Biotin-Dependent Carboxylation Catalyzed by Transcarboxylase Is a Stepwise Process†

Stephen J. O'Keefe and Jeremy R. Knowles*
Department of Chemistry, Harvard University, Cambridge, Massachusetts 02138
Received March 4, 1986; Revised Manuscript Received June 13, 1986

ABSTRACT: To investigate the mechanism of the carboxylation of pyruvate to oxalacetate catalyzed by the enzyme transcarboxylase, we have measured the $^{D}(V/K)$ and $^{13}(V/K)$ isotope effects. Comparison of the double-reciprocal plots of the initial velocities with [$^{1}H_3$]pyruvate and with [$^{2}H_3$]pyruvate as substrate yields a deuterium isotope effect on V_{max}/K_m of 1.39 ± 0.04. The ^{13}C kinetic isotope effect on the carboxylation of pyruvate to oxalacetate has been measured by the competitive method and is 1.0227 ± 0.0008. To determine whether the removal of the proton from pyruvate and the addition of the carboxyl group occur in the same or in different steps, the double-isotope fractionation test has been used. When [$^{2}H_3$]pyruvate replaces [$^{1}H_3$]pyruvate as the substrate, the observed $^{13}(V/K)$ isotope effect falls from 1.0227 to 1.0141 ± 0.001. The latter value is in excellent agreement with the value of 1.0136, predicted for a stepwise pathway. We may conclude, therefore, that the carboxylation of pyruvate catalyzed by transcarboxylase proceeds by a stepwise mechanism involving the intermediate formation of the substrate carbanion.

There are seven known biotin-dependent carboxylases that are responsible for a variety of carboxylation reactions in metabolism (Moss & Lane, 1971; Wood & Barden, 1977). The overall carboxylations are comprised of two half-reactions. In most instances, the first partial reaction is the ATP-dependent carboxylation of biotin by HCO_3^- to form the N^1-carboxybiotin intermediate and the second partial reaction involves the transfer of the carboxyl group from the intermediate carboxybiotin to the substrate

$$\text{ATP} + HCO_3^- + \text{enz–biotin} \rightleftharpoons \text{ADP} + P_i + \text{enz–biotin–}CO_2^-$$

$$\text{enz–biotin–}CO_2^- + \text{substrate} \rightleftharpoons \text{enz–biotin} + \text{product}$$

Evidence that these half-reactions are independent has come from the isolation of the carboxybiotinyl–enzyme intermediate (Kaziro & Ochoa, 1961; Wood et al., 1963a) and from the observation of the expected isotopic exchange reactions (Halenz & Lane, 1961; Scrutton et al., 1965; Northrop & Wood, 1969). Steady-state kinetic analysis (Northrop, 1969; Barden et al., 1972) has shown that some biotin-containing enzymes proceed via a ping-pong mechanism. The separation and purification of the three subunits of acetyl–CoA carboxylase (Guchait et al., 1974a; Polakis et al., 1974) allowed the direct demonstration of the existence of different active sites for the two half-reactions. The biotin carboxylase subunit catalyzes the carboxylation of biotin bound to its carrier protein, and the carboxyltransferase subunit catalyzes the carboxylation of acetyl-CoA by the carboxylated biotin-carrier protein.

To discover the nature of the carboxybiotin intermediate, Lynen et al. (1959; 1961) exploited the fact that 3-methylcrotonyl-CoA carboxylase can use free biotin as substrate in the first half-reaction. The carboxylated intermediate was methylated with diazomethane and identified as N^1-(methoxycarbonyl)-D-biotin methyl ester (Knappe et al., 1961), suggesting that the actual intermediate was N^1-carboxybiotin. While model studies (Caplow, 1965; Caplow & Yager, 1967) led to the suggestion (Bruice & Hegarty, 1970) that enzyme-catalyzed carboxylation occurs at the ureido *oxygen* of biotin, the elegant studies of Guchait et al. (1974b) confirmed the identity of the intermediate as N^1-carboxybiotin.

The stereochemical course of the reaction catalyzed by propionyl-CoA carboxylase was found to proceed with retention of configuration at the carbon center undergoing carboxylation (Rêtey & Lynen, 1965), and this stereochemical result prompted the proposal of a six-electron electrocyclic transition state for the carboxylation reaction (Figure 1A). All biotin-dependent carboxylases subsequently examined have been shown also to proceed with retention of stereochemistry (Rose 1970; Cheung et al., 1975). In further support of the concerted mechanism, Prescott & Rabinowitz (1968) found that propionyl-CoA did not incorporate ^{3}H from solvent without overall turnover of the enzyme and enzymatically synthesized [2-^{3}H]propionyl-CoA did not lose the isotopic label unless all substrates were present. Indeed, the rate of tritium washout was equal to the carboxylation turnover rate. Analogous findings have been reported by Mildvan et al. (1966) with pyruvate carboxylase and by Cheung et al. (1975) with transcarboxylase.

To examine the concertedness of biotin-dependent carboxylations, the reaction of β-fluoropropionyl-CoA with propionyl-CoA carboxylase and with transcarboxylase was examined (Stubbe & Abeles, 1977; Stubbe et al., 1980). These enzymes catalyze the elimination of HF from the fluorinated substrate to form acrylyl-CoA. Yet this elimination of HF occurs at the same rate as ATP hydrolysis for propionyl-CoA carboxylase and at the same rate as oxalacetate decarboxylation for transcarboxylase, which is precisely what would be expected if proton removal and carboxylation were coupled processes. Nevertheless, the balance of the evidence led Abeles and his group to prefer a stepwise pathway in which a substrate proton is abstracted to generate an enolate anion that in a second step attacks the carboxyl group of N^1-carboxybiotin (Figure 1B). Earlier, indeed, Sauer et al. (1975) had proposed a stepwise mechanism for biotin-dependent carboxylations in which enzyme-bound CO_2 generated from N^1-carboxybiotin was attacked by the substrate carbanion (Figure 1C).

In a more direct test of the substrate carbanion as a discrete intermediate in these reactions, Kuo & Rose (1982) examined

† Supported by the National Science Foundation.

0006-2960/86/0425-6077$01.50/0

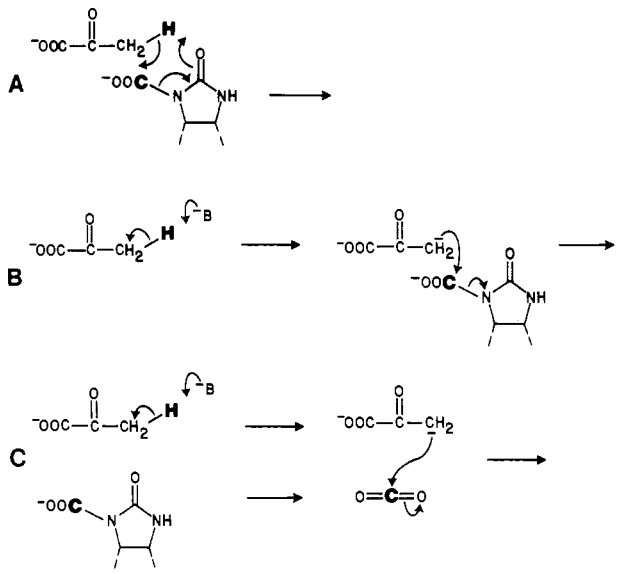

FIGURE 1: Pathways for biotin-dependent carboxylations. The carbon and the hydrogen directly involved in the isotope effects are shown in heavy type.

