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Pyruvate Carboxylase from a Thermophilic *Bacillus*: Some Molecular Characteristics†

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ABSTRACT: Analysis of the native enzyme and of the subunits produced upon its denaturation shows that pyruvate carboxylase from a thermophilic *Bacillus* is a tetramer with a molecular weight (mean value) of 558 000 and that the four polypeptide subunits are probably identical. The three functions (carboxyl carrier, carboxylation, and carboxyl transfer) in the pyruvate carboxylation reaction must therefore reside in this quarter-molecular polypeptide. The enzyme molecule contains four atoms of zinc and four molecules of D-biotin, and in the electron microscope the disposition of its four subunits presents a rhombic appearance. Reaction of the denatured enzyme with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) reveals 10 sulfhydryl groups/subunit. In the native enzyme less than one of these groups reacts with DTNB. By contrast, all of these groups (11/subunit) of the native chicken liver pyruvate carboxylase are accessible to DTNB. The thermophile enzyme is also more resistant to other sulfhydryl reagents and to denaturation under certain conditions than the avian enzyme.

Pyruvate carboxylase [pyruvate:CO_2 ligase (ADP), EC 6.4.1.1], which catalyzes the generation of oxaloacetate by carbon dioxide fixation on pyruvate, is an enzyme that contains a divalent metal and covalently linked D-biotin and is of great strategic importance in the metabolic network of a number of organisms. The enzyme species from vertebrate tissues and yeast, which are activated by acetyl coenzyme A, are large tetramers of molecular weights in the range $(4.8–6) \times 10^5$, whereas those from the bacteria *Azotobacter vinelandii* and *Pseudomonas citronellolis*, which are not activated by acetyl coenzyme A, are approximately half this size (Scrutton & Young, 1972; Scrutton & Taylor, 1974; Taylor et al., 1975). The *Pseudomonas* enzyme has further been shown to consist of four subunits, two of one kind and two of another. The activity of the larger pyruvate carboxylases may be regulated by effector metabolites other than acetyl coenzyme A, such as acetoacetyl coenzyme A and L-aspartate, which act as inhibitors (Utter et al., 1975).

Our examination of the enzymes mediating carbon dioxide fixation on C_3 metabolites in thermophiles has shown that this function is fulfilled by pyruvate carboxylase in a moderately thermophilic *Bacillus* species but apparently by phosphoenolpyruvate carboxylase in the extremely thermophilic bacterium *Thermus aquaticus* (Sundaram et al., 1969; Sundaram, 1973; Bridger & Sundaram, 1976). The thermophile pyruvate carboxylase has been purified to homogeneity, and a number of its kinetic and regulatory properties have been detailed (Cazzulo et al., 1970b; Libor et al., 1975, 1978). In the present study we have elucidated several of its molecular characteristics such as molecular size, biotin content, identity and content of bound metal, subunit structure, number of sulfhydryl groups, and arrangement of the subunits as seen in the electron microscope.

Materials and Methods

D-[*carbonyl*-^{14}C]Biotin, obtained from the Radiochemical Centre, Amersham, was purified by the method of Wright et al. (1954). Nonradioactive biotin from Sigma (London) Chemical Co. was recrystallized twice from water before use. Guanidine hydrochloride from the British Drug Houses Ltd. was purified by the procedure of Nozaki (1972). Ammonium sulfate (specially low in heavy metals) was purchased from British Drug Houses Ltd., Sepharose 6B was from Pharmacia, and protein markers were from Boehringer (London) Ltd.; phosphocellulose was a Whatman product. Acetyl coenzyme A was prepared by reacting coenzyme A with acetic anhydride as described previously (Cazzulo et al., 1970b). All other chemicals were purchased from commercial sources.

Pyruvate carboxylase was isolated by methods previously described and was assayed spectrophotometrically at 43 °C (Cazzulo et al., 1970b; Libor et al., 1978). Protein contents were determined by the methods of Lowry et al. (1951) and Warburg & Christian (1941) and by the microbiuret method

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described by Munkres & Richards (1965).

