Reconstitution of the Pyruvate Dehydrogenase Multienzyme Complex from *Bacillus stearothermophilus*†

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ABSTRACT: The pyruvate dehydrogenase multienzyme complex from *Bacillus stearothermophilus* was inactivated and disassembled into its component enzymes by treatment with 2 M potassium iodide. The purified lipoate acetyltransferase (E2) core retained its icosahedral structure, and the lipoamide dehydrogenase (E3) component remained catalytically active, while the pyruvate decarboxylase (E1α and E1β) did not show appreciable residual activity. As a consequence, no overall pyruvate dehydrogenase complex activity was recovered when various amounts of the four component enzymes were mixed in 0.2 M potassium phosphate buffer, pH 7.6, containing 5 mM ethylenediaminetetraacetic acid (EDTA) and 0.5–5 mM dithiothreitol at 0, 25, or 40 °C. Inactivation and dissociation could also be achieved by incubation in 1 M glycine/H_3PO_4 buffer, pH 2.0–2.3, in the presence of 1 mM EDTA at 0 °C or by treatment with 8 M urea or 6 M guanidine hydrochloride in the presence of 1 mM EDTA at room temperature. Subsequent dilution of the denatured enzyme solution into 0.2 M potassium phosphate buffer, pH 7.6, containing 5 mM EDTA and 0.5–5 mM dithiothreitol produced substantial (~30%) recovery of pyruvate dehydrogenase complex activity under appropriate conditions of protein concentration and temperature. Reassembly of the complex was indicated by analytical ultracentrifugation, polyacrylamide gel electrophoresis, and electron microscopy. The kinetics of reactivation suggest that reshuffling of the reassembled subunits, not regeneration of the lipoate acetyltransferase (E2) core, or refolding of lipoamide dehydrogenase (E3) is rate determining in generating the active pyruvate dehydrogenase complex. It is evident that in vitro assembly of the multienzyme system is a spontaneous process that has no obligatory proteolytic processing step.

Pyruvate dehydrogenase is a multienzyme complex that catalyzes the overall reaction

$$CH_3CO\text{–}COOH + CoASH + NAD^+ \rightarrow CH_3CO\text{–}SCoA + NADH + H^+ + CO_2$$

The reaction involves two intermediate states with thiamin pyrophosphate and lipoic acid as coenzymes and FAD[1] as the cofactor reoxidizing the reduced lipoic acid. Three enzymes function successively in the process: decarboxylation (catalyzed by E1, pyruvate decarboxylase, EC 1.2.3.1), lipoate acetyl transfer (catalyzed by E2, the respective transferase, EC 2.3.1.12), and lipoamide reoxidation (catalyzed by E3, the FAD-dependent lipoamide dehydrogenase, EC 1.6.4.3). FAD is finally restored by reduction of NAD^+ [reviewed by Reed (1974) and Perham (1975)].

Pyruvate dehydrogenase complexes from different organisms have been found to differ in their size and quaternary structure (Reed, 1974; Koike & Koike, 1976), but in all cases the E2 component forms a structural core to which multiple copies of the E1 and E3 components are bound tightly but noncovalently such that the substrate, fixed to E2 by a lipoyllysine swinging arm, may be transferred from one catalytic center to the other (Ambrose & Perham, 1976; Grande et al., 1976; Green & Oda, 1961; Koike et al., 1963). The E2 core of the pyruvate dehydrogenase complex from *Escherichia coli* appears to comprise 24 polypeptide chains arranged with octahedral symmetry (Reed, 1974; Danson et al., 1979) whereas that of the pyruvate dehydrogenase complex from *Bacillus stearothermophilus* appears to comprise 60 polypeptide chains arranged with icosahedral symmetry (Henderson et al., 1979). The E1 component of the *B. stearothermophilus* complex consists of two types of polypeptide chain, E1α and E1β, which aggregate with E2 and E3 to form a spherical particle of about 40-nm diameter and M_r about 10×10^6 (Henderson et al., 1979; Henderson & Perham, 1980).

In this paper we analyze the requirements for the spontaneous assembly of the *B. stearothermophilus* enzyme complex from its unfolded subunits. For the folding and association of simple oligomeric enzymes, a general mechanism involving sequential first- and second-order processes has been observed (Jaenicke, 1978, 1979; Jaenicke & Rudolph, 1980). Depending on the enzyme, as well as the conditions of denaturation–renaturation, the rate of reconstitution may vary from close to those of diffusion-controlled assembly reactions to those of very slow processes governed by folding reactions with high energies of activation. In the case of the most simple multienzyme complex, tryptophan synthase from *E. coli*, reshuffling of the recombined β_2 species has been found to be rate determining (Groha et al., 1978). Owing to the low specific activity of the separated β_2 protomers of that enzyme, the interdependence of the folding and association of the polypeptide chains within the heterologous quaternary structure could not be analyzed quantitatively.

