The Nature of the Carbon Dioxide Substrate and Equilibrium Constant of the 6-Phosphogluconate Dehydrogenase Reaction

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1. It was shown that dissolved CO_2 and not HCO_3^- or H_2CO_3 is the primary substrate for reductive carboxylation with 6-phosphogluconate dehydrogenase from sheep liver. 2. The equilibrium constant of the reaction was measured in solutions of various ionic strengths and at several temperatures, and the free energy and heat of reaction were determined.

The equilibrium constant and standard free-energy data for the oxidative decarboxylation reaction catalysed by 6-phosphogluconate dehydrogenase have not been reported previously. Such data are important for studies of the mechanisms of energy transformations in living matter (cf. Burton & Krebs, 1953). Equilibrium constants at physiological temperature and ionic strength for reactions catalysed by dehydrogenases can be used in some circumstances to obtain information about the redox state of cellular compartments (Williamson, Lund & Krebs, 1967). Since 6-phosphogluconate dehydrogenase appears to be located exclusively in the cytoplasm of liver cells (Glock & McLean, 1953), the equilibrium constant might be used to estimate the cytoplasmic $\text{NADP}^+/\text{NADPH}$ concentration ratio in this way (Veech, Eggleston & Krebs, 1969). The constant is also of value for correlation with initial-rate parameters in kinetic studies of enzyme reaction mechanisms (Alberty, 1953).

In the present work, the equilibrium constant has been measured at several temperatures and ionic strengths. To formulate the equilibrium, and for studies of the mechanism of the enzyme-catalysed reaction and of pH effects on the rate, it is necessary to know which of the three forms of carbon dioxide in solution is the actual substrate of the enzyme. Experiments that answer this question are also described.

MATERIALS AND METHODS

Enzymes. 6-Phosphogluconate dehydrogenase was prepared from sheep liver as described by Villet & Dalziel (1969). Carbonic anhydrase from bovine erythrocytes was purchased from Koch-Light Laboratories Ltd., Colnbrook, Bucks.

Coenzymes and substrates. NADP^+ , NADPH and sodium 6-phosphogluconate were purchased from Boehringer Corp. (London) Ltd., London W.5. Ribulose 5-phosphate used in some experiments was a generous gift from C. F. Boehringer und Soehne G.m.b.H., Tutzing, Germany, but for most experiments it was prepared by the method of Srere, Cooper, Tabachnick & Racker (1958), and assayed as 50% (w/w) pure by the cysteine-carbazole reaction (Dische & Borenfreund, 1951). The main impurity was probably xylulose phosphate. NADP^+ was assayed as described by Klingenberg (1963), and NADPH by E_{340} measurements. 6-Phosphogluconate was assayed spectrophotometrically; the reaction mixture was 1.1 ml. of 0.1 M-glycine adjusted to pH 8.9 with NaOH , 0.4 ml. of 0.1 M- MgCl_2 , 0.4 ml. of 2 mM- NADP^+ , 0.1 ml. of 6-phosphogluconate solution (about 0.8 mg./ml.) and 20 μg . of 6-phosphogluconate dehydrogenase.

For kinetic studies of the reductive carboxylation reaction, saturated solutions of CO_2 were prepared by bubbling the gas from a Thermos flask containing solid CO_2 into water or 0.36 M- KHCO_3 solution contained in narrow-necked flasks immersed in a water bath at 17°. The concentrations of these solutions are 40 ± 2 mM at atmospheric pressure (Harned & Davis, 1943), and the pH of the solution in 0.36 M- KHCO_3 is 7.35.

The same source of pure CO_2 was used for equilibrium measurements with large CO_2 concentrations. In addition, equilibrium measurements were made with a smaller CO_2 concentration by passing $\text{CO}_2 + \text{N}_2$ (20:80) from a cylinder (British Oxygen Co. Ltd.).

Buffers and salts. Salts were A.R. grade from British Drug Houses Ltd., Poole, Dorset. For kinetic studies of the reductive carboxylation reaction, 0.1 M-triethanolamine hydrochloride- NaOH buffer, pH 7.35 (Hems, 1959), was used. The hydrochloride was purchased from Boehringer Corp. (London) Ltd. For equilibrium measurements, reaction mixtures were buffered at pH 6.45–7.7 by the addition of the appropriate concentration of KHCO_3 calculated from the dissolved CO_2 concentration and pK_1' 6.4 for H_2CO_3 . The ionic strength was also varied at pH 6.95 by the addition of KCl .