Polyacrylamide Gel Electrophoresis. For the native enzyme, the method of Ornstein & Davis (1961) was followed. The acrylamide concentration in the running gel was 5 or 7.5%; the gels were prepared in 0.5 × 7.5 cm tubes, and electrophoresis was carried out at a current strength of 2 mA/gel. After electrophoresis the gels were stained for protein with Amido Black [0.1% in 7% (v/v) acetic acid] and then destained with 7% acetic acid. The denatured enzyme was electrophoresed in polyacrylamide gels, prepared in 0.5 × 15 cm tubes, containing sodium dodecyl sulfate ($NaDodSO_4$) by the procedure of Laemmli (1970). After electrophoresis at 2 mA/gel, the gels were stained with Coomassie Brilliant Blue and then destained with a water–methanol–acetic acid mixture. The enzyme was denatured, before electrophoresis, by one of the following methods: (a) it was added to a boiling sample buffer containing 1% $NaDodSO_4$ and mercaptoethanol (Laemmli, 1970) and 0.3 mg/mL of the protease inhibitor phenylmethanesulfonyl fluoride, and the mixture was boiled for 5 min in order to ensure complete dissociation of the protein and prevent proteolytic degradation of the subunits produced; (b) it was carboxymethylated in 7.5 M urea or 6 M guanidine hydrochloride (Weber et al., 1972); and (c) it was succinylated in 7.5 M urea (Jaenicke et al., 1968). Slicing and drying of gels for autoradiography were done as described by Fairbanks et al. (1965). For measurement of radioactivity in the gels, they were sliced transversely along their length, the slices were treated with NCS protein solubilizer (Basch, 1968), and the radioactivity was determined in a Packard Tricarb scintillation spectrometer, Model 2405, by using Bray's solution (Bray, 1960) or a Triton–toluene scintillant.

Determination of Biotin Content. The thermophilic *Bacillus* was grown at 55 °C in a salts medium supplemented with sodium succinate as carbon source (Sundaram et al., 1969) and devoid of exogenous biotin. Cells from this culture were inoculated into a similar medium containing [^{14}C]biotin (50 μg/L, 0.05 μCi/μg) and grown to late exponential phase at 55 °C. The cells were harvested, and the pyruvate carboxylase was isolated from them in a homogeneous state as judged by the results of electrophoresis of the native and denatured enzymes. The radioactivity in the pure enzyme was determined in a Packard scintillation spectrometer. The biotin content of the enzyme protein was calculated from the specific radioactivity of the [^{14}C]biotin determined by using internal standards.

Confirmation of the result from the above experiment was provided by direct microbiological assay of the biotin, using *Neurospora crassa* as a test organism, in the pure enzyme isolated from cells grown in a biotin-rich medium. The salts medium of Horowitz & Beadle (1943) was used, and the assay was carried out as recommended by Snell (1950). The basal medium for the assay was treated with activated charcoal to remove any traces of biotin, and all glassware was soaked in chromic acid and then thoroughly rinsed with water. The enzyme protein was hydrolyzed for the assay by treatment with 3 M sulfuric acid for 30 min at a steam pressure of 15 psi. It was established that these hydrolysis conditions released the maximum amount of biotin from the protein.

Identification and Determination of Bound Metal. The glassware used in this experiment was rinsed in 5 mM ethylenediaminetetraacetate (EDTA). The purified pyruvate carboxylase preparation, 8 mL containing 4 mg of protein and 144 units of activity (micromoles of NADH oxidized per minute in the spectrophotometric assay at 43 °C), was dialyzed at 4 °C for a total of 24 h against two successive 2-L lots of 50 mM potassium phosphate buffer (pH 7) containing 2 mM EDTA. The dialyzed enzyme was assayed for magnesium, manganese, zinc, and cobalt by using a Perkin-Elmer 405B atomic absorption spectrophotometer. The second lot of dialysate buffer, when analyzed by the same method, did not contain significant amounts of these four metals. Standards for the assay were aqueous solutions of salts of the metals (Analar grade); these stock standards were diluted in the phosphate–EDTA buffer for the preparation of calibration curves. In the case of zinc, an additional standard was prepared from the metal (Analar grade) dissolved in dilute hydrochloric acid. The calibration curves were each linear in the range 0–1 μg of metal. Control tests showed that the calibration for each metal was unaffected by the presence of the other three metals or of bovine serum albumin (500 μg/mL).

Determination of the Molecular Weight of the Native Enzyme. A column (2.5 × 40 cm) of Sepharose 6B was equilibrated at 4 °C with 50 mM Tris-HCl buffer (pH 7.6) containing 1 mM EDTA and 150 mM KCl, and after the application of the protein sample elution was carried out with the same buffer mixture at a flow rate of 20 mL/h. The column was calibrated with marker proteins and the Stokes' radius (a) of the thermophile pyruvate carboxylase was obtained by interpolating its elution volume in the standard curve relating the Stokes' radii of the marker proteins to their elution volumes (Ackers, 1964; Scrutton & Taylor, 1974). Substitution of this value for the Stokes' radius in the Einstein equation, $D = RT/N6\pi\eta a$ (Ackers, 1970), yielded the standard value ($D^0_{20,w}$) for the diffusion coefficient. The sedimentation coefficient ($s^0_{20,w}$) of the enzyme was derived from the sedimentation velocity in 20 mM potassium phosphate 0.2 M KCl buffer, pH 7, determined in a Beckman E analytical ultracentrifuge. The centrifugation was carried out at 20 °C and 60 000 rpm in a double sector cell at a series of protein concentrations (1.28–5.5 mg/mL). The molecular weight (M_r) of the pyruvate carboxylase was calculated from the Svedberg equation, $M_r = RTs^0_{20,w}/[D^0_{20,w}(1 - \bar{v}\rho)]$; the partial specific volume, $\bar{v}$, was assumed to be 0.74 mL/g, a value generally applicable to most globular proteins.

