Dissociation and Characterization of Enzymes from a Multienzyme Complex Involved in CO_2 Fixation†

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ABSTRACT: A multienzyme complex from *Euglena*, molecular weight about 360,000, containing phosphoenolpyruvate carboxylase, malate dehydrogenase, and acetyl-coenzyme A carboxylase has been dissociated into active constituent enzymes. The respective molecular weights are 183,000, 67,000, and 127,000. The malate dehydrogenase contained in the complex is electrophoretically distinct from other malate dehydrogenase isozymes found in *Euglena*. The K_m for HCO_3^- of the free and complexed acetyl-CoA carboxylase is 4.2–5.4 m*M*, and the substrate dependency for acetyl-CoA describes a sigmoidal relationship. The HCO_3^- K_m for the free phosphoenolpyruvate carboxylase is 7.3–5.4 m*M* while that for the same enzyme contained in the complex is 0.7–1.3 m*M*. Both the free and complexed forms of phosphoenolpyruvate carboxylase have a K_m for phosphoenolpyruvate of 0.9–1.7 m*M*. The latter enzyme in both the complex and free forms is stimulated by NADH, acetyl-CoA, and ATP. In the free phosphoenolpyruvate carboxylase, the stimulation passes through a maximum depending on effector concentration. The effect of NADH is to increase V_{max} while K_m values remain unmodified.

A multienzyme complex containing phosphoenolpyruvate carboxylase, malate dehydrogenase, and acetyl-coenzyme A carboxylase was isolated from *Euglena* by Wolpert and Ernst-Fonberg (1975). The system was obtained from cells broken by two different methods and was shown to exist in relatively crude extracts of cells as well as being purified as a homogeneous protein preparation. A portion of the reaction sequence catalyzed resembled that proposed by Black with regard to the Hatch–Slack cycle (Utter and Kolenbrander, 1972) where phosphoenolpyruvate carboxylase was proposed to capture CO_2 which was transferred to ribulose-1,5-diphosphate carboxylase by way of malate and malic enzyme. The enzymes implicated in the latter cycle have not been isolated in association with one another. The *Euglena* multienzyme complex was proposed to function as a means for trapping CO_2 via phosphoenolpyruvate carboxylase for eventual use in fatty acid biosynthesis through the action of malate dehydrogenase, acetyl-CoA carboxylase, and malic enzyme (which was not a component of the complex). The latter enzyme was shown to be present in *Euglena* and would serve to free the captured CO_2 for use by acetyl-CoA carboxylase (to form malonyl-CoA) as well as to produce NADPH, the other substrate for fatty acid biosynthesis. The system may function as a means of controlling the levels of substrates for fatty acid biosynthesis in an organism which does not possess an allosteric acetyl-CoA carboxylase which is subject to control via physical modification.

In this report, the multienzyme complex has been dissociated into its constituent enzymes and has been shown to contain 1 mol each of acetyl-CoA carboxylase, malate dehydrogenase, and phosphoenolpyruvate carboxylase per mol of complex. Kinetic studies of the free and complexed phosphoenolpyruvate carboxylase have provided additional support for the rationale of the existence of the complex in that the K_m for HCO_3^- increased several-fold upon dissociation of the multienzyme complex. Acetyl-CoA, ATP, and NADH have been shown to stimulate the activity of phosphoenolpyruvate carboxylase in both forms. Details of these experiments are in the subsequent text.

Experimental Section

Materials and Reagents. $NaH^{14}CO_3$ was from New England Nuclear. Imidazole was recrystallized as described by Wolpert and Ernst-Fonberg (1975); the same source cites the origin of proteins used as molecular weight standards. DEAE-cellulose was Whatman DE-52. Buffer A was 0.02 *M* imidazole (pH 7.0), 0.007 *M* in 2-mercaptoethanol.

Assay of Phosphoenolpyruvate Carboxylase. The incorporation of $H^{14}CO_3^-$ into oxaloacetate was measured routinely in a final volume of 0.25 ml in the presence of: imidazole buffer, pH 7.5, 0.1 *M;* phosphoenolpyruvate, 3 m*M;* dithiothreitol, 2 m*M;* $NaH^{14}CO_3$, 25 m*M* (specific activity, 0.6); bovine serum albumin, 100 μg; and $MgCl_2$, 8 m*M*. Oxaloacetate, 0.875 mg, in 3 *N* HCl was added immediately at termination of reaction. The reaction apparatus was the same as that described for the assay of acetyl-CoA carboxylase. Reaction was initiated by the addition of phosphoenolpyruvate.