Rate measurements. Progress curves for the reductive carboxylation of ribulose 5-phosphate at 17° and pH 7.35 were obtained by measurements, with a recording fluorimeter (Dalziel, 1962), of the decrease of fluorescence

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accompanying the oxidation of NADPH. The reaction mixtures, made up in fluorimeter tubes with solutions free from dust and debris, comprised 3.0 ml. of 0.1 M-triethanolamine buffer, pH 7.35, 0.15 ml. of 0.3 mM-NADPH, 0.2 ml. of 12 mM-ribulose 5-phosphate, 0.2 ml. of 6-phosphogluconate dehydrogenase solution (0.18 mg./ml.), and water to give a total volume of 4 ml. after the addition of carbon dioxide substrate, which was added last to initiate the reaction. Stock buffer solutions and water were first freed from dissolved CO₂ by passing CO₂-free N₂ through them, and the reaction mixture was treated in the same way immediately before the addition of enzyme and carbon dioxide substrate. The blank reaction before the latter addition was then negligible.

Carbon dioxide was added as a saturated solution of CO₂ in 0.36 M-KHCO₃ (pH 7.35), or as an unbuffered solution of CO₂ in water, or as an unbuffered solution of KHCO₃. The shapes of the progress curves in the last two cases show whether dissolved CO₂ or HCO₃⁻ is the primary substrate of the enzyme. The principle of the method is described in detail by Dalziel & Londesborough (1968), and depends on the relative slowness of the hydration and dehydration steps in the interconversion of CO₂ and HCO₃⁻. The half-reaction time for the equilibration of these two species under the conditions of these experiments is 41 sec., and at equilibrium the ratio [HCO₃⁻]/[CO₂] is 9. The amounts of unbuffered CO₂ and KHCO₃ added do not alter the pH of the reaction mixture by more than 0.05 pH unit, and are also small compared with the *K_m* for carbon dioxide. With large concentrations of the other substrates, the rate of carboxylation is then proportional to the concentration of dissolved CO₂ or HCO₃⁻, whichever is the true substrate, and will increase or decrease during the first 2 min. after reaction is initiated as these forms of carbon dioxide come to equilibrium.

Equilibrium measurements. The technique was essentially that described by Londesborough & Dalziel (1968) for equilibrium studies of the isocitrate dehydrogenase reaction. The initial reaction mixture contained 6-phosphogluconate and NADP⁺ and was saturated with either 100% or 20% CO₂ at atmospheric pressure. For the lower CO₂ concentration, 105 μM-NADPH was also added to the initial reaction mixture. Equilibrium was reached in 15–20 min. with 18 μg. of enzyme in 3 ml. of reaction mixture. Trebling the enzyme concentration had no effect on the apparent equilibrium constant.

The concentration of NADPH at equilibrium was calculated from the final *E*₃₄₀. The partial pressure of CO₂ was calculated from the barometric pressure and saturated water vapour pressure at the temperature of the experiment, and the dissolved CO₂ concentration from the solubility data of Harned & Davis (1943).

The equilibrium constant for the reaction:

was calculated from the equation:

$$K_p = \frac{[\text{NADPH}][\text{ribulose 5-phosphate}^{2-}]}{[\text{NADP}^+][6\text{-phosphogluconate}^{3-}]} \times \frac{p}{760} \quad (2)$$

where *p* is the partial pressure of CO₂ in mm. Hg.

The equilibrium constant for the reaction:

was calculated from:

$$K_c = \frac{[\text{NADPH}][\text{ribulose 5-phosphate}^{2-}][\text{CO}_2]}{[\text{NADP}^+][6\text{-phosphogluconate}^{3-}]} \quad (4)$$

The relation between these two formulations of the equilibrium constant and the thermodynamic quantities derived from them is discussed by Londesborough & Dalziel (1968).

RESULTS

Nature of the carbon dioxide substrate. Apparent *K_m* values for the three substrates involved in the reductive carboxylation reaction were determined in the usual way from initial-rate measurements at 17° and pH 7.35. For NADPH, the apparent *K_m* with 0.1 mM-ribulose 5-phosphate and 20 mM-CO₂–18 mM-KHCO₃ is 0.3 μM. For ribulose 5-phosphate, with the same carbon dioxide concentration and 11 μM-NADPH, the value is 30 μM. For carbon dioxide, with 0.6 mM-ribulose 5-phosphate and 11 μM-NADPH, the apparent *K_m* value is 15 mM-CO₂, or 135 mM-HCO₃⁻. The results of initial-rate measurements from which this value was determined are shown in Fig. 1.