Determination of the Subunit Molecular Weight. The pyruvate carboxylase was denatured by a variety of methods, and the molecular weights of the subunit species produced were determined by $NaDodSO_4$–polyacrylamide gel electrophoresis as recommended by Weber et al. (1972).

Analysis of Pyruvate Carboxylase Denatured in Guanidine Hydrochloride. A Sepharose 6B column (1.5 × 40 cm) equilibrated with 6 M guanidine hydrochloride in 20 mM acetate buffer (pH 4.6) was prepared by the method of Kenneth & Wayne (1972). Protein samples were carboxymethylated and boiled for 5 min in the guanidine hydrochloride–acetate buffer and subjected to gel filtration through the Sepharose column; elution was carried out with the same buffer mixture at a flow rate of 3 mL/h, and the effluent fractions were monitored for protein by determination of absorbance at 225 nm. For the measurement of sedimentation coefficient, the thermophile carboxylase was carboxymethylated in 6 M guanidine hydrochloride and equilibrated with the denaturant in 0.1 M sodium acetate buffer (pH 4.6) containing 1 mM dithiothreitol by extensive dialysis. The sedimentation velocity was determined at 25 °C and a speed of 507 000 rpm in a Beckman Model E analytical ultracentrifuge by using a single sector cell.

Determination of Sulfhydryl Groups. The purified thermophile carboxylase in 50 mM Tris-HCl buffer (pH 7.4)

containing 1 mM EDTA and 0.1 M NaCl was treated with 0.1% $NaDodSO_4$, 6 M guanidine hydrochloride, or 7.5 M urea dissolved in the same buffer. 5,5′-Dithiobis(2-nitrobenzoate) (DTNB) (0.5 mM) was added (Ellman, 1959), and the absorbance at 412 nm was measured against an appropriate reagent blank.

Identification of the Amino Terminal Group. Carboxymethylated pyruvate carboxylase (ca. 3 mg, 0.02 μmol, of protomer) dissolved in 6 M guanidine hydrochloride was treated with sodium bicarbonate (0.1 M) and 5 times its weight of dansyl chloride dissolved in acetone, and the dansylation reaction was carried out according to the method of Gray & Hartley (1963). After removal of excess dansyl chloride and hydrolysis of the protein, the dansylated amino acid in the hydrolysate was identified by thin-layer chromatography on polyamide sheets by using standards of authentic dansylated amino acids by the procedure described by Hartley (1970).

Electron Microscopy. Purified pyruvate carboxylase (1 mg/mL, 36–40 EU/mL) in 50 mM Tris buffer, pH 7.5, was diluted to either 20 or 80 μg/mL in 50 mM phosphate buffer, pH 7.5. The addition of acetyl coenzyme A (0.5 mM) to this preparation resulted in a significant increase in the frequency of intact tetrameric structures on the electron micrographs. In the procedure adopted as standard, the enzyme sample was treated with an equal volume of glutaraldehyde (E. M. grade, triple distilled; 5% (v/v) in 50 mM phosphate buffer, pH 7.5) in order to cross-link the subunits of the protein.

Ultrathin, hydrophilic carbon support films, prepared by evaporating carbon onto narrow strips of freshly cleaved, high-grade mica in an oil-free, turbomolecular-pumped vacuum system, were floated onto a clean water surface. The floating carbon film, still attached to the upper part of the mica strip to facilitate location, was picked up on copper support grids. Droplets of the enzyme solution were left for 30 s on the freshly prepared films, and excess liquid was then removed with a micropipet. Negative contrasting was carried out before the remaining enzyme solution had dried (Valentine et al., 1966). In the standard procedure the stain used was 2–4% sodium silicotungstate, pH 7–7.4.

Specimens were examined in a JEOL JEM 100B electron microscope fitted with a high-resolution, eucentric goniometer stage. Images were recorded at magnifications in the range 40000–100000. For calibration of the magnification, catalase crystals (Wrigley et al., 1972) and replicas of cross-lined diffraction gratings were used. Measurements of the dimensions of enzyme particles were made on electron micrographs by using a Mitutoyo PJ-250 C optical comparator.

For simulation of the negative staining, a plasticine model of the tetrameric structure seen on electron micrographs was embedded in plaster of Paris and X-ray radiographs were taken with the model in selected orientations (Caspar, 1966).