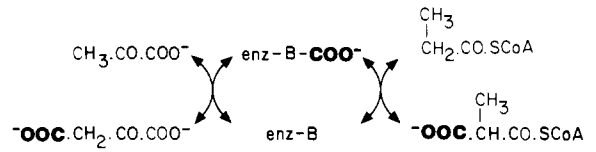

FIGURE 2: Reaction catalyzed by transcarboxylase. The enzyme is enz and biotin is B.

exogenous enolpyruvate, a presumed intermediate in the stepwise pathway for transcarboxylase, as a substrate for this enzyme. It was shown that transcarboxylase catalyzes the stereospecific ketonization of enolpyruvate but only in the presence of methylmalonyl-CoA. The stereochemical results indicated, however, that a negligible fraction of the product malate was formed by direct carboxylation of enolpyruvate. Thus although enolpyruvate appears to be a substrate for transcarboxylase in reprotonation, it is not carboxylated. This is a disconcerting result because earlier experiments had shown that proton removal from pyruvate is at least partially rate-limiting (Cheung et al., 1975; Rose et al., 1976), and a true enolpyruvate intermediate should partition both back to pyruvate *and* forward to oxalacetate.

In the light of these uncertainities, we have chosen to examine the question of concertedness in the reaction catalyzed by transcarboxylase by the double-isotope fractionation method (Hermes et al., 1982, 1984a,b; Belasco et al., 1983). Transcarboxylase from *Propionibacterium shermanii* (Swick & Wood, 1960) catalyzes the reversible exchange of a carboxyl group from *S*-methylmalonyl-CoA to pyruvate to produce propionyl-CoA and oxalacetate (Figure 2) and is unique among biotin-containing enzymes in that the carboxylations are not driven by ATP and HCO_3^-. The kinetic mechanism of the enzyme is two-site ping-pong (Northrop, 1969) and the reaction has an overall equilibrium constant of about 1 (Wood et al., 1969).

We report here the determination of the $^{13}(V/K)$ isotope effect[1] on the reaction catalyzed by transcarboxylase using either [$^1H_3$]pyruvate or [$^2H_3$]pyruvate and the value of the $^D(V/K)$ isotope effect for these substrates under the same conditions. These data allow the application of the double-isotope fractionation method (Hermes et al., 1982, 1984a,b; Belasco et al., 1983) as a test for concertedness in this reaction.

Experimental Procedures

Materials. Avidin, CoA,[2] dithiothreitol, Dowex-50 (H^+ form), DTNB, EDTA, Hepes, (*RS*)-methylmalonyl-CoA, NADH, $NADP^+$, Pipes, PMSF, and sodium pyruvate were from Sigma Chemical Co. (St. Louis, MO). [1-^{14}C]Acetic anhydride (20 mCi/mmol) was from Amersham Corp. (Chicago, IL). *n*-Butyllithium in hexane was from Alfa Products (Danvers, MA). Cellulose plates with fluorescent indicator (0.16 mm, 20 cm × 20 cm) were from Kodak (Rochester, NY). AG1-X8 (acetate form, 200–400 mesh), Bio-Gel A1.5m (100–200 mesh), and Amberlite IR120 (H^+) were from Bio-Rad Corp. (Rockville Center, NY). D_2O (99.8 atom %) was from Merck and Co. (Rahway, NJ). All other chemicals and solvents were of commercial reagent grade or better.

Citrate synthase (porcine heart), glutathione reductase (yeast), lactate dehydrogenase in 50% glycerol (rabbit muscle), malate dehydrogenase in 50% glycerol (porcine heart), and malic enzyme (chicken liver) were from Sigma Chemical Co. (St. Louis, MO).

Propionibacterium freundreichii (*Propionibacterium shermanii* ATCC 9615) was obtained from the American Type Culture Collection (Rockville, MD) and grown according to Leadlay (1981). Transcarboxylase was isolated by a modification of Wood et al. (1977). After $(NH_4)_2SO_4$ precipitation, the protein pellet was taken up in a minimum of 0.2 M sodium acetate buffer, pH 5.5, containing EDTA (0.1 mM), PMSF (0.1 mM), and NaN_3 (0.02% (w/v)) and loaded on a Bio-Gel A1.5m column (4.5 × 90 cm) equilibrated with the above buffer. The column was eluted with the above buffer, and fractions containing transcarboxylase having a specific catalytic activity greater than 10 U/mg were concentrated with an Amicon PM-10 membrane (76 mm, nominal M_r rejection 10000) in an Amicon series 80 ultrafiltration cell. The concentrated enzyme solution was rechromatographed on the same Bio-Gel column as described above. The final enzyme preparation had a specific catalytic activity of 17 U/mg when isolated and was stored at −70 °C until use. At the time of the kinetic experiments the specific catalytic activity had fallen to 10 U/mg. Unless otherwise noted, the activity of transcarboxylase was measured by following the procedure of Wood et al. (1969). The enzyme is completely specific for pyruvate and oxalacetate but is slightly promiscuous for the CoA thioester. Malonyl-CoA is reported to react at 50–100% of the V_{max} determined with (*RS*)-methylmalonyl-CoA (Wood et al., 1963b; Hoving et al., 1985). Assays containing malonyl-CoA (1mM) in place of (*RS*)-methylmalonyl-CoA were performed at 30 °C.

Acetyl-CoA was synthesized according to Simon & Shemin (1953). [1-^{14}C]Acetyl-CoA was purified according to Sedgwick & Cornforth (1977). A portion of the labeled sample was completely converted by citrate synthase to [^{14}C]citrate, and the reaction was followed at 412 nm by removal of CoA

[1] The nomenclature used is that of Northrop (1977), in which isotope effects on kinetic or thermodynamic parameters are defined by leading superscripts. Thus, 13 and D refer to ^{13}C and ^{2}H, respectively. When necessary following subscripts are used. For example, $^{13}(V/K)_D$ is the ^{13}C isotope effect on V_{max}/K_m with [$^2H_3$]pyruvate as the substrate.

[2] Abbreviations: CoA, coenzyme A; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid; NADH, nicotinamide adenine dinucleotide, reduced form; $NADP^+$, nicotinamide adenine dinucleotide phosphate; Pipes, 1,4-piperazinediethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; TLC, thin-layer chromatography.

with DTNB. The conversion mixture was added to 3 mM HCl (100 mL) containing acetyl-CoA (1 μmol) and the solution was applied to a DEAE–cellulose column (1.5 × 15 cm). The column was eluted with a linear gradient (100 mL + 100 mL, 0–0.1 M) of LiCl in 3 mM HCl. No radioactive contaminant was detected in the acetyl-CoA peak. The samples of [$^1H_3$]pyruvate and [$^2H_3$]pyruvate used in the determination of the deuterium isotope effect were synthesized according to Cook et al. (1980) and stored frozen in H_2O or D_2O (as appropriate) at −70 °C.

