately 0.05 mol of oxygen into the quinone oxygen position, with the remaining ca. 1.0 mol of oxygen presumably incorporated into H_2O.

DISCUSSION

Implications of the Mass Spectral Data for the Carboxylase Reaction. In Dowd's mechanism, $^{18}O_2$ either can add to the C3 position of dihydrovitamin K_1 and proceed down path A (Figure 2) or can add to the C1 position and follow path B. The hydroperoxy anion formed by A_1, pK_a of ~13, is the putative base that abstracts the *pro-S* γ-glutamyl proton from glutamate in the coupled carboxylation/oxygenation reaction. The neutral peroxy adduct thus formed is converted to the epoxide with complete loss of one ^{18}O atom to form $H_2{}^{18}O$ and ^{18}O-vitamin K epoxide giving a KO (M + 2) peak which can be quantitated by mass spectrometry. In the basicity enhancement pathway B, the $^{18}O_2$ forms a peroxyanion adduct to C1 which then is converted to the dioxetane intermediate by path B_2. The dioxetane then rearranges to form the alkoxide which has a pK_a of ~20. This base, which can abstract the *pro-S* γ-glutamyl proton from glutamate, has an enhanced basicity of 7 pK_a units. The neutralized alkoxide [1,1-dihydroxyvitamin K (2*R*,3*S*)-epoxide] can dehydrate by either path B_5 or B_6 yielding doubly labeled ^{18}O-vitamin K

epoxide (M + 4) or singly labeled ^{18}O-vitamin K epoxide (M + 2) and a molecule of $H_2{}^{18}O$.

What conclusions can now be made given these ^{18}O incorporation data, and importantly, what are the limitations of these conclusions? The first issue to be addressed is the extent of uncoupled KO/Gla formation occurring during the reactions. Although reaction conditions were carefully chosen which limited the amount of uncoupled KO formation it is certainly possible that the 0.05 mol atoms of ^{18}O incorporated into the quinone oxygen formed entirely as a result of uncoupled KO formation. For instance, the enzyme reaction could proceed down path B_1–B_3 to form the alkoxide intermediate which could abstract a proton from solvent rather than from the γ-methylene of glutamate. The 1,1-dihydroxyvitamin K (2*R*,3*S*)-epoxide thus formed could then proceed by path B_5 forming the KO product with its quinone oxygen derived exclusively from $^{18}O_2$. The remaining 95% of the flux of overall reaction would then proceed through a separate coupled γ-glutamyl proton abstraction reaction via path A_1–A_3 or another O_2-dependent coupled reaction mechanism yielding KO product with 0.95 mol atoms of unexchanged ^{16}O at the quinone oxygen position. Any number of complex branching reaction schemes can be generated which are consistent with the 0.05 mol atoms of ^{18}O-quinone oxygen being incorporated via a minor side reaction not involved in glutamate proton abstraction. It seems improbable, however, that two such different paths of O_2 activation would be occurring and in particular leaves unsolved the dilemma of the essential role of O_2 activation in the overall γ-carboxylation reaction.

A much simpler and more optimistic interpretation of the present data in favor of Dowd's model chemistry is that after the tertiary alkoxide abstracts the γ-glutamyl proton from glutamate, it stereospecifically dehydrates at a ratio of 0.95:0.05 in favor of the labeled oxygen (path B_6 versus B_5), thus leaving 0.05 mol atoms of residual ^{18}O at the 1-oxo position. As Dowd et al. (1991) have pointed out, such stereospecific loss (90%) of ^{18}O from (*R*)- and (*S*)-[1-^{18}O]propane-1,2-diol is observed in the analogous coenzyme B_{12} dependent diol dehydrase reaction (Retey et al., 1966). Furthermore, it is conceivable that the tertiary alkoxide stereospecifically ejects $^{18}OH^-$ into the active site which could then abstract the γ-glutamyl proton, though this must also be done stereospecifically.

The critical issue, then, is that any proposed enzyme mechanism which would account for the appearance of ^{18}O at the quinone oxygen *must* proceed via a common 1,1-dihydroxy-(or alkoxide) vitamin K (2*R*,3*S*)-epoxide intermediate irrespective of the degree of uncoupled KO formation. For instance, if the alkoxide generated by path B_3 fails to abstract a glutamate γ-methylene proton and instead is protonated by solvent, as would happen in an uncoupled reaction, the ^{18}O-quinone-enriched KO product must necessarily have proceeded through path B_1–B_5 or at least through the alkoxide/diol intermediate. These data indicate that carboxylase must be able to form the alkoxide/1,1-dihydroxyvitamin K epoxide intermediate independent of any evidence that this occurs along a coupled but distinct pathway to formation of a Gla coproduct. Thus, the experiments presented here do not focus on the extent of uncoupled carboxylation in the overall enzyme reaction. Instead, they deal directly with the fate of the 2 oxygens from molecular oxygen and the likely adducts formed with vitamin K which are excellent candidates for the putative enzyme-generated base. It is this strong base which must perform the difficult C–H cleavage at the unactivated γ-methylene locus of glutamyl residues prior to carboxylation.