Results

Molecular Weight of Native Enzyme. The complete calibration of the Sepharose 6B column with the various protein markers was achieved over several runs, and the elution volumes in each run were related to the void volume (determined with Blue Dextran 2000) in that run for the purpose of normalization. The Stokes' radius of the thermophile pyruvate carboxylase was derived to be 76 Å from the standard curve constructed with the following marker proteins: bovine thyroglobulin, 81 Å; *Escherichia coli* β-galactosidase, 68 Å; bovine liver glutamate dehydrogenase, 64 Å; rabbit muscle aldolase, 47 Å; pig muscle lactate dehydrogenase, 42 Å; and pig heart malate dehydrogenase, 28 Å. The Stokes' radii of the standards were either taken from the literature or calculated from the published values of their diffusion coefficients by applying the Einstein equation. The diffusion coefficient of the thermophile carboxylase, $D^0_{20,w}$, calculated from its Stokes' radius by this same equation, was 2.81×10^{-7} cm²/s. The sedimentation coefficient, $s^0_{20,w}$, determined in the ultracentrifuge, was 15.85 S. Substitution of these s and D numbers in the Svedberg equation yielded a value of 558000 for the molecular weight of the native thermophile pyruvate carboxylase. In good agreement with this result was the direct estimate of the molecular weight of 600000 ± 10% made from the gel filtration data by using the molecular weights of the marker proteins (Andrews, 1965).

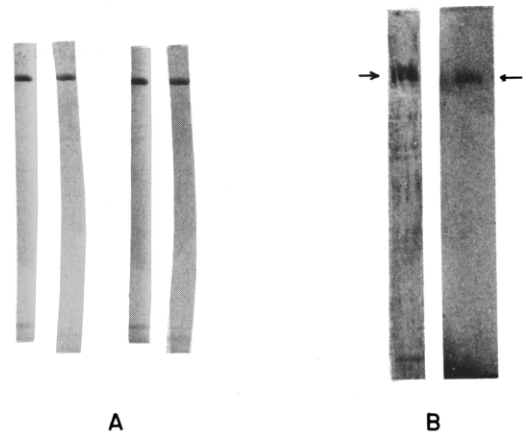

FIGURE 1: Electrophoretic analysis of denatured pyruvate carboxylase in $NaDodSO_4$–polyacrylamide. (A) From left to right: enzyme treated with $NaDodSO_4$–mercaptoethanol; enzyme carboxymethylated in guanidine hydrochloride; enzyme carboxymethylated in urea; enzyme succinylated in urea. Ten micrograms of denatured protein was run in each gel. (B) [^{14}C]Biotin-labeled pyruvate carboxylase (100 μg), after carboxymethylation in 6 M guanidine hydrochloride, was electrophoresed in $NaDodSO_4$–polyacrylamide. The gel was stained for protein and sliced longitudinally. The dried gel slice is shown on the left and the autoradiograph on the right. Arrows indicate the protein band and the radioactive band, respectively.

Subunit Molecular Weight and N-Terminal Group. The enzyme was electrophoresed in $NaDodSO_4$–polyacrylamide after denaturation by boiling in $NaDodSO_4$–mercaptoethanol, by carboxymethylation in 7.5 M urea or in 6 M guanidine hydrochloride, or by succinylation in 7.5 M urea. Irrespective of the denaturation procedure used, a single major polypeptide band was seen after the electrophoresis (Figure 1). The molecular weight of this polypeptide, determined by the method of Weber & Osborn (1969), ranged between 140000 and 155000 in the different experiments, in all of which this subunit species migrated slightly behind the β-galactosidase subunit. When the thermophile enzyme was labeled with [^{14}C]biotin, autoradiographs prepared from the gels featured a single radioactive band which corresponded with the single major polypeptide band visualized with the Coomassie Blue stain (Figure 1). Direct determination of radioactivity in transverse sections of the gels confirmed this observation. These results suggest that the pyruvate carboxylase consists of four polypeptide chains of similar size. This conclusion is supported by the following observations.

When the radioactive enzyme, after carboxymethylation in 6 M guanidine hydrochloride, was fractionated by gel filtration through Sepharose 6B equilibrated with 20 mM sodium acetate buffer, pH 4.6, containing 6 M guanidine hydrochloride, a radioactive component was identified in the breakthrough fraction, which was not separated from the subunit of β-galactosidase, a marker protein used, but was clearly separated from the subunits of smaller marker proteins such as bovine serum albumin, lactate dehydrogenase, aldolase,

Table I: Biotin Content of Thermophilic *Bacillus* Pyruvate Carboxylase[a]

expt	amount of biotin/mg of pyruvate carboxylase (μg)	amount of biotin/140 000 g of pyruvate carboxylase[b] (mol)
1	1.557	0.89
2	1.392	0.80
3	1.42	0.81
4	1.38	0.79
5	1.61	0.92

[a] In experiments 1–3 the biotin content of the pyruvate carboxylase was calculated from the radioactivity of the [^{14}C]biotin-labeled enzyme. In experiments 4 and 5, the biotin content was determined by microbiological assay. The enzyme was purified by the method described by Cazzulo et al. (1970b) or by the method described by Libor et al. (1978). The molecular weight of the subunit of the carboxylase is taken to be 140 000. [b] Mean value = 0.84 mol.