Enzymic activity also was measured spectrophotometrically by the rate of decrease in absorbance at 340 nm in a Cary 118 equipped with a thermostated cell holder. Temperature, 30°, was maintained with a Haake circulating water bath. The assay was like that described above except that NADH, 0.18 m*M*, and malate dehydrogenase, 1.5 units, were present. Reaction velocity was expressed as nanomoles/15 min per milliliter of enzyme.

For all carboxylation reactions, solutions were prepared from distilled water which had been boiled for 30 min to drive off dissolved CO_2 and then, following preparation of

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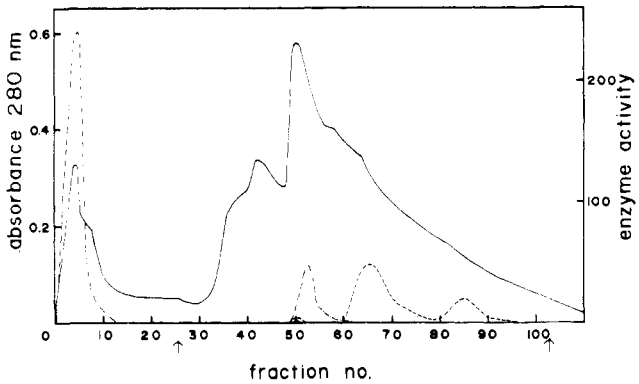

FIGURE 1: Dissociation of multienzyme complex by ion exchange chromatography. A DEAE-cellulose column was prepared and operated under the conditions described in the text; the arrows indicate the region of the linear gradient. Fractions of 10 ml were collected and measured for absorbance at 280 nm (solid line), malate dehydrogenase activity (dotted and dashed line, μmol/ml per 15 min), acetyl-CoA carboxylase activity (dashed line, nmol/ml per 15 min × 10), and phosphoenolpyruvate carboxylase activity (dotted line, nmol/ml per 15 min). The initial peak of malate dehydrogenase activity is isozymic forms which are not associated with the multienzyme complex. The second peak, eluted within the gradient, is that from the complex.

the solution, chilled and purged with N_2. Residual HCO_3^- was estimated spectrophotometrically in the presence of all reaction components except added HCO_3^-; reaction proceeded until absorbance became constant (54 min). By this method, the concentration of residual HCO_3^- was calculated to be 0.218 m*M*.

Assay of Acetyl-CoA Carboxylase. The assay procedure employed in the preceding article (Wolpert and Ernst-Fonberg, 1975) was modified so that the entire operation was done in a single vessel. Reactions were done in glass liquid scintillation counting vials with 15-mm tops which were sealed with serum stoppers. The serum stoppers were linked in series via two 18 gage hypodermic needles in each stopper. The counting vials were secured in a metal rack in a 30° circulating water bath in a fume hood. All reaction components except ATP were added. Following a 3–5-min warming period, reaction was initiated by the addition of ATP, and the serum stoppers were secured during reaction. After 10 min, 0.1 ml of 3 *N* HCl was injected, and residual $^{14}CO_2$ was removed by purging with air for 20 min. The $^{14}CO_2$ was trapped in 40% KOH. The solutions were dried at 55° for 45 min. Radioactivity was measured by adding 0.1 ml of H_2O, 3 ml of ethanol, and 15 ml of toluene which contained 15.1 g/gal of 2,5-diphenyloxazole and counting in a Packard Model 3320 liquid scintillation counter. Quenching was corrected by the channels ratio method.

The enzyme assay, final volume 0.25 ml, contained: imidazole buffer, pH 7.5, 0.1 *M;* ATP, 4 m*M;* $MgCl_2$, 8 m*M;* bovine serum albumin, 100 μg; dithiothreitol, 2 m*M;* acetyl-CoA, 0.24 m*M;* and $H^{14}CO_3^-$, 25 m*M* (specific activity, 0.6). Acetyl-CoA and $H^{14}CO_3^-$ were varied as indicated.