In Figs. 2 and 3, progress curves for the reaction with 0.6 mM-ribulose 5-phosphate and 11 μM-NADPH initiated with three forms of carbon dioxide substrate are shown. When reaction is started by the addition of 0.2 mM-CO₂–1.8 mM-KHCO₃, the progress curve is linear for the first 2 min. (Fig. 2, curve A). For the reaction initiated by the addition of 2 mM-CO₂ as an unbuffered solution in water, the

Fig. 1. Variation of the reciprocal initial rate of reductive carboxylation of ribulose 5-phosphate at 17° with the reciprocal of the concentration of dissolved CO₂. Reaction mixtures contained 11 μM-NADPH and 0.6 mM-ribulose 5-phosphate in 0.075 M-triethanolamine buffer, pH 7.35. The CO₂ was added as a saturated solution (0.04 M) in equilibrium with KHCO₃ (0.36 M).

Fig. 2. Progress curves for the reductive carboxylation of ribulose 5-phosphate with $9\mu\text{g.}$ of 6-phosphogluconate dehydrogenase/ml. Reaction was initiated by 0.2mm-CO_2 - 18mm-KHCO_3 (curves *A* and *D*) or 2mm-CO_2 (curves *B* and *C*). For curve *C* carbonic anhydrase (2mg.) was added 15sec. after the CO_2 , and for curve *D* carbonic anhydrase was added immediately before the CO_2 - KHCO_3 mixture. Other conditions were as in Fig. 1. The ordinate shows the fluorescence of the reaction mixture in recorder-chart units ($10\text{units} = 0.09\mu\text{M-NADPH}$).

slope of the progress curve (curve *B*) is initially about ten times that of curve *A*, as would be expected with substrate concentrations much smaller than K_m . During the first 1.7min. , the slope decreases to a constant value equal to that of curve *A*, consistent with the conversion of 90% of the dissolved CO_2 added into HCO_3^- , inactive as substrate. The effect of adding carbonic anhydrase to a similar reacting mixture 15sec. after the unbuffered CO_2 solution is to accelerate the attainment of the same constant rate (curve *C*). A control experiment (curve *D*) shows that carbonic anhydrase is without effect on the reaction started with an equilibrium mixture of CO_2 and KHCO_3 .

Progress curves for the reaction started with 20mm-KHCO_3 added as an unbuffered solution essentially free of CO_2 , with two enzyme concentrations, are shown in curves *A* and *B* of Fig. 3. There is an increase of rate for about 1min. , after which the rate remains constant and equal to that obtained at once when reaction is started with 2mm-CO_2 - 18mm-KHCO_3 (curves *C* and *D*). The rate in the latter case with the lower enzyme concentration is

Fig. 3. Progress curves for the reductive carboxylation of ribulose 5-phosphate. Reaction was initiated by the addition of 20mm-KHCO_3 (curves *A* and *B*) or 2mm-CO_2 - 18mm-KHCO_3 (curves *C* and *D*). The enzyme concentration was $4.5\mu\text{g./ml.}$ for curves *A* and *C* and $9.0\mu\text{g./ml.}$ for curves *B* and *D*. Other conditions were as in Fig. 2.

approximately equal to that obtained initially with 2mm-CO_2 , in the absence of KHCO_3 , and with the same enzyme concentration (Fig. 2, curve *B*).

Equilibrium measurements. The results of equilibrium measurements at 25° with various reactant concentrations and at different ionic strengths and pH values are shown in Table 1. Each value for the equilibrium concentration of NADPH is the mean of two determinations. The equilibrium constant is evidently independent of the reactant concentrations, and does not vary significantly with pH from 6.45 to 7.7 , nor with ionic strength from 0.036 to 0.45 . The mean value of 2.38atm. for K_p can therefore be equated with K_a , the thermodynamic equilibrium constant for reaction (1) at 25° .