Thiophenylmalonic acid was synthesized by a modification of the procedure of Brooks (1978). A solution (2.7 M) of *n*-butyllithium in hexane (14.7 mL) was added to a solution of diisopropylamine (5.6 mL, 0.04 mol) in tetrahydrofuran (70 mL) at −40 °C under N_2. After 15 min, the resulting solution of lithium diisopropylamide was cooled to −78 °C and a solution of thiophenyl acetate (0.03 mol) in tetrahydrofuran (5 mL) was added to the reaction mixture over 90 min. Dry ice (20 g) was then added to the mixture and the solution was allowed to warm to 0 °C. The reaction mixture at 0 °C was added to aqueous 1 M $NaHSO_4$ (75 mL) at 0 °C and the product was extracted into $CHCl_3$ (3 × 75 mL). The solvent was removed under reduced pressure and the residue was redissolved in $CHCl_3$ (30 mL). The organic layer was extracted into saturated aqueous $NaHCO_3$ (2 × 25 mL) at 0 °C. This extract was immediately acidified to pH 2.5 with 6 N HCl at 0 °C and the product reextracted into $CHCl_3$ (3 × 25 mL). The chloroform extracts were washed with brine and dried over Na_2SO_4, and the solvent was then removed under reduced pressure.

The resulting solid residue was dissolved in a minimum of $CHCl_3$ and the solution treated with charcoal. The solution was filtered through Celite, hexane was added, and the solution was cooled to 0 °C until crystallization began and then placed in a bath at −78 °C. The resulting crystals were filtered, washed with cold hexane, and dried overnight in a vacuum desiccator: yield, 30%, based on thiophenyl acetate; mp 73–75 °C; proton NMR ($CDCl_3$) δ 9.90 (br s), 7.44 (s), 3.72 (s). Literature values: mp 72–73 °C; proton NMR ($CDCl_3$) δ 10.73 (s), 7.42 (s), 3.70 (s) (Wilson & Hess, 1974). The signal of the carboxylic acid proton at δ 9.90 is concentration dependent.

Malonyl-coenzyme A was synthesized by a modification of the procedure of Vagelos (1963). Thiophenyl malonate (1.2 mmol) was added to a solution (24 mL, 40 mM) of CoA in 0.2 M $KHCO_3$, pH 8.0, at room temperature, and N_2 was bubbled through the mixture. After 60 min, if the nitroprusside test (Toennies & Kolb, 1951) was positive, a second equivalent of thiophenyl malonate was added. After a further 30 min, the reaction mixture was once again tested with the nitroprusside reagent. During the reaction, the pH was maintained at about pH 7.5 by the addition of solid K_2CO_3. When the nitroprusside test was negative, the reaction mixture was acidified to pH 2–3 with Dowex-50 (H^+). The Dowex-50 resin was removed by filtration and washed with H_2O. The combined filtrates were washed with ether (3 × 20 mL) and then with $CHCl_3$ (3 × 20 mL). The solution was stored frozen at −70 °C. The malonyl-CoA synthesized in this manner contained no detectable thiol or acetyl-CoA contaminant.

Methods. Ultraviolet–visible absorption measurements were made on a Perkin-Elmer 554 spectrophotometer. Scintillation counting was performed on a Beckman LS 1801 instrument.

Determination of Substrate Concentrations. Pyruvate concentrations were determined according to Bergmeyer (1974). Malate concentrations were determined according to Hermes et al. (1982) except that 5 mM $NADP^+$ was substituted for 3-acetylpyridine adenine dinucleotide phosphate. If the malate solution contained inorganic phosphate, then a solution of malic enzyme that had been dialyzed against 50 mM Pipes, pH 7.0, containing dithiothreitol (1 mM) and glycerol (50% v/v) (Grissom & Cleland, 1985) was used in the assay. Malonyl-CoA was assayed with transcarboxylase and pyruvate (10 mM) in 75 mM Hepes buffer, pH 8.0. Acetyl-CoA was assayed with citrate synthase, according to Tubbs and Garland (1969).

Thioester Exchange Reaction. The reaction mixture (0.4 mL) in an optical cuvette contained [1-^{14}C]acetyl-CoA (2.4 μmol), malonyl-CoA (2.4 μmol), 0.3 M potassium phosphate buffer, pH 6.9, and malate dehydrogenase (3.8 U). The exchange reaction was initiated by the addition of transcarboxylase (0.04 U). The turnover rate under these conditions was measured by the addition of pyruvate (0.064 μmol) to the reaction mixture and recording the decrease in A_{340nm}.

To follow the exchange reaction, portions of the reaction mixture (5 μL) were quenched into an avidin solution (50 μL of a solution of 1.1 mg/mL, 4 °C) at 10-min intervals. After 4 min at 4 °C, the quenched samples were acidified with acetic acid (10 μL of a solution of 40% v/v). The acidified samples were concentrated to dryness in a rotary concentrator and dissolved in H_2O (5 μL), and the solutions were applied to cellulose TLC plates (4 × 20 cm). The plates were developed in butanol:acetic acid:H_2O:triethylamine:58% NH_3(w/v) (50:20:24:4:2 by volume). After the TLC plates dried, they were cut into 0.5- or 1-cm strips and each strip was placed in a plastic scintillation vial. Ready-Solv scintillation cocktail was added to each vial, and the radioactivity was measured.

Determination of the Deuterium Isotope Effect. The [$^1H_3$]- and [$^2H_3$]pyruvate samples were synthesized in identical fashion except H_2O was substituted for D_2O in the appropriate reaction. The [$^2H_3$]pyruvate was stored in D_2O until addition to the assay mixture to prevent nonenzymic washout of the label. To correct for any solvent isotope effect, all reaction mixtures were brought to 10% (v/v) D_2O. Reaction mixtures comprised assay buffer (0.8 mL of 0.44 M potassium phosphate buffer, pH 6.9, containing malonyl-CoA (1.2 mM), NADH (0.3 mM), and malate dehydrogenase (20 units) and appropriate amounts of H_2O and D_2O. The pyruvate sample (5–50 μL) was added next to last, to a final volume of 1 mL. The cuvettes (1-cm pathlength) were incubated at 30 °C for 4 min, and the reaction was initiated by the addition of transcarboxylase (10 μL of a solution of 1.7 U/mL). Under the conditions of the assay, the nonenzymatic washout of the deuterium label was negligible. The initial reaction velocities were determined by monitoring the decrease in A_{340nm} with time and correcting for the nonenzymatic decrease that had been measured before the addition of enzyme. V/K isotope effects were determined by comparing the slopes of double-reciprocal plots.

Determination of $^{13}(V/K)_{pyruvate}$. The ^{13}C isotope effects on the carboxylation of pyruvate were determined by the method of O'Leary (1980) using the natural abundance of ^{13}C in the carboxyl group of malonyl-CoA. In a typical reaction mixture, malonyl-CoA (250 μmol) and acetyl-CoA (250 μmol) were incubated with transcarboxylase (20 U) at 30 °C in 0.35 M potassium phosphate buffer, pH 6.8 (41.5 mL). The reaction was initiated by the addition of pyruvate (6 μmol), and 1 μmol was added at each subsequent minute for 44 min. The product oxalacetate was converted to malate in situ with NADH (125 μmol) and malate dehydrogenase (500 units). The reaction was followed by monitoring the change in A_{340nm}.

At the end of the reaction time period, the mixture was quenched by rapidly passing the solution through an Amicon PM-10 membrane to remove the enzymes. The extent of reaction was determined by measuring the concentrations of malonyl-CoA and of malate in the quenched reaction mixture. For reactions that were taken to completion, a tenfold excess of pyruvate over malonyl-CoA was used, and acetyl-CoA was omitted from the reaction mixture.