All enzyme kinetic data were processed in a Wang 600 by a series of programs obtained from Ignacy Fonberg, Computer Consultant. Statistical analyses and calculated linear regressions for Lineweaver–Burk and Eadie–Hofstee plots and least-squares rectangular hyperbolae for Michaelis–Menten plots were done with all sets of data.

Assay of Malate Dehydrogenase. Malate dehydrogenase activity was measured at 30° by the decrement at 340 nm detected by a Cary 118. The reaction solution included in a final volume of 0.25 ml: NADH, 0.18 m*M;* malic acid, 0.8 m*M;* and enzyme sufficient to decrease the absorbance at a rate of 0.05–0.25/min

Dissociation of Multienzyme Complex. All manipulations were done at 4°. The enzyme complex obtained from 17.5 g of *Euglena* was purified through DEAE-cellulose chromatography as described by Wolpert and Ernest-Fonberg (1975) and concentrated by precipitation at 60% saturation $(NH_4)_2SO_4$. The precipitate was collected by centrifugation, dissolved in 10 ml of buffer A, and desalted on a G-25 column equilibrated in buffer A. The resulting solution (22 ml) was clarified by centrifugation at 48,000*g* for 15 min, and was then diluted (about fourfold) with 0.001 *M* imidazole buffer (pH 7.0), 0.007 *M* in 2-mercaptoethanol, until the conductivity equaled that of buffer A. The protein (150 mg) solution was pumped onto a DEAE-cellulose column, 2.5 cm × 10 cm, equilibrated with buffer A. The column was washed with equilibration buffer until the absorbance at 280 nm was less than 0.05 and then an 800-ml linear gradient from 0.02 *M* (buffer A) to 0.3 *M* (buffer A which was 0.28 *M* in NaCl) was applied. Finally, the column was washed with 100 ml of the 0.3 *M* buffer. Fractions of 10 ml were collected, and the flow rate was 11 ml/cm^2 per hr. Aliquots were assayed for malate dehydrogenase, phosphoenolpyruvate carboxylase, and acetyl-CoA carboxylase activities throughout the chromatogram. Fractions comprising the various enzyme activities were pooled and precipitated by dialysis against 60% saturation $(NH_4)_2SO_4$ in 0.1 *M* imidazole buffer (pH 7.0), 0.007 *M* in 2-mercaptoethanol. The enzymes were stored at 4° as slurries in the $(NH_4)_2SO_4$ solution until further use.

Estimation of Molecular Weight. Each of the enzyme concentrates from DEAE-cellulose chromatography was collected by centrifugation, dissolved in buffer A (1.5 ml, 10–45 mg of protein), and applied to a column (2.5 × 22 cm) of Bio-Gel A-1.5 calibrated with proteins of known molecular weight. The column was equilibrated and run in buffer A which was 0.01 *M* in NaCl at a flow rate of 5 ml/cm^2 per hr, and 2-ml fractions were collected and assayed for the appropriate enzyme activity and absorbance at 280 nm.

Electrophoresis on Cellulose Acetate. Millipore Phoroslides, 25 × 76 mm, were used as a medium for electrophoresis in a Millipore Phoroslide Cell Apparatus. Samples of 0.3 μl were applied to the strips, and electrophoresis was carried out at 100 V for 20–40 min at room temperature. Phoroslides were stained with a malate dehydrogenase specific preparation at 38° which contained the following: Tris buffer, pH 7.9, 0.2 *M*, 8 ml; NAD^+ (5 mg/ml), 0.4 ml; nitroblue tetrazolium (1 mg/ml of H_2O), 1 ml; phenazinemethosulfate (1.6 mg/ml of H_2O), 1 ml; and 0.05 *M* L-malic acid, 1.6 ml. The general procedure was that described by Markert (1968).

Results

Dissociation of the Multienzyme Complex. Application of the multienzyme complex to a DEAE-cellulose column under conditions where it was retained led to its dissociation (Figure 1). A clean separation of the constituent enzymic activities was not obtained, but they were no longer behaving as a single complex of proteins. This was shown by gel filtration chromatography of the DEAE-purified material (Figure 2) where three separate peaks of activity were obtained. Presumably, when the complex was bound to the DEAE-cellulose, the interaction with the resin in conjunction with high salt resulted in its dissociation whereupon all