Table II: Metal Content of Thermophilic *Bacillus* Pyruvate Carboxylase[a]

metal	amount of metal/mg of pyruvate carboxylase (μg)	amount of metal/140 000 g of pyruvate carboxylase (g-atom)
zinc	0.45	0.96
magnesium	0.02	0.115
manganese	0.01	0.025
cobalt	0	0

[a] The molecular weight of the subunit of the carboxylase is taken to be 140 000.

and myoglobin. Electrophoretic analysis in NaDodSO$_4$–polyacrylamide showed that the molecular weight of this radioactive polypeptide was about one-quarter that of the molecular weight of the native carboxylase.

The sedimentation coefficient of the carboxymethylated polypeptide at 25 °C (s_{25}) in 6 M guanidine hydrochloride, determined in the ultracentrifuge, was 1.4 S at a protein concentration of 3.5 mg/mL. An estimated value for the sedimentation coefficient at zero protein concentration, s^0, can be obtained from the data of Tanford et al. (1967) on a set of 12 proteins. Tanford et al. point out that the quantity $s^0(1 - \phi'\rho)$ should vary systematically with molecular weight or chain length of the polypeptide (ϕ' is the effective specific volume of the protein and ρ the solvent density). Fitting the sedimentation coefficient of the carboxymethylated pyruvate carboxylase obtained by us into the data of Tanford et al., we find that it is compatible with a molecular weight of ~150 000 for the subunit of the enzyme, which was determined by the other methods.

Qualitative end-group analysis revealed a single dansylamino acid, dansylserine, in the hydrolysate of the dansylated pyruvate carboxylase. This indicates, but does not prove, the existence of a single amino acid, serine, at the N terminus of the enzyme. Although not conclusive evidence, this finding is consistent with the pyruvate carboxylase molecule containing only one kind of subunit.

Biotin and Metal Content. Three separate experiments in which the radioactivity of pure pyruvate carboxylase samples isolated from cells of the thermophile grown in a salts medium supplemented with [^{14}C]biotin was determined showed that the value for the biotin content of the enzyme protein approximated to 1 molecule/subunit (Table I). The validity of this result rests on the assumption that no significant amount of endogenously synthesized biotin was incorporated into the enzyme under the growth conditions employed. Experimental support for this assumption was provided by the finding that the specific pyruvate carboxylase activity in cell-free extracts prepared from cells harvested from the salts–succinate culture devoid of exogenous biotin that was used to inoculate the [^{14}C]biotin-containing salts medium was never more than 0.2% of that in extracts of cells grown in a biotin-rich medium. Direct microbiological assay of the biotin in an acid hydrolysate of pure pyruvate carboxylase corroborated the value for the biotin content obtained above (Table I).

Atomic absorption spectrophotometric analysis of pure pyruvate carboxylase, typical results of which are presented in Table II, revealed that the value for the zinc content of the enzyme was nearly 1 atom/subunit. Magnesium and manganese were present in negligible amounts, and cobalt was absent; these three metals have been detected in some of the other pyruvate carboxylases (Scrutton & Young, 1972).

Effect of Sulfhydryl Reagents and Denaturants. This study was undertaken in view of the greater resistance to denaturants and inhibitors that several thermophile enzymes possess in comparison with their mesophilic counterparts. At 20 °C *p*-hydroxymercuribenzoate at a 10-fold molar concentration inactivated the thermophile pyruvate carboxylase 50% and at 50-fold concentration the pyruvate carboxylase was completely inactivated. In similar conditions DTNB inhibited 0–25%, fresh enzyme preparations being inhibited less than aged ones. Iodoacetate at a (2×10^4)-fold molar concentration inactivated the enzyme slowly, 90% in 48 h, and under similar conditions *N*-ethylmaleimide produced 30% inactivation. Neither of the substrates, pyruvate and ATP, nor the allosteric activator, acetyl coenzyme A, afforded significant protection against the inactivation. In similar studies the chicken liver enzyme was

Table III: Comparative Denaturability of Thermophilic *Bacillus* and Chicken Liver Pyruvate Carboxylases

denaturing condition	pyruvate carboxylase from thermophilic *Bacillus*	pyruvate carboxylase from chicken liver[a]
low temperature	not inactivated	reversibly inactivated
pH	85% recovery of original enzyme activity after exposure for 2 min to pH in the range 5–11 and readjustment to pH 7.5	irreversible inactivation when exposed to pH 6.2; 50% of activity recovered after exposure to pH 7.95
urea (0.4 M)	no effect on activity for at least 75 min	70% of activity lost in 75 min; inactivation reversible
urea (6 M)	inactivation; after exposure to denaturant for 2 h, 95% of the original activity regained upon dilution or removal by gel filtration of the urea	irreversible inactivation
sodium dodecyl sulfate (0.025%)	immediate irreversible inactivation	inactivation
guanidine hydrochloride (6 M)	irreversible inactivation	inactivation

[a] Data on the chicken liver enzyme are from Irias et al. (1969) and Utter et al. (1975).