After ultrafiltration, inorganic phosphate was removed from the reaction mixture by precipitation with LiOH (14.5 mmol) and filtration under vacuum. Excess Li^+ was then adsorbed on a column (1.5 × 15 cm) of Amberlite IR-120(H^+), which was washed with water (100 mL). From the combined eluant, malate was isolated according to O'Leary et al. (1981). The pH of the column eluant was adjusted to pH 7 and applied to a column (1.5 × 15 cm) of AG1X8 (formate). After the column was washed with water (50 mL), it was developed with a linear gradient of formic acid (300 mL × 300 mL, 0–6 M) and fractions (20 mL) were collected. Each fraction was evaporated to dryness under reduced pressure, and the residue was taken up in a small amount of water. Fractions that contained malate were pooled. To the pooled fractions, 0.4 M Hepes buffer, pH 8.0 (2 mL), was added, and the volume was adjusted to 20 mL with water. This solution was treated with charcoal (1 g), which was then removed by filtration through Celite. The resulting filtrate was then evaporated to dryness under reduced pressure. Typical recoveries of malate were 80–95%.

The purified malate was decarboxylated by the procedure of Hermes et al. (1982). The solid residue, obtained as described above, was dissolved in 0.4 M Hepes buffer, pH 8.0 (4 mL), and added to a solution (5 mL) containing $Mg(OAc)_2$ (10 mM), oxidized glutathione (50 mM), NADH (25 mM), and dithiothreitol (0.4 mM) as described by O'Leary (1980), except that a smaller reaction vessel of 50 mL was used. The reaction mixture was sparged overnight with CO_2-free N_2. A dialysis bag containing malic enzyme (20 U) and lactate dehydrogenase (40 U) in 0.4 M Hepes, pH 8.0 (0.3 mL), was placed in the reaction vessel, and the sparging was continued for 2 h. After the vessel was sealed the dialysis bag was punctured with the syringe needle used for sparging, and the reaction was initiated by the addition of glutathione reductase (30 U) and $NADP^+$ (1 μmol). The reaction was quenched after 18 h by the addition of concentrated H_2SO_4 (0.45 mL). The resulting CO_2 was isolated on a high-vacuum line by a modification of the procedure of O'Leary (1980). After several freeze–evacuate–thaw cycles to remove N_2, the CO_2 was isolated under passive vacuum without freezing the solution. Assay of the spent reaction mixture showed that, using the above procedure, all decarboxylations were more than 99.6% complete. The ^{13}C:^{12}C ratio in the collected CO_2, corrected for ^{17}O content (Craig, 1957), was determined by Krueger Enterprises, Cambridge, MA, on an Isogas 903 isotope ratio mass spectrometer. Samples of malate deriving from the complete reaction of malonyl-CoA were treated identically.

Data Analysis. The velocity of the thioester exchange reaction was determined according to eq 1, where F is the fraction of isotopic equilibrium achieved, t is the time of the

$$v^* = -\frac{1}{t}\frac{[A][B]}{([A] + [B])} \ln (1 - F) \quad (1)$$

measurement, and A and B are malonyl-CoA and acetyl-CoA, respectively (Segel, 1975). F was determined for each time point as (dpm in malonyl-CoA)/(total dpm recovered), since the concentrations of the thioesters were equal.

The data from the determination of the deuterium isotope effect were first plotted to check the linearity of the double-reciprocal plots and then were fitted to eq 2 and 3 by the

$$v = VA/[(K + A)(1 + F_1E_{V/K})] \quad (2)$$

$$v = VA/[K(1 + F_1E_{V/K}) + A(1 + F_1E_V)] \quad (3)$$

least-squares method, using a BASIC translation of the appropriate Fortran program of Cleland (1979). Equation 2 assumes equal isotope effects on V and V/K, while eq 3 treats the isotope effects independently. F_1 is the fraction of deuterium label in the substrate and $E_{V/K}$ and E_V are the isotope effects minus one for V/K and V, respectively.

^{13}C isotope effects were determined with eq 4, where R_f is the ratio of ^{13}C to ^{12}C in CO_2 at the fraction of reaction f and R_0 is the ^{13}C:^{12}C mass ratio at $f = 1.0$.

$$^{13}(V/K) = \ln (1 - f)/\ln [1 - f(R_0/R_f)] \quad (4)$$

RESULTS

Kinetic Deuterium Isotope Effects on the Transcarboxylase Reaction. When [$^1H_3$]pyruvate and [$^2H_3$]pyruvate are varied at a fixed malonyl-CoA concentration, isotope effects (obtained by direct comparison of the initial velocities with deuterated and unlabeled substrates) are observed on both V_{max} (1.40 ± 0.07) and V_{max}/K_m (1.39 ± 0.04). Cheung et al. (1975) had previously measured the isotope effects on V_{max} and V_{max}/K_m with methylmalonyl-CoA as the cosubstrate and found a value of 2.1 for both. The discrepancies among these determinations may be attributed to differences in pH, buffer, solvent composition, and cosubstrate. For example, it is not clear whether the earlier workers maintained a constant amount of D_2O in reactions of both [$^1H_3$]pyruvate and of [$^2H_3$]pyruvate. The level of D_2O could cause discrepancies in the value of $^D(V/K)$, in light of the known solvent isotope effect in the reaction catalyzed by transcarboxylase (P. F. Leadlay, personal communication).

Velocity of the Thioester Exchange Reaction. The exchange rate between [^{14}C]acetyl-CoA and unlabeled malonyl-CoA was measured by quenching samples from the reaction mixture at 10-min intervals, separating the thioesters by TLC, and measuring the radioactivity in each thioester band. The velocity at each time point was calculated according to Segel (1975). The average rate for the exchange reaction was 1.037 μmol mL^{-1} min^{-1}. The velocity of the overall forward reaction was measured in the same reaction mixture. Pyruvate was added to a final concentration of 0.16 mM and the product of the reaction, oxalacetate, was converted in situ to malate with malate dehydrogenase and NADH. The decrease in A_{340nm} with time provides the velocity of the forward reaction. The measured reaction rate was 0.011 μmol mL^{-1} min^{-1}.

Since the rate at which acetyl-CoA is carboxylated to generate malonyl-CoA must be at least as fast as the rate of the thioester exchange reaction, and since, at the low pyruvate concentrations used, the rate of the overall reaction represents the rate of carboxylation of pyruvate, acetyl-CoA is carboxylated more than 10 times faster than pyruvate. The first half-reaction is therefore essentially at equilibrium, and the second half-reaction, the carboxylation of pyruvate, limits the rate of enzyme turnover. Thus, the ^{13}C isotope effect that is measured indeed relates to the carboxylation of pyruvate by carboxybiotin.