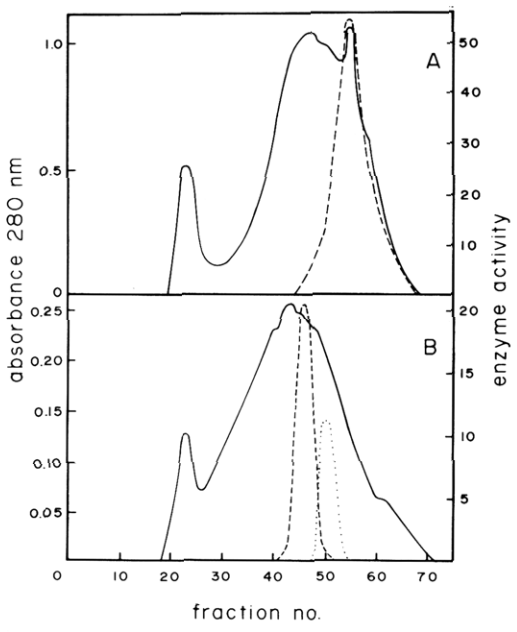

FIGURE 2: Gel filtration molecular weight determination of the dissociated enzymes of the multienzyme complex. Bio-Gel A-1.5, 2.5 × 30 cm, equilibrated in buffer A which was 0.01 *M* in NaCl, flow rate 10 ml/hr per cm^2; 2-ml fractions were used. Samples of 2 ml were layered under the buffer column. The solid line represents absorbance at 280 nm: (A) malate dehydrogenase from pooled and concentrated DEAE-cellulose fractions 45–60; enzyme activity shown by dashed line as μmol/15 min per ml; (B) pooled and concentrated fractions 61–80 from DEAE-cellulose chromatography; acetyl-CoA carboxylase shown by dotted line and phosphoenolpyruvate carboxylase shown by dashed line. Both activities given as nmol/ml per 15 min.

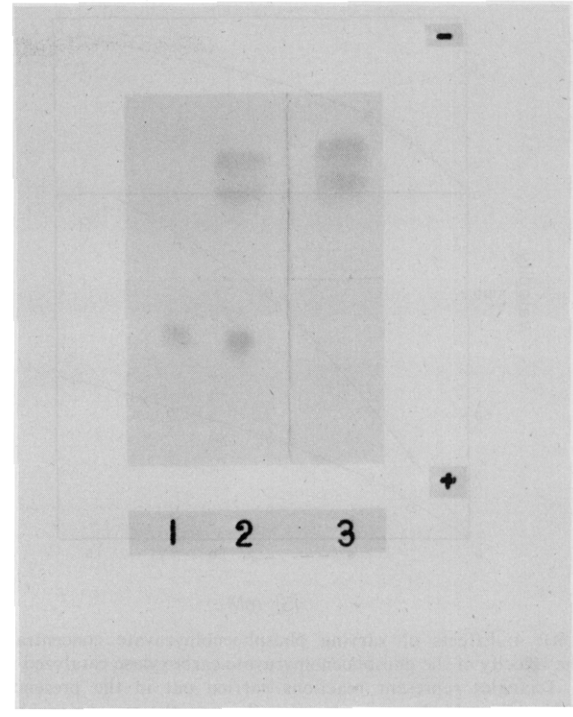

FIGURE 3: Electrophoretogram of *Euglena* malate dehydrogenases. Details of electrophoresis and staining are given in the text. Malate dehydrogenase obtained from the dissociated multienzyme complex is shown in 1. The isozymes which were not retained by DEAE-cellulose are in 3, while 2 shows the pattern obtained from crude extract of cells.

of the malate dehydrogenase activity from the complex (about fraction 53) was eluted. Practically all of the acetyl-CoA and phosphoenolpyruvate carboxylases were retained, and with still greater ionic strength, the majority of the acetyl-CoA carboxylase was eluted. Finally, the major portion of phosphoenolpyruvate carboxylase was eluted along with the remainder of the other carboxylase. Possibly there was some nonionic interaction of the two enzymes which was not disrupted readily by the conditions of increasing ionic strength employed. Alternatively, the acetyl-CoA carboxylase may have been interacting primarily ionically with the other carboxylase rather than with the resin, and when the phosphoenolypyruvate carboxylase released from the column, the remaining interacting acetyl-CoA carboxylase was eluted with it. Nevertheless, gel filtration chromatography (Figure 2) showed that the conditions did result in a separation of the two carboxylases. When a steeper gradient or faster flow rate was employed, the acetyl-CoA carboxylase was spread diffusely through fractions 60–100 indicating that the ionic interaction with phosphoenolpyruvate carboxylase which was suggested may be kinetically significant (Cann, 1970).