In their early experiments, Sadowski et al. (1977) unequivocally demonstrated that the epoxide oxygen of the vitamin K product derived from O_2. In these studies, they used crude microsomal preparations and analyzed the vitamin K epoxide products of reactions carried out in either 100% $^{16}O_2$ or 99% $^{18}O_2$ by EI-MS. A reanalysis of the Sadowski data was recently done by Dowd et al. (1991). They concluded that the 470 (M + 4) peak of vitamin K epoxide formed in the presence of 99% $^{18}O_2$ was enhanced 4-fold above natural abundance (6.6%), implying that 17% incorporation of ^{18}O into the quinone oxygen occurred by path B_1–B_5. Likewise, their analysis revealed that the 425 (M + 2) peak was also enhanced 4-fold above natural abundance (5.7%). Thus, if correct, these data would suggest that we have underestimated the flux of oxygen through path B_1–B_5 by 2–3-fold. Closer examination of the data would indicate otherwise. As discussed above, it is possible that these enhanced abundances are exaggerated by hydrogen radical recombination (eqs 1–3) incurred during electron ionization. Indeed, in the $^{16}O_2$ experiment the 467 (M + 1) and the 424 (M + 1) peak intensities are 40% and 39% of M, respectively. The expected natural abundancies are 35.3% and 33.0%, respectively. In the $^{18}O_2$ experiment the 469 (M + 1) peak has normal intensity (0.7% above natural abundance); however, the 423 (M + 1) peak is 10% above natural abundance. Clearly these EI-MS data suffer from the same limitations which we encountered and which we addressed by performing multiple experiments and by monitoring the dependence of intensity on sample concentration (i.e., Figure 5). In fact, some of our single EI-MS experimental results showed even higher apparent ^{18}O incorporation (25–30%) at elevated sample concentrations. We were also able to corroborate the EI-MS results using the recently available low energy SFC-NICI-MS technique. Nonetheless, the Sadowski data are qualitatively in agreement with our results, namely, that the flux of oxygen by path B_1–B_5 is small relative to the flux by path A or B_1–B_4, B_6.

Structural and Functional Similarities Between Carboxylase and the Dioxygenase Soybean Lipoxygenase 1. The carboxy-terminal portion of carboxylase shares approximately 19% sequence homology with the lipoxygenase 1 isozyme from soybean seeds over amino acid residues 468–666 of carboxylase (Wu et al., 1991). The implied structural similarities between the two enzymes may be quite significant since the lipoxygenase is a dioxygenase that converts 1,4-*cis*,*cis*-pentadiene-containing polyunsaturated fatty acids to the corresponding 1,3-*cis*,*trans*-conjugated monoperoxy fatty acids in the presence of O_2 (Vliegenthart & Veldink, 1982). The data presented here indicate that the vitamin K carboxylase can also function as an intramolecular dioxygenase. Lipoxygenase is an extremely efficient catalyst with k_{cat} values of 232 s^{-1} at 4 °C and utilizes an essential non-heme Fe (Egmond et al., 1977). One can easily envision a free radical mechanism in which the ferrous form of lipoxygenase complexes with O_2 [Enz-Fe^{2+} + O_2 → Enz-$(Fe\text{-}O_2^{\bullet})^{2+}$] which can then effect hydrogen abstraction. It will be interesting to see if pure carboxylase does in fact contain stoichiometric amounts of a redox-active transition metal.

Conclusions. The novel 5% incorporation of *both* atoms of O_2 is experimentally significant considering the intrinsic nonlability of a quinone oxygen under physiological conditions. These experimental findings put real constraints on various aspects of mechanism, including the particular mode of activation of dioxygen to form a potentially strong base, and the role of vitamin KH_2. Although we have not unequivocally demonstrated that the incorporation of 0.05 mol atoms of ^{18}O

at the quinone oxygen position occurs during the coupled KO/Gla formation, *any* O_2-dependent enzyme-catalyzed ^{18}O incorporation is indicative of an unusual oxygen transfer which is entirely consistent with Dowd's recently proposed mechanism (path B_1–B_5). Thus, the chemistry of the dioxygen activation is perhaps a more fundamental catalytic aspect of the enzyme's machinery and the CO_2 fixation is a late stage reaction-terminating consequence from a point of view of chemistry. From a physiological and hematological standpoint, the carboxylation outcome is the crucial productive event. If the carboxylation does proceed by a glutamate carbanion mechanism, it seems likely, given our dioxygenation data, that the carbanion is formed by the kind of oxygen-driven chemistry shown in Figure 2.

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