The results of equilibrium measurements with $I\ 0.12$ at five temperatures are shown in Fig. 4 as a plot of $\log K_p$ against the reciprocal of the absolute temperature. From the slope, the heat of reaction (1), ΔH° , is estimated at 13.75kcal./mole , by using the Van't Hoff isochore:

$$d(\log K_a)/d(1/T) = -\Delta H^\circ/2.3R$$

DISCUSSION

Progress curves for reductive carboxylation of ribulose 5-phosphate recorded after starting the reaction with dissolved CO_2 and unbuffered potassium hydrogen carbonate solution show that the former is the primary substrate for the enzyme and that HCO_3^- is a much poorer substrate, and possibly not a substrate at all. The time-course of the reaction initiated with unbuffered CO_2

Table 1. 6-Phosphogluconate dehydrogenase equilibrium in potassium bicarbonate buffers at 25°

The pH and the ionic strength (0.036–0.12) were changed by varying the concentration of KHCO_3 . For ionic strengths greater than 0.12, KCl was also added. The concentrations of NADP^+ and 6-phosphogluconate are the initial values and those of NADPH, and the partial pressure of CO_2 (p), are the equilibrium values. Values of K_p are calculated from eqn. (2), and values of K_c from eqn. (4).

<i>I</i>	pH	Concn. of NADP (μM)	Concn. of 6-phosphogluconate (μM)	Concn. of NADPH (μM)	p (mm. Hg)	K_p (atm.)	K_p (av.) (atm.)	K_c (M)
0.036	6.45	83.8	53.8	39.0	0.969	2.22	2.22	0.074
0.048	7.30	59.1	67.4	146.0*	0.194	2.38	2.38	0.079
0.084	6.80	83.8	53.8	39.8	0.969	2.49	2.49	0.083
0.096	6.90	83.8	53.8	39.6	0.969	2.42	2.42	0.081
0.120	6.95	84.5	56.0	40.6	0.968	2.36	2.41	0.080
		56.3	70.0	38.8	0.968	2.66		
		57.6	67.4	37.8	0.970	2.39		
		84.5	210.0	69.5	0.968	2.22		
		84.5	35.0	29.1	0.968	2.50		
		90.6	53.8	40.3	0.968	2.32		
0.120	7.70	60.4	67.4	146.0*	0.194	2.22	2.22	0.074
0.250	6.95	55.4	35.7	26.4	0.967	2.50	2.33	0.076
		55.4	142.8	46.8	0.969	2.54		
		84.5	56.0	42.7	0.968	2.16		
		84.5	56.0	40.5	0.968	2.33		
		90.6	53.8	39.6	0.969	2.10		
0.350	6.95	84.5	56.0	40.4	0.968	2.30	2.40	0.075
		84.5	56.0	41.3	0.968	2.60		
		90.6	53.8	40.3	0.968	2.32		
0.450	6.95	90.6	53.8	40.1	0.968	2.25	2.25	0.069
Mean							2.38	

* 105 μM -NADPH was added initially.

Fig. 4. Effect of temperature on the equilibrium constant, K_p , of the 6-phosphogluconate dehydrogenase reaction.

solution is consistent with this conclusion and with the conversion of 90% of the added CO_2 into HCO_3^- at the rate predicted from kinetic data for the hydration of CO_2 and the dehydration of carbonic acid (Dalziel & Londesborough, 1968). For one experiment, the theoretical progress curve was calculated on the assumption that dissolved CO_2 is the sole substrate, by using the initial-rate parameters for the reductive carboxylation reaction and the velocity constants for the hydration and dehydration reaction. The differential equation was solved numerically with a digital computer (Villet, 1968). The predicted progress curve agreed reasonably well with the experimental curve. The

effects of carbonic anhydrase and the time-course of the reaction initiated with unbuffered potassium hydrogen carbonate solution also support these conclusions. However, in the latter experiments the rate of reductive carboxylation increases to a constant value more quickly than is expected from the known rate of equilibration of the two forms of carbon dioxide. The discrepancy appears to be outside the experimental error, and the reason for it is not known.

These findings are in accordance with the results of similar experiments with NADP-linked isocitrate dehydrogenase of ox heart mitochondria and 'malic' enzyme of wheat germ (Dalziel & Londesborough, 1968). All three enzymes catalyse formally analogous oxidative decarboxylations, with NADP as coenzyme, and dissolved CO_2 is the primary product of the reactions, by the principle of microscopic reversibility.