In Figure 2, the DEAE-cellulose dissociated proteins show noncoinciding enzymic activities with discrete peaks on gel filtration chromatography. Prior to dissociation, all three enzymic activity profiles coincided at a molecular weight of about 360,000 (Wolpert and Ernst-Fonberg, 1975). Subsequent to dissociation, the following molecular weights were obtained for the constituent enzymes: malate dehydrogenase, 67,000; acetyl-CoA carboxylase, 127,000; and phosphoenolpyruvate carboxylase, 183,000.

Euglena contains isozymic forms of malate dehydrogenase (Chancellor-Maddison and Noll, 1963), and an electrophoretogram of extract purified through 0–60% saturation with $(NH_4)_2SO_4$ showed three separate forms of this activity (Figure 3). The malate dehydrogenase which did not bind to DEAE-cellulose was seen as two bands of positively charged material at pH 8.4. The negatively charged malate dehydrogenase was the species found in the multienzyme complex. The electrophoretic mobility of the latter material was identical for the dissociated form and for the malate dehydrogenase in the complex. Thus, it appears that a unique form of malate dehydrogenase relative to other isozymes in the cell is present in the multienzyme complex.

Table I: Effect of NADH on Phosphoenolpyruvate Carboxylase Activity.[a]

NADH (m*M*)	Velocity[b]
0	9.3
0.27	13.7
0.53	13.2
0.80	16.8
1.33	12.8
2.66	11.6
5.33	8.8

[a] The enzyme was free phosphoenolpyruvate carboxylase purified through DEAE-cellulose chromatography. [b] Velocity was nanomoles of $H^{14}CO_3^-$ incorporated/minute.

Phosphoenolpyruvate Carboxylase. Both the complexed and free forms of the enzyme required phosphoenolpyruvate, HCO_3^-, and $MgCl_2$ for activity. The reaction rate was linear with enzyme concentration and with time (for at least 30 min). Product inhibition up to 20.98 m*M* oxaloacetate was not observed. The activity of the free phosphoenolpyruvate carboxylase was stimulated by the presence of acetyl-CoA, NADH, or ATP. As shown in Table I, the

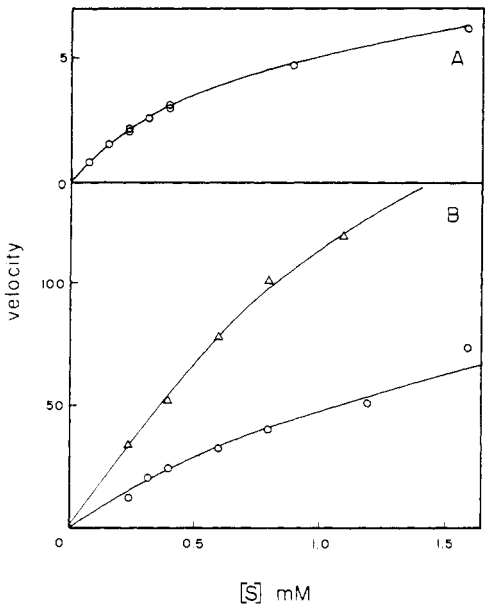

FIGURE 4: Effects of varying phosphoenolpyruvate concentrations on the velocity of the phosphoenolpyruvate carboxylase catalyzed reaction. Triangles represent reactions carried out in the presence of NADH, 0.75 mM. Experiments were done on the enzyme within the multienzyme complex (A) which had been purified to homogeneity and on the phosphoenolpyruvate carboxylase dissociated from the multienzyme complex by ion-exchange chromatography (B). The details of the assay procedure are described in the text. The curves are least-squares rectangular hyperbolae calculated from the experimental points shown.