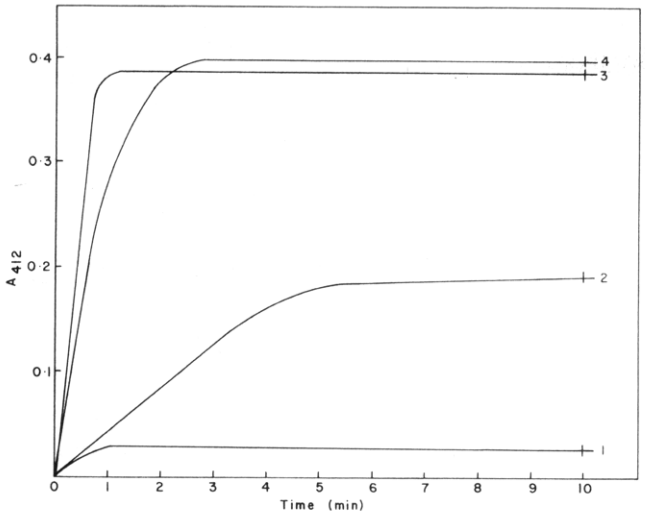

FIGURE 2: Reaction of the sulfhydryl groups of pyruvate carboxylase with DTNB. Native and denatured (as indicated) samples of pyruvate carboxylase were treated with DTNB (0.5 mM), and the absorbance at 412 nm was monitored for 10 min in a recording spectrophotometer. The protein concentration was 430 μg/mL. Assuming a molecular weight of 560 000 for the native enzyme, the absorbance change of 0.4 observed with the $NaDodSO_4$-denatured enzyme corresponds to the titration of 40 sulfhydryl groups/molecule of enzyme. (1) Native enzyme; (2) 7.5 M urea-denatured enzyme; (3) 6 M guanidine hydrochloride denatured enzyme; (4) 0.1% $NaDodSO_4$-denatured enzyme.

considerably more sensitive to these sulfhydryl reagents (Palacian & Neet, 1970).

Table III presents a comparative summary of the response of the thermophilic *Bacillus* and chicken liver pyruvate carboxylases to various denaturing treatments. Under several of these conditions the thermophile carboxylase was markedly less susceptible to denaturation; a notable feature is that it could be efficiently renatured after denaturation with 6 M urea.

Sulfhydryl Groups. In view of the marked resistance of the thermophilic *Bacillus* carboxylase to sulfhydryl reagents in comparison with the chicken liver enzyme, the number of sulfhydryl groups and their reactivity in the thermophile enzyme were examined. Upon treatment of the native carboxylase with DTNB at room temperature or 45 °C, less than 1 sulfhydryl group/enzyme molecule reacted with this reagent. In 7.5 M urea, 5 sulfhydryl groups reacted. In 0.1% $NaDodSO_4$ or 6 M guanidine hydrochloride, the reaction indicated 10 sulfhydryl groups/subunit of the enzyme molecule (Figure 2). The corresponding number for the chicken liver enzyme works out to 11/subunit of the tetrameric molecule on the basis of its recently revised molecular weight (Scrutton & Utter, 1965; Utter et al., 1975).

Electron Microscopical Appearance. Electron micrographs of samples of negatively stained, cross-linked thermophile pyruvate carboxylase showed clearly defined tetramers together with irregular denatured particles. Typical fields of view obtained after negative staining by the standard procedure appear in Figure 3. Most of the clearly defined tetramers exhibit a rhombic or near-rhombic projection and in this respect resemble the yeast pyruvate carboxylase tetramers observed by Valentine (1968) and by Wrigley (personal communication). Measurements of the dimensions of the rhombic outlines indicated a longer axis of 15 ± 1 nm and a shorter axis of 13 ± 1 nm, averaged over 20 particles. An approximate estimate of the size of the quarter-molecular subunit, derived from these values and the volume/molecular weight ratios given by Stryer (1968), is 140 000. This is in good agreement with the results from our electrophoresis and sedimentation experiments.