Kinetic ^{13}C Isotope Effects on the Transcarboxylase Reaction. Malonyl-CoA and carboxybiotin were brought to isotopic equilibrium in the presence of acetyl-CoA. Pyruvate

Table I: ^{13}C Isotope Effects on Pyruvate Carboxylation

substrate	extent of reaction[a] (%)	^{13}C:^{12}C ratio ($\times 10^5$) in CO_2[b] partial conversion[c]	complete conversion[d]	$^{13}(V/K)$[e]
[$^1H_3$]pyruvate	18.3	1021.44	1043.24	1.0233
	21.1	1023.58	1043.69	1.0222
	12.6	1022.68	1044.25	1.0226
				mean value 1.0227 ± 0.0008
[$^2H_3$]pyruvate	18.8	1031.67	1043.80	1.0131
	25.0	1031.78	1045.27	1.0151
				mean value 1.0141 ± 0.001

[a] Defined as 100[[L-malate]/{[L-malate] + [malonyl-CoA]}]. The concentrations of these reactants and products were determined after quenching the reaction. [b] Derived from the malate product. These ratios have been adjusted for the contribution of ^{17}O (Craig, 1957). [c] The ^{13}C:^{12}C ratio of C-4 of L-malate after reaction to the extent shown in the previous column. [d] Malonyl-CoA was allowed to react to completion to generate a sample of malate containing a carboxyl group with the same isotopic content as the starting thioester. [e] Calculated according to eq 3.

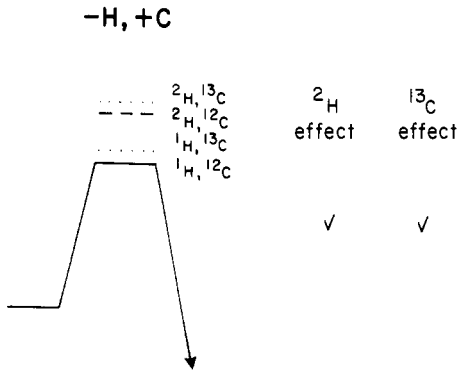

FIGURE 3: Free energy profile for a hypothetical concerted carboxylation reaction. Free energy differences deriving from isotopic substitution are shown in the transition state *only* for purposes of illustration.

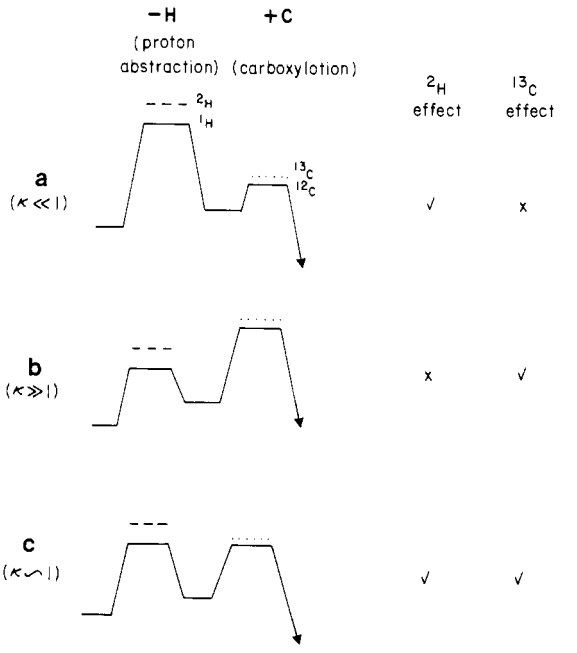

FIGURE 4: Free energy profiles for hypothetical stepwise carboxylation reactions. Free energy differences deriving from isotopic substitution are shown in the transition state *only* for purposes of illustration. Case a: proton abstraction is cleanly rate limiting. The carbanionic intermediate partitions completely to product ($\kappa \ll 1$), and only a ^2H isotope effect is observed. Case b: carboxyl transfer is cleanly rate limiting. The carbanionic intermediate is in preequilibrium with the substrate ($\kappa \gg 1$), and only a ^{13}C kinetic isotope effect is observed. Case c: proton abstraction and carboxyl transfer are each partially rate limiting. The partitioning of the intermediate is balanced ($\kappa \approx 1$). Upon deuteration of the substrate, the partitioning of the intermediate forward to product is increased. The change in the partitioning results in a decrease in the observed ^{13}C isotope effect.

was added slowly, and the product oxalacetate was converted irreversibly to malate in situ with malate dehydrogenase and NADH. Since pyruvate was added to the reaction mixture at the rate it was consumed, the concentration was never greater than 0.16 mM, and the first half-reaction was at equilibrium at all times. At the end of the reaction time period, the malate was isolated and then decarboxylated with malic enzyme according to Hermes et al. (1982). For determination of the isotopic content of the carboxyl group of the malonyl-CoA at time zero, 50 μmol of malonyl-CoA was *completely* converted to malate in the presence of a large excess of pyruvate.

The $^{13}(V/K)_H$ and $^{13}(V/K)_D$ isotope effects were determined by a comparison of the ^{13}C:^{12}C ratios of CO_2 according to eq 4. The results are summarized in Table I.

Discussion

The mechanism of the carboxylation reactions catalyzed by biotin-dependent carboxylases has been a controversial issue for many years. The first proposals (Rêtey & Lynen, 1965; Mildvan et al., 1966; Prescott & Rabinowitz, 1968) suggested that these reactions were concerted and involved the simultaneous cleavage of the carbon–hydrogen bond and formation of the new carbon–carbon bond. More recently, however, it has been argued (Stubbe & Abeles, 1977; Stubbe et al., 1980; Kuo & Rose, 1982) that a stepwise pathway via a carbanion intermediate is more consistent with the available evidence. Yet the data are not entirely without ambiguity, and we sought a method that would allow an unequivocal distinction between concerted and stepwise pathways to be made.

Double-Isotope Fractionation. In the case of the carboxylation reactions considered here, a carbon–hydrogen bond is broken and a carbon–carbon bond is formed. If the reaction were concerted (and provided that the rate-determining transition state was that involving the covalency changes), then the reaction would show both a ^2H and a ^{13}C primary kinetic isotope effect (Figure 3). If, however, the reaction were stepwise, with the proton abstraction step preceding the carboxyl-transfer step, then the reaction would show either one primary kinetic isotope effect [i.e., a ^2H effect (Figure 4a) or a ^{13}C effect (Figure 4b)] if the intermediate carbanion partitioned unevenly or both isotope effects if the carbanion partitioned evenly (Figure 4c). From Figures 3 and 4, it is clear that the existence of one isotope effect but not the other requires a stepwise reaction. Yet if both ^2H and ^{13}C kinetic isotope effects are observed, the reaction could be concerted (Figure 3) *or* "balanced" stepwise (Figure 4c). As is discussed below, the distinction between these possibilities can be made by investigating how the isotope effect on the second step is affected by changing the isotope in the first step. For a carboxylation reaction, this requires the determination of the ^{13}C isotope effect by using ^1H substrate and using ^2H substrate.