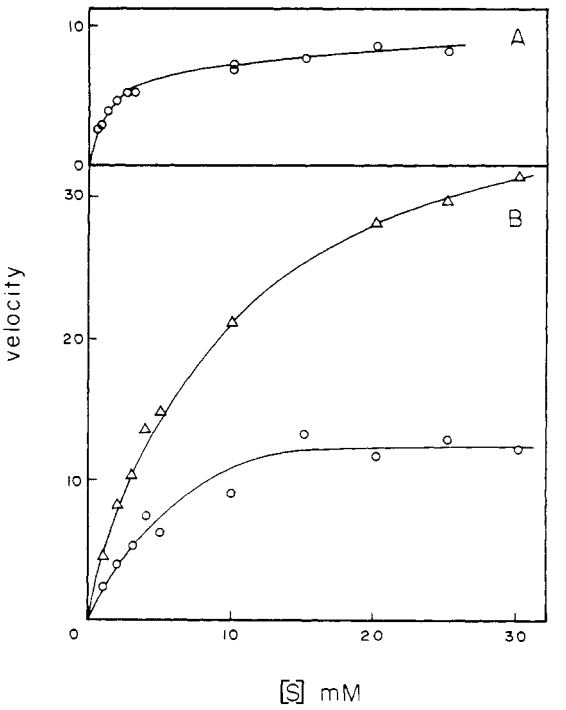

FIGURE 5: Effects of varying HCO_3^- concentration on the velocity of the reaction catalyzed by phosphoenolpyruvate carboxylase. Details are given in the text and in the legend for Figure 4.

stimulation by NADH passed through a maximum with increasing concentration of effector. Other instances of maxima in activation curves have been reported (Elliott, 1953; Kachmar and Boyer, 1952; Kuby et al., 1954). The highest velocity was seen in the vicinity of 0.8 mM NADH. Stimulation of the enzyme in the complex by these compounds was reported in the preceding paper (Wolpert and Ernst-Fonberg, 1975).

The dependency of the reaction rate on phosphoenolpyruvate concentration is shown in Figure 4, where in A data were obtained with the enzyme complex, and in B data were from the free phosphoenolpyruvate carboxylase. The lower curve in B and the curve in A were obtained in the absence of effector and yielded similar K_m values of 0.9–1.7 mM for phosphoenolpyruvate. The upper curve in B shows the results with 0.75 mM NADH included in the assay mixture and stimulation of rate is evident. The effect of NADH on phosphoenolpyruvate carboxylase was to increase the V_{max} from 16.12 in the absence of effector to 38.96 under the conditions cited, an increase of 242%; K_m was unaffected.

Dependency on HCO_3^- concentration is shown in Figure 5 where A and B again represent the phosphoenolpyruvate carboxylase in the complex and in the free forms, respectively. Comparison of the curves in A and the lower one in B which represent data obtained in the absence of effectors shows that the curve in A saturates with substrate more readily than the lower curve in B. The K_m for HCO_3^- of the free enzyme was 7.3–9.8 mM while that of the enzyme in the complex was 0.7–1.3 mM, almost an order of magnitude difference. Comparison of the curves in Figure 5B, with and without effector, again shows that NADH effected the enzyme by increasing the V_{max} by 242% while the K_m was unaffected.

Incubation of phosphoenolpyruvate carboxylase (either the free enzyme or the purified multienzyme complex) for 30 min in the presence of $H^{14}CO_3^-$ and oxaloacetate (4 mM) gave acid stable radioactivity, R_f 0.37. Oxaloacetate had an R_f of 0.37 in the same thin-layer chromatography system (Wolpert and Ernst-Fonberg, 1975). The exchange reaction was not due to the presence of oxaloacetate decarboxylase since the phenomenon was observed with the homogeneous multienzyme complex as well as with the free phosphoenolpyruvate carboxylase purified through DEAE-cellulose chromatography. Also, no $^{14}CO_2$ was incorporated in the presence of pyruvate, the product of oxaloacetate decarboxylase catalyzed reaction. Since the exchange occurred in the absence of nucleoside phosphates, the presence of phosphoenolpyruvate carboxykinase was ruled out.

Acetyl-CoA Carboxylase. The HCO_3^- dependence of both the free enzyme and the complexed form was the same. Each relationship described a rectangular hyperbola, and K_m values of 4.2–5.4 mM were obtained. Varying acetyl-CoA concentration yielded a sigmoidal relationship between the substrate concentration and the velocity of the reaction catalyzed by the free enzyme.