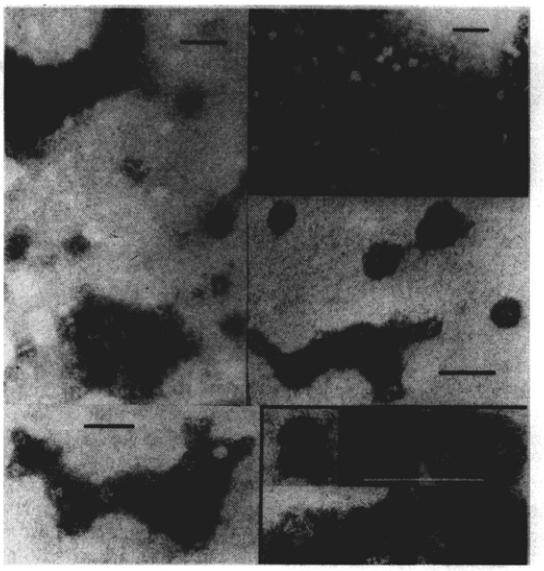

FIGURE 3: Electron microscopical appearance of thermophile pyruvate carboxylase. Typical fields of view are shown. The enzyme specimen was cross-linked with glutaraldehyde and negatively stained with sodium silicotungstate. Scale marker = 25 nm.

Treatment of the enzyme with glutaraldehyde prior to the negative staining increased the frequency of tetrameric structures on the electron micrographs, presumably by cross-linking the subunits and so preventing the dissociation of the tetramer. A similar observation has been made with yeast pyruvate carboxylase (N. G. Wrigley, personal communication). The addition of bacitracin (25 mM) as a wetting agent produced images of an unacceptably grainy appearance. Several negative stains besides sodium silicotungstate were tested: 0.3–2% uranyl acetate, pH 4.8; 1% uranyl formate, pH 5; 1% uranyl oxalate, pH 7; 1–2% ammonium molybdate, pH 6; and 1% phosphotungstic acid, pH 6.5–7.2. All of these proved inferior to sodium silicotungstate. However, similar tetrameric structures were observed with the various stains. This finding coupled with the following facts suggests strongly that these structures represent pyruvate carboxylase molecules: the enzyme used for the electron microscopy was highly purified as indicated by the appearance of a single protein band in polyacrylamide after electrophoresis in both the native and denatured states; a large number of specimens processed for electron microscopy from different enzyme preparations contained tetrameric structures similar in appearance and dimensions; enzyme samples treated with avidin, the protein that specifically binds biotin, yielded on electron micrographs, without the glutaraldehyde fixing, large structures which presumably were aggregates produced through cross-linking by avidin; the adding of acetyl coenzyme A, the allosteric activator of the carboxylase, and fixing with glutaraldehyde improved the frequency of tetramers on electron micrographs.

To simulate the electron microscope projections of pyruvate carboxylase, a model of the tetramer was constructed in plasticine. Each "monomer" in this model was a prolate ellipsoid with an axial ratio of ~1.4. A "dimer" was constructed by joining two monomers with their major axes coplanar and approximately at right angles in such a way that it possessed dihedral symmetry. The interlocking of two dimers, with one dimer lying in a horizontal plane and the other in a vertical plane, yielded a "tetramer" with 222 (*D2*) symmetry (Figure 4). Such a pseudotetrahedral arrangement

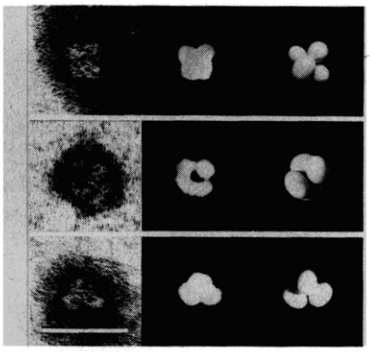

FIGURE 4: X-ray radiographic simulation of the electron microscope images of thermophile pyruvate carboxylase. On the left are three commonly observed electron microscope projections of the pyruvate carboxylase molecule. On the right is a plasticine model of the tetrameric molecule, constructed as explained in the text, and in the middle are X-ray radiographs of the model, embedded in plaster of Paris, in orientations which appear to correspond to those of the tetramer on the electron micrographs. Scale marker = 25 nm.

of the ellipsoid monomers can explain the frequently observed rhombic appearance of tetramers (Figure 3) even though the monomers are identical (Valentine, 1968). As illustrated in Figure 4, the model embedded in plaster of Paris and exposed to X-rays in various orientations gave radiographs closely resembling some of the commonly observed electron-optical images of the pyruvate carboxylase molecule.