For a *stepwise* mechanism with two partially rate-determining transition states, an observed isotope effect may be expressed in terms of fractionation factors as $\phi_{gs}/\phi_{1,2}$ (Albery & Knowles, 1976), where ϕ_{gs} is the ground-state fractionation factor and $\phi_{1,2}$ is the "mixed" transition-state fractionation factor. The "mixed" transition-state fractionation factor (Belasco et al., 1983) is the weighted average of the individual fractionation factors for transition states 1 and 2. The weighting factor, κ, is the partition ratio, k_{-1}/k_2, of the intermediate that is flanked by the two transition states 1 and 2 for the isotopically *un*labeled species. On the basis that the isotopes fractionate independently of each other (Kresge, 1964; Hegarty & Jencks, 1975), four fractionation factors are required to describe the transition states for a stepwise carboxylation reaction. The fractionation factors $^{13}\phi_1$, $^{13}\phi_2$, $^{D}\phi_1$, and $^{D}\phi_2$ describe the fractionation of carbon and of hydrogen in transition states 1 and 2, respectively. With ^{1}H substrate, the mixed transition-state fractionation factor for carbon is

$$(^{13}\phi_{1,2})_H = \frac{1 + \kappa}{^{13}\phi_1^{-1} + \kappa\,^{13}\phi_2^{-1}} \quad (5)$$

Transition state 1 involves the removal of a proton from pyruvate, and since the carboxyl group is at rest (on the N^1 of biotin) in this transition state, $^{13}\phi_1$ will have a value close to 1. On the other hand, transition state 2 involves the carboxylation step in which carbon is in flight, and $^{13}\phi_2$ will be less than 1. Analogously, $^{D}\phi_1$ will be less than 1, since deuterium is in flight in transition state 1, whereas $^{D}\phi_2$ will be close to 1 (presuming that the abstracted proton is then bound to an oxygen or nitrogen base, see below) since the deuterium is at rest in transition state 2. For the "balanced" stepwise case, therefore, $^{13}\phi_{1,2}$ will be less than $^{13}\phi_{gs}$ and $^{D}\phi_{1,2}$ will be less than $^{D}\phi_{gs}$, these differences being expressed in observable ^{13}C and ^{2}H primary kinetic isotope effects. Each of the observed isotope effects [$^{D}\phi_{gs}/^{D}\phi_{1,2}$ and $^{13}\phi_{gs}/(^{13}\phi_{1,2})_H$] will be less than the *intrinsic* isotope effects ($^{D}\phi_{gs}/^{D}\phi_1$ and $^{13}\phi_{gs}/^{13}\phi_2$), of course, since neither of the isotopically sensitive transition states is cleanly rate limiting.

For a *concerted* mechanism, in contrast, the observed effects of substitution either of hydrogen by deuterium or of ^{12}C by ^{13}C will be the intrinsic effects, and the two kinetic isotope effects will equal $^{D}\phi_{gs}/^{D}\phi_{ts}$ and $^{13}\phi_{gs}/^{13}\phi_{ts}$, respectively. [This requires, of course, that the concerted transition state (ts) be cleanly rate limiting.]

What will happen to the observed ^{13}C isotope effect if we use ^{2}H substrate rather than ^{1}H substrate? If the reaction is *concerted*, there will be no change in $^{13}\phi_{gs}/^{13}\phi_{ts}$, since the two isotopes fractionate independently (see Figure 3: Kresge, 1964; Hegarty & Jencks, 1975). In the *stepwise* case, however, substrate deuterium will change the relative free energies of the two transition states and make the ^{13}C-sensitive step less rate limiting than it was for the ^{1}H substrate (see Figure 4c). The partitioning of the carbanionic intermediate back to substrate will decrease by a factor[3] $^{D}\phi_1/^{D}\phi_2$. The mixed transition-state fractionation factor now becomes

$$(^{13}\phi_{1,2})_D = \frac{1 + (^{D}\phi_1/^{D}\phi_2)\kappa}{^{13}\phi_1^{-1} + (^{D}\phi_1/^{D}\phi_2)\kappa\,^{13}\phi_2^{-1}} \quad (6)$$

Upon deuteration of the substrate, the step involving carbon–carbon bond formation becomes less rate limiting: the forward partitioning increases, $^{13}\phi_{1,2}$ rises towards unity, and the observed carbon isotope effect ($^{13}\phi_{gs}/^{13}\phi_{1,2}$) falls.

Transcarboxylase. When the rates of transcarboxylase-catalyzed carboxylation of [$^{1}H_3$]pyruvate and of [$^{2}H_3$]pyruvate are compared, an intermolecular kinetic isotope effect, $^{D}(V/K)$, of 1.4 is observed. When the ^{13}C isotope effect is measured on the transcarboxylase half-reaction in which pyruvate is carboxylated to oxalacetate [the other half-reaction must be maintained at equilibrium by using saturating concentrations of acetyl-CoA and subsaturating levels of pyruvate, since the carbon center under scrutiny in the pyruvate carboxylation half-reaction is delivered to biotin in the first half-reaction], a value for $^{13}(V/K)_H$ of 1.0227 ± 0.0008 is obtained (see Table I). When the determination of $^{13}(V/K)_D$ is made with [$^{2}H_3$]pyruvate, the observed isotope effect falls from 1.0227 to 1.0141 ± 0.001 (Table I). According to the discussion above, a fall in the value of $^{13}(V/K)$ when deuterated substrate is used is diagnostic of a stepwise reaction. Importantly, the value of $^{13}(V/K)_D$ can be *predicted* with eq 7 (Hermes et al., 1982). $^{D}K_{eq}$ may be estimated from the known fractionation factor of ^{2}H on pyruvate (Cleland, 1980) and from the assumption that the base that removes the proton has a fractionation factor near unity. [This is true for nitrogen and oxygen centers. Only thiols have fractionation factors that are not near unity. If transcarboxylase were to use a thiolate base having a ground-state fractionation factor near 0.5 (Schowen, 1977; Szawelski et al., 1982), the predicted value for $^{13}(V/K)_D$ would be 1.0274. This is almost twice the observed value, so the above assumption is evidently justified.] Strictly, eq 7 only holds for the second half-reaction of transcarboxylase, but the observed $^{13}(V/K)$ isotope effect is equal to $^{13}(K_{eq})_A\,^{13}(V/K)_B$ (where the subscripts A and B refer to the first and second half-reactions), since the carboxyl group is delivered to pyruvate from N^1-carboxybiotin and not from malonyl-CoA. Substitution into eq 7 yields

$$\frac{^{13}(V/K)_H - ^{13}(K_{eq})_A}{^{13}(V/K)_D - ^{13}(K_{eq})_A} = \frac{^{D}(V/K)}{^{D}K_{eq}} \quad (8)$$

It is expected, however, that $^{13}(K_{eq})_A$ will not be very different from 1, and the difference in the value of $^{13}(V/K)_D$ predicted from eq 7 and 8 will be small.

The predicted value for $^{13}(V/K)_D$ from eq 7 is 1.0136, which is in excellent agreement with the measured value of 1.0141. The conclusion is that the carboxylation of pyruvate catalyzed by transcarboxylase is a stepwise process.[4]

While our results rule out a concerted process for the biotin-dependent carboxylation catalyzed by transcarboxylase, we cannot differentiate between the two stepwise pathways presented in Figure 1. Arguments may be made in favor of each mechanism. Thus, N^1-carboxybiotin breaks down to form

[3] The deuterium isotope effect on the back-reaction $^{H}k_{-1}/^{D}k_{-1}$ is equal to $^{D}\phi_e/^{D}\phi_1$, where $^{D}\phi_e$ is the deuterium fractionation factor of the abstracted proton that is now on the enzyme. From this equivalence, $^{D}k_{-1} = (^{D}\phi_1/^{D}\phi_e))^{H}k_{-1}$. Analogously, $^{D}k_2 = (^{D}\phi_2/^{D}\phi_e)^{H}k_2$. Thus, the new partition ratio, $^{D}k_{-1}/^{D}k_2 = (^{D}\phi_1/^{D}\phi_2)\kappa$. Since $^{D}\phi_1$ is the fractionation factor for a proton in flight in transition state 1, it is expected to have a value less than 1. On the other hand, $^{D}\phi_2$ is the fractionation factor for a proton at rest in transition state 2, and it is expected to have a value near 1. The partitioning of the intermediate back to substrate is, therefore, decreased when ^{2}H is substituted for ^{1}H.