Discussion

A multienzyme complex obtained as a homogeneous protein by Wolpert and Ernst-Fonberg (1975) which contained the activities acetyl-CoA carboxylase, phosphoenolpyruvate carboxylase, and malate dehydrogenase has been dissociated into the cited constituent enzymes. The molecular weight of the complex was 360,000. The molecular weights of the individual enzymes have been shown by gel filtration and dimethyl suberimidate cross-linking (Wolpert, 1975) to be about 127,000, 183,000, and 67,000, the sum of which is 377,000. This excellent agreement between the whole and the sum of the parts indicates that the multienzyme complex consists of 1 mol each of acetyl-CoA carboxylase, phosphoenolpyruvate carboxylase, and malate dehydrogenase, respectively.

The parameters obtained for the individual enzymes comprising the complex were in general agreement with those available for similar enzymes isolated from other organisms. The molecular weight of 67,000 for malate dehydrogenase obtained from the multienzyme complex is identical with that of the noncomplexed form of the enzyme isolated from *Euglena* (Wolpert and Ernst-Fonberg, 1975). The latter, however, was shown to differ in electrophoretic mobility. A value of approximately 60,000 for the molecular weight of this enzyme from a multiplicity of sources including the *Euglena* isozymes was reported from a survey done by Murphey and colleagues (1967). Purification and examination of phosphoenolpyruvate carboxylase from five separate sources (Utter and Kolenbrander, 1972) gave molecular weights ranging from 200,000 for the enzyme from *Salmonella typhimurium* to 750,000 for the one from spinach. Molecular weights of intact acetyl-CoA carboxylases from plant and bacterial forms have not been reported.

The *Euglena* phosphoenolpyruvate carboxylase, like those described from other sources (Utter and Kolenbrander, 1972), is a complicated enzyme. It was shown to be activated by ATP, acetyl-CoA, and NADH. In an earlier study of the enzyme from *Euglena* (Ohmann and Plhak, 1969), it was concluded that these compounds as well as many others tested did not stimulate; however, the materials were present at a concentration of 5 m*M*. Since the data in Table I show that stimulation occurred within a narrow range of NADH concentration, stimulation would not have been observed under the experimental conditions employed by Ohmann and Plhak (1969). NADH at maximum stimulation concentration increased the V_{max} of the enzyme by about 240% while the K_m values for phosphoenolpyruvate and bicarbonate were unaffected. Acetyl-CoA stimulation of the enzymes from two bacteria, *S. typhimurium* (Maeba and Sanwal, 1969) and *Escherichia coli* (Smith, 1970), was due to both a lowering of the K_m for phosphoenolpyruvate and an increase in V_{max}. Stimulation of the phosphoenolpyruvate carboxylases from plant sources has not been observed (Utter and Kolenbrander, 1972). However, in view of the small range of concentration through which stimulation was seen in the enzyme from *Euglena*, it is possible that the appropriate effector concentrations were not employed in the plant systems investigated in this manner. The *Euglena* enzyme K_m for phosphoenolpyruvate reported here was 0.9–1.7 m*M*, which is in excellent agreement with the value of 1.6 m*M* reported by Ohmann and Plhak (1969) for the same enzyme.

Unlike phosphoenolpyruvate carboxylases from spinach (Bandurski, 1955), *Acetobacter* (Benziman, 1969), and peanut cotyledons (Maruyama and Lane, 1962), the enzyme from *Euglena* catalyzed some exchange between $H^{14}CO_3^-$ and oxaloacetate. There are other instances of contradictory findings with regard to reaction mechanisms for this enzyme from various sources. The enzyme from maize has been shown by Waygood and colleagues (1969) to utilize CO_2 as the reacting species while Maruyama et al. (1966) showed that the enzyme from peanut cotyledons used HCO_3^-. The ionic form of the substrate required by the enzyme from *Euglena* has not been determined.

A convincing indication that the existence of the multienzyme complex has a marked effect on the catalytic efficiency of phosphoenolpyruvate carboxylase is seen in the K_m values for HCO_3^- for the enzyme in the two forms. The free phosphoenolpyruvate carboxylase had a K_m of 7.3–9.8 m*M* for bicarbonate while the same enzyme bound in a multienzyme complex had a K_m of 0.7–1.32 m*M*. This finding further supports the intracellular existence of the multienzyme complex described by Wolpert and Ernst-Fonberg (1975) and its proposed function of capturing CO_2 for eventual use by acetyl-CoA carboxylase, another component of the multienzyme complex.

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