Discussion

The present study shows that the thermophilic *Bacillus* pyruvate carboxylase is a tetramer consisting of polypeptide subunits of similar size. That the four subunits are identical, though not unequivocally established, is strongly suggested by the following considerations. The values for the contents of biotin and of zinc in the enzyme are close to 1 molecule and 1 atom, respectively, per subunit, and only one kind of amino acid has been detected at the amino terminus. The most convincing evidence comes from the finding (Libor et al., 1975; Buckley et al., 1979) that the thermophile pyruvate carboxylase binds to Sepharose–avidin, a strong specific affinity adsorbent for biotin and biotin–proteins, and that the protein eluted from the affinity column with buffer containing 6 M urea possesses only a very low level of enzyme activity but is reconstituted in good yield to virtually homogeneous active enzyme upon removal of the urea; this makes unlikely the existence in the enzyme molecule of a subunit not containing biotin. It has been suggested that the three distinct functions (carboxyl carrier, carboxylation, and carboxyl transfer) in the pyruvate carboxylation reaction might be fulfilled by separate polypeptide components of the complete enzyme, as in the acetyl coenzyme A carboxylase and methylmalonyl coenzyme A:pyruvate carboxytransferase systems (Vagelos, 1971). This is not borne out by our findings, which imply that, in the thermophilic *Bacillus* at any rate, the three functions reside in the large quarter-molecular protomeric polypeptide. Barden et al. (1975) have reached a similar conclusion with several tetrameric mesophile pyruvate carboxylases and surmise that dissimilar subunits reported by other workers (McClure et al., 1971; Warren & Tipton, 1974) may be artifacts produced by proteolysis.

The molecular size and subunit structure of the thermophilic *Bacillus* pyruvate carboxylase are quite similar to those of its acetyl coenzyme A activated counterparts from mesophilic sources, mammalian, avian, and yeast. The metal component of the thermophile enzyme, zinc, is also a constituent of the yeast enzyme but not of any of the vertebrate pyruvate carboxylases (Scrutton & Young, 1972). The apparently rhombic arrangement of the subunits revealed by electron microscopy, which probably is the consequence of the pseudotetrahedral disposition of ellipsoid subunits with a 222 (*D*2) symmetry as suggested by the results of the X-ray radiographic simulation (Figure 4), also seems to be a common feature of the yeast and *Bacillus* enzymes. Previous investigations have indicated that the subunits of the vertebrate pyruvate carboxylases are at the corners of a square rather than of a rhombus (Utter et al., 1975). More recent studies suggest, however, that these carboxylases may also have a rhombic profile (M. F. Utter, personal communication). A detailed examination of the regulatory properties of the thermophilic *Bacillus*, yeast, mammalian, and avian pyruvate carboxylases has shown that the *Bacillus* enzyme shares important characteristics with each of the other three species (Libor et al., 1978).

A thermophile enzyme sometimes (Bridgen & Harris, 1973) has a significantly lower content of sulfhydryl groups than its mesophilic counterpart. The thermophile pyruvate carboxylase, however, appears to contain nearly as many sulfhydryl groups (10) per subunit as the chicken liver enzyme (11). A paucity of these groups therefore need not characterize all enzymes from thermophiles. The virtual lack of reaction between the native thermophile carboxylase and DTNB suggests that all of the sulfhydryl groups are situated internally in the protein structure. By contrast, all of the sulfhydryl groups of the chicken liver enzyme are accessible to DTNB. Denaturation with 7.5 M urea exposes only half of these groups of the thermophile enzyme (Figure 2), which indicates that under these conditions the protein structure is not completely unfolded. This would explain the nearly complete recovery of enzyme activity, which is not possible in the avian system, upon the removal of urea (Table III). The rapid inactivation of the thermophile pyruvate carboxylase by low concentrations of $NaDodSO_4$ (Table III) contrasts with the relative stability of enzymes such as glyceraldehyde-3-phosphate dehydrogenase from *Bacillus stearothermophilus* at a $NaDodSO_4$ concentration as high as 1% (Suzuki & Harris, 1975). This difference presumably stems from the fact that the dehydrogenase has a low isoelectric point, whereas the isoelectric point of the carboxylase is close to neutral.

The thermophilic *Bacillus* enzyme is the only acetyl coenzyme A activated species of bacterial pyruvate carboxylase that has been characterized in great detail. A pyruvate carboxylase, subject to acetyl coenzyme A activation, has been isolated from the mesophilic bacterium *Arthrobacter globiformis*, but not in a homogeneous state (Gurr & Jones, 1977). The *Bacillus* carboxylase is also one of the very few enzymes larger than 500 000 daltons that have been examined from thermophilic sources. In view of the ease with which it can be isolated (Libor et al., 1975), of its relative hardiness to thermal and chemical denaturation, and of its many similarities to cognate enzymes from yeast and vertebrates (Cazzulo et al., 1970b; Libor et al., 1978), the thermophile pyruvate carboxylase has proved to be a versatile experimental system. Another attraction is that only in this system is it possible to study with purified components the conversion of the biotin-free, enzymically inactive apoenzyme to the biotin-containing, enzymically active holoenzyme (Cazzulo et al., 1969, 1970a, 1971; Sundaram et al., 1971).

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