The enthalpy change for reaction (1), calculated from the effect of temperature on the equilibrium constant, is 13.75 kcal./mole, identical with that estimated for the analogous reaction catalysed by isocitrate dehydrogenase (Londesborough & Dalziel, 1968). The enthalpy change for reaction (3) is the sum of this value and the heat of solution of CO_2 , $\Delta H^0 = -4.72$ kcal./mole (Harned & Davis, 1943).

Table 2. *Free-energy data for the 6-phosphogluconate dehydrogenase reaction*

The data are calculated from the mean value of the equilibrium constant, K_p , measured in $\text{KHCO}_3\text{--KCl}$ solutions of various concentrations and found to be independent of ionic strength. ΔG^0 values are for unit activity of all reactants in aqueous solution at 25°, except that CO_2 is the gas at 1 atm. $\Delta G'$ and E'_0 refer to a standard state of the H^+ ion of pH 7.0, and $\Delta G''$ and E''_0 refer in addition to a standard state for CO_2 of 0.05 atm. These quantities were calculated from ΔG^0 as described by Londesborough & Dalziel (1968).

Reactants or oxidant (+ H_2 gas)	Products or reductant	ΔG^0 (kcal./mole)	$\Delta G'$ (kcal./mole)	$\Delta G''$ (kcal./mole)	E'_0 (v)	E''_0 (v)
6-Phosphogluconate ³⁻ + NADP ⁺	Ribulose 5-phosphate ²⁻ + NADPH + CO_2	-0.51	-0.51	-2.28	+0.011	+0.050
Ribulose 5-phosphate ²⁻ + CO_2	6-Phosphogluconate ³⁻ + H^+	+6.44	-3.11	-1.34	-0.346	-0.385

There was no significant variation of the equilibrium constant of reaction (1) with ionic strength. If the Debye-Hückel limiting law can be applied to 6-phosphogluconate³⁻, ribulose 5-phosphate²⁻ and NADPH⁴⁻, this finding means that the activity coefficient of the zwitterion NADP^{4-} is given by:

$$-\log \gamma = 5.5 \sqrt{I}$$

corresponding to an 'effective charge' of -3.5. From the small effect of ionic strength on the equilibrium constant of the isocitrate dehydrogenase reaction, Londesborough & Dalziel (1968) estimated the 'effective charge' as -3.4. The difference presumably indicates different deviations by the multivalent substrate ions from the Debye-Hückel limiting law.

The value for K_p at 25°, 2.38 atm., is therefore the thermodynamic dissociation constant for reaction (1). From the reaction isotherm for 25°:

$$\Delta G^0 = -1.364 \log K_a = -0.15 \text{ kcal./mole}$$

From this value and $\Delta G^0 = 5.93 \text{ kcal./mole}$ for the reduction of NADP⁺ (Engel & Dalziel, 1967), the standard free-energy change for the oxidative decarboxylation of 6-phosphogluconate:

is -6.44 kcal./mole. These and other free-energy data are summarized in Table 2 and were calculated for the different standard states of the H^+ ion and of CO_2 by relations given by Londesborough & Dalziel (1968).

A few measurements of the equilibrium constant were also made with NADPH, ribulose 5-phosphate and carbon dioxide as initial reactants. The values were rather variable and three to four times as large as those reported, possibly because of impurities in the ribulose 5-phosphate. It may be noted that a kinetic estimate of the equilibrium constant K_e from initial-rate parameters for the forward and reverse reactions at 25° gave a value of 0.070 M (R. H. Villet & K. Dalziel, unpublished work), in

satisfactory agreement with the direct estimate of 0.079 M (Table 1).

The value of the equilibrium constant at 38°, pH 6.95 and I 0.12, is 7.00 atm. or 0.170 M. Since the equilibrium constant is independent of pH and of ionic strength, this is the value under physiological conditions.

The equilibrium constant is about an order of magnitude smaller than that for the isocitrate dehydrogenase reaction (Londesborough & Dalziel, 1968). For both reactions, however, and for the analogous oxidative decarboxylation of malate, the substrate couples are much more powerful reducing systems than those of most NAD-linked dehydrogenases, especially if a standard state of CO_2 of 0.05 atm. for the former is adopted as well as a standard state of pH 7.0 for the H^+ ion involved in the latter reactions. The role of NADPH in synthetic reactions has been correlated with these facts (Klingenberg & Bücher, 1960), and also provides an explanation for the specificity of most dehydrogenases for one or other of two coenzymes that have almost identical redox potentials (Krebs & Veech, 1969).

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