[4] Attwood and Cleland (P. V. Attwood and W. W. Cleland, private communication) have recently arrived at the same conclusion for pyruvate carboxylase by measuring the ^{13}C isotope effects for the enzymic decarboxylation of oxalacetate in H_2O and D_2O. These investigators have shown that the $^{13}(V/K)$ isotope effect falls on going from H_2O solvent into D_2O solvent.

CO_2 *more* rapidly in the presence of transcarboxylase than in its absence (Wood et al., 1963b), and we must expect that nucleophilic attack on CO_2 will be much easier than on the carboxyl group of N^1-carboxybiotin (Sauers et al., 1975). Yet there are reports in the literature of two biotin-dependent *de*carboxylases, methylmalonyl-CoA decarboxylase (Galivan & Allen 1968a,b) and oxalacetate decarboxylase (Stern, 1967). If these enzymes follow the microscopic reverse of the biotin-dependent carboxylation reaction, the benefits of the mechanism of Sauers et al. (1975) are not obvious. Why should an enzyme catalyze the breakdown of its substrate to CO_2 only to form N^1-carboxybiotin, which will be broken down to CO_2 and biotin again in a later step? The resolution of this and other questions on the role of biotin in these carboxylases and decarboxylases must await further experiments.

ACKNOWLEDGMENTS

We are indebted to Joel Belasco for germinal contributions to the method of double-isotope fractionation and to W. John Albery, W. W. Cleland, Peter Leadlay, and Jeffrey Hermes for their contributions and helpful discussions.

REFERENCES

Albery, W. J., & Knowles, J. R. (1976) *Biochemistry 15*, 5588–5600.

Barden, R. E., Fung, C., Utter, M. F., & Scrutton, M. C. (1972) *J. Biol. Chem. 247*, 1323–1333.

Belasco, J. G., Albery, W. J., & Knowles, J. R. (1983) *J. Am. Chem. Soc. 105*, 2475–2477.

Bergmeyer, H. U., Ed. (1974) *Methods of Enzymatic Analysis*, 2 ed., Vol. 3, pp 1446–1451, Verlag Chemie, New York.

Brooks, D. (1978) Ph.D. Thesis, University of Edmonton, Alberta, Edmonton.

Bruice, T. C., & Hegarty, A. F. (1970) *Proc. Natl. Acad. Sci. U.S.A. 65*, 805–809.

Caplow, M. (1965) *J. Am. Chem. Soc. 87*, 5774–5785.

Caplow, M., & Yager, M. (1967) *J. Am. Chem. Soc. 89*, 4513–4521.

Cheung, Y. F., Chien, H. F., & Walsh, C. T. (1975) *Biochemistry 14*, 2981–2986.

Chuang, M., Ahmad, F., Jacobson, B. & Wood, H. G. (1975) *Biochemistry 14*, 1611–1619.

Cleland, W. W. (1979) *Methods Enzymol. 63*, 103–138.

Cleland, W. W. (1980) *Methods Enzymol. 64*, 104–125.

Cook, P. F., Blanchard, J. S., & Cleland, W. W. (1980) *Biochemistry 19*, 4853–4858.

Craig, H. (1957) *Geochimica. Cosmochim. Acta 12*, 133–149.

Grissom, C. B., & Cleland, W. W. (1985) *Biochemistry 24*, 944–948.

Guchait, R. B., Polakis, S. E., Dimroth, P., Stoll, E., Moss, J., & Lane, M. D. (1974a) *J. Biol. Chem. 249*, 6633–6645.

Guchait, R. B., Polakis, S. E., Hollis, D., Fenselau, C., & Lane, M. D. (1974b) *J. Biol. Chem. 249*, 6646–6656.

Halenz, D. R., & Lane, M. D. (1961) *Biochim. Biophys. Acta 48*, 426–427.

Hegarty, A. F., & Jencks, W. P. (1975) *J. Am. Chem. Soc. 97*, 7188–7189.

Hermes, J. D., Roeske, C. A., O'Leary, M. H., & Cleland, W. W. (1982) *Biochemistry 21*, 5106–5114.

Hermes, J. D., Morrical, S. W., O'Leary, M. H., & Cleland, W. W. (1984a) *Biochemistry 23*, 5479–5488.

Hermes, J. D., Tipton, P. A., Fisher, M. A., O'Leary, M. H., & Cleland, W. W. (1984b) *Biochemistry 23*, 6263–6275.

Hoving, H., Crysell, B., & Leadlay, P. F. (1985) *Biochemistry 24*, 6163–6169.

Kaziro, Y., & Ochoa, S. (1961) *J. Biol. Chem. 236*, 3131–3136.

Knappe, J., Ringelmann, E., & Lynen, F. (1961) *Biochem. Z. 335*, 168–176.

Kresge, A. J. (1964) *Pure Appl. Chem. 8*, 243–258.

Kuo, D. J., & Rose, I. A. (1982) *J. Am. Chem. Soc. 104*, 3235–3236.

Leadlay, P. F. (1981) *Biochem. J. 197*, 413–419.

Lynen, F., Knappe, J., Lorch, E., Jutting, G., & Ringelmann, E. (1959) *Angew. Chem. 71*, 481–486.

Lynen, F., Knappe, J., Lorch, E., Jutting, G., Ringelmann, E., & Lachance, J. P. (1961) *Biochem. Z. 335*, 123–167.

Mildvan, A. S., Scrutton, M. C., & Utter, M. F. (1966) *J. Biol. Chem. 241*, 3488–3498.

Moss, J., & Lane, M. D. (1971) *Adv. Enzymol. Relat. Areas Mol. Biol. 35*, 321–442.

Northrop, D. B. (1969) *J. Biol. Chem. 244*, 5808–5819.

Northrop, D. B. (1977) in *Isotope Effects on Enzyme-Catalyzed Reactions* (Cleland, W. W., O'Leary, M. H., & Northrop, D. B., Eds.) pp 122–152, University Park Press, Baltimore, MD.

Northrop, D. B., & Wood, H. G. (1969) *J. Biol. Chem. 244*, 5820–5827.

O'Leary, M. H. (1980) *Methods Enzymol. 64*, 83–104.

O'Leary, M. H., Rife, J. E., & Slater, J. D. (1981) *Biochemistry 20*, 7308–7314.

Polakis, S. E., Guchait, R. B., Zwergel, E. E., Lane, M. D., & Cooper, T. G. (1974) *J. Biol. Chem. 249*, 6657–6667.

Prescott, D. J., & Rabinowitz, J. L. (1968) *J. Biol. Chem. 243*, 1551–1557.

Rêtey, J., & Lynen, F. (1965) *Biochem. Z. 342*, 256–271.

Rose, I. A. (1970) *J. Biol. Chem. 245*, 6052–6056.

Rose, I. A., O'Connell, E. L., & Solomon, F. (1976) *J. Biol. Chem. 251*, 902–904.

Sauers, C. K., Jencks, W. P., & Groh, S. (1975) *J. Am. Chem. Soc. 97*, 5546–5553.

Schowen, R. L. (1977) in *Isotope Effects on Enzyme-Catalyzed Reactions* (Cleland, W. W., O'Leary, M. H., & Northrop, D. B., Eds.) pp 64–99, University Park Press, Baltimore, MD.

Scrutton, M. C., Keech, D. B., & Utter, M. F. (1965) *J. Biol. Chem. 240*, 574–581.

Sedgwick, B., & Cornforth, J. W. (1977) *Eur. J. Biochem. 75*, 465–479.

Segel, I. H. (1975) *Enzyme Kinetics*, pp 860–864, Wiley, New York.

Simon, E. J., & Shemin, D. (1953) *J. Am. Chem. Soc. 75*, 2520.

Stern, J. R. (1967) *Biochemistry 6*, 3545–3551.

Stubbe, J., & Abeles, R. H. (1977) *J. Biol. Chem. 252*, 8338–8340.

Stubbe, J., Fish, S., & Abeles, R. H. (1980) *J. Biol. Chem. 255*, 236–242.

Swick, R. W., & Wood, H. G. (1960) *Proc. Natl. Acad. Sci. U.S.A. 46*, 28–41.

Szawelski, R. J., Wharton, C. W., & White, S. (1982) *Biochem. Soc. Trans. 10*, 232–233.

Toennies, G., & Kolb, J. J. (1951) *Anal. Chem. 23*, 823–826.

Tubbs, P. K., & Garland, P. B. (1969) *Methods Enzymol. 13*, 535–551.

Vagelos, P. R. (1963) *Methods Enzymol. 6*, 544–549.

Wilson, G. E., Jr., & Hess, A. (1974) *J. Org. Chem. 39*, 3170.

Wood, H. G., & Barden, R. E. (1977) *Annu. Rev. Biochem. 46*, 385–413.

Wood, H. G., Lochmüller, H., Riepertinger, C., & Lynen, F. (1963a) *Biochem. Z. 337*, 247–266.
Wood, H. G., Allen, S. H. G., Stjernholm, R., & Jacobson, B. (1963b) *J. Biol. Chem. 238*, 547–556.
Wood, H. G., Jacobson, B., Gerwin, B. I., & Northrop, D. B. (1969) *Methods Enzymol. 13*, 215–230.
Wood, H. G., Chiao, J., & Poto, E. M. (1977) *J. Biol. Chem. 252*, 1490–1499.

Phosphate Inhibition of the Copper- and Zinc-Containing Superoxide Dismutase: A Reexamination†

Wayne F. Beyer, Jr., Yang Wang, and Irwin Fridovich*
Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710
Received March 20, 1986

ABSTRACT: Phosphate was reported to be an inhibitor of copper- and zinc-containing superoxide dismutase (SOD) [de Freitas, D. M., & Valentine, J. S. (1984) *Biochemistry 23*, 2079–2082]. Thus SOD activity, in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.4), was decreased by approximately 50% when the assay was made 10 mM in phosphate, and the ionic strength was adjusted with sodium fluoride. The inhibitory effect of phosphate was attributed to the neutralization of the positive charge on the guanidino residue of Arg-141. We have reexamined the effects of phosphate inhibition of SOD and found that the enzyme has identical activity in phosphate or HEPES buffer when the ionic strength is adjusted with NaBr. The putative inhibitory effect of phosphate appears to have been due to fluoride inhibition of the superoxide generating system of xanthine/xanthine oxidase. We have confirmed this result by using a photochemical generation of O_2^- in addition to the enzymatic generation of O_2^-. Chemical modification of the lysine residues to homoarginines does not affect the activity of the enzyme and does not impart a phosphate sensitivity. Chemical modification with phenylglyoxal caused approximately 80% inactivation of the native enzyme and 90% inactivation of the *O*-methylisourea-modified enzyme. Our results suggest that phosphate does not inhibit the copper- and zinc-containing superoxide dismutase (Cu,Zn-SOD) beyond the expectations of its effect on ionic strength.

The Cu,Zn-SOD[1] is one of a family of SODs that provides a defense against oxygen toxicity by catalyzing the dismutation of O_2^- to H_2O_2 plus O_2 (Fridovich, 1983, 1986). Electrostatic guidance was proposed as one explanation for the great catalytic efficiency of this enzyme (Koppenol, 1981). Raising the ionic strength depressed the catalytic activity of Cu,Zn-SOD, in accord with the expectations of electrostatic facilitation (Malinowski & Fridovich, 1979; Cudd & Fridovich, 1982a; Rigo et al., 1975). The structure of the bovine enzyme, deduced from X-ray diffraction data, indicated that the cationic residues Arg-141, Lys-120, and Lys-134 are located 5, 12, and 13 Å, respectively, from the active site Cu(II) and suggested that they provide for electrostatic attraction of the anionic substrate (Tainer et al., 1982). Covalent modification of these residues suppresses the catalytic activity of Cu,Zn-SOD (Malinowski & Fridovich, 1979; Cudd & Fridovich, 1982a; Marmocchi et al., 1983; Cocco et al., 1982).

Increasing the concentration of phosphate was seen to decrease the activity of Cu,Zn-SOD, and this was interpreted as an effect due to ionic strength (Cudd & Fridovich, 1982a; Rigo et al., 1975). More recently, de Freitas and Valentine (1984) reported that phosphate inhibited Cu,Zn-SOD, when the ionic strength was held constant, by addition of NaF to the HEPES buffer. These authors concluded that phosphate inhibits Cu,Zn-SOD by binding to Arg-141. In the course of their work de Freitas and Valentine considered several salts that might be used to maintain ionic strength as phosphate was varied. In the first paragraph under Results of their paper they stated, "SOD activities and anion binding affinities were higher when the ionic strength was adjusted with trifluoromethanesulfonate rather than with fluoride, but with the former, little or no effect of added phosphate on the properties of the protein was observed...". This suggested to us that the reported effect of phosphate might well be an artifact derived from the influence of fluoride on the assay system. We have reinvestigated the effects of phosphate and now present results indicating that it does not inhibit, Cu,Zn-SOD beyond the expectations of its contribution to ionic strength.

Materials and Methods

Bovine liver Cu,Zn-SOD (lyophilized powder) was a generous gift from Diagnostic Data, Inc. (Mountain View, CA). Phenylglyoxal, *O*-methylisourea sulfate salt, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), xanthine, 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris), horse heart cytochrome *c* (type III), 6-hydroxydopamine hydrobromide, methionine, riboflavin, Triton X-100, 2-(*N*-cyclohexylamino)ethanesulfonic acid (CHES), bicine, and nitroblue tetrazolium [2,2′-di-*p*-nitrophenyl-5,5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4′-diphenylene)ditetrazolium chloride (NBT)] were used as received from Sigma. Potassium phosphate dibasic trihydrate and potassium phosphate monobasic were

† This work was supported by research grants from the National Science Foundation, the U.S. Army Research Office, and the Council for Tobacco Research, Inc.
* Address correspondence to this author.

[1] Abbreviations: Cu,Zn-SOD, copper- and zinc-containing superoxide dismutase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; 6-HDA, 6-hydroxydopamine; NBT, nitroblue tetrazolium; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; KP_i, potassium phosphate; EDTA, ethylenediaminetetraacetic acid.

0006-2960/86/0425-6084$01.50/0