With the pyruvate dehydrogenase complex, however, the overall reaction and the partial activities of the constituent enzymes can be investigated. In view of the thermophilic nature of *B. stearothermophilus*, the stability of the enzyme is an additional property of considerable importance. The wide temperature range accessible for reconstitution experiments

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[1] Abbreviations: CoA, coenzyme A; EDTA, ethylenediaminetetraacetic acid; FAD, flavin adenine dinucleotide; $NaDodSO_4$, sodium dodecyl sulfate; PDH, pyruvate dehydrogenase; PMSF, phenylmethanesulfonyl fluoride; TPP, thiamin pyrophosphate; NAD^+, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; NMR, nuclear magnetic resonance.

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allows us to study the specific requirements for structure formation in a system that is adapted to extreme environmental conditions. Regarding the structural dynamics of proteins (Gurd & Rothgeb, 1979), the high mobility of significant parts of the polypeptide chain may determine the yield and rate of reconstitution (Zettlmeissl et al., 1982). As indicated by recent NMR studies, polypeptide chain mobility is a typical feature of the 2-oxo acid dehydrogenase complexes (Perham et al., 1981; Wawrzynczak et al., 1981; Perham & Roberts, 1981; Duckworth et al., 1982). Given the structure of the assembled enzyme complex, one would expect reconstitution to require a strictly defined tertiary structure of the subunits in order to form the E2 core to which E1α, E1β, and E3 are then specifically bound. The question of whether the folding reactions of the various components mutually affect each other during the process of the overall recovery of enzymatic activity thus becomes of interest. Similarly the effect of the various ligands (cofactors) on the yield and rate of reactivation requires consideration.

Materials and Methods

Enzyme and Reagents. Pyruvate dehydrogenase complex was purified from *B. stearothermophilus* according to Henderson & Perham (1980). For avoidance of proteolysis all purification steps were performed in the presence of 1–5 mM phenylmethanesulfonyl fluoride (PMSF) and 5 mM EDTA. No alterations of the specific activity of the enzyme complex (2.9 IU/mg in the PDH complex assay at 30 °C) or in the pattern of $NaDodSO_4$–polyacrylamide gel electrophoresis (E1α, E1β, E2, E3) were observed upon storage of the enzyme in the presence of the given additives at −20 °C over several weeks.

In the analytical ultracentrifuge the major species, sedimentation coefficient of about 70 S, showed a certain degree of asymmetry and anomalous broadening; faster sedimenting species did not exceed 7% of the total enzyme. For all reconstitution experiments a stock solution of the enzyme containing 34 mg/mL was used. Cofactors (NAD^+, FAD, TPP, CoA) were purchased from Boehringer, Mannheim, West Germany, and Sigma, St. Louis, MO, respectively. All chemicals were of AR grade purity. Doubly distilled water was used throughout.

Enzyme Assays. Protein concentrations were estimated from the absorbance at 280 nm ($A^{0.1\%}_{280nm}$ = 1.0 $cm^2 \cdot mg^{-1}$), correcting for turbidity according to Englander & Epstein (1957). The overall catalytic activity of the complex was determined in 0.2 M potassium phosphate, pH 7.0, containing 5 mM EDTA and 1 mM dithiothreitol ("standard buffer"), in the presence of 2.6 mM NAD^+, 0.2 mM TPP, 0.1 M pyruvate, 6.5 mM CoA, and 1 mM $MgCl_2$, by measuring the NADH absorbance at 340 nm. The catalytic activity of lipoamide dehydrogenase (E3) was determined similarly but with dihydrolipoamide as substrate (sp act. ~100 IU/mg, based on $A^{0.1\%}_{280nm}$ = 1.0 $cm^2 \cdot mg^{-1}$).

Analysis of Quaternary Structure. For characterization of the quaternary structure of the enzyme in its native and its deactivated state, analytical ultracentrifugation and quasi-elastic light scattering were applied. Sedimentation velocity and synthetic boundary experiments were performed in a Beckman Spinco Model E analytical ultracentrifuge with schlieren optics and ultraviolet absorption (12-mm double-sector cells, rotors An-D and An-F Ti).

For quasi-elastic light scattering (90° relative to the incident beam) the broadening of the Rayleigh line (λ_0 = 6328 nm) was measured by a digital autocorrelator with a Spectra-Physics He–Ne 15-mW laser (P. Johnson, unpublished results). Solvents and solutions were cleaned by repeated filtration through 0.45-μm Millipore filters and maintained at constant temperature (20 ± 1 °C) during the experiment. For correction of the viscosity of the solvents, relative viscosities were measured in an Ostwald viscometer (180 s for water, 20 °C).

Dissociation and Inactivation of the Native Enzyme Complex. Use was made of four different approaches: (a) dissociation and resolution of the components according to Henderson et al. (1979), dissolving the complex in 2 M KI in 50 mM sodium phosphate, pH 7.0, 10 mM 2-mercaptoethanol, 2 mM EDTA, and 0.02% NaN_3 at room temperature, and subsequently separating the components on Sepharose CL-6B (column 1.5 × 93 cm); (b) acid dissociation at 0 °C by means of 0.1–2-h incubation at pH 2.3, making 1:2 to 1:10 dilutions of the stock enzyme solution with 1 M glycine/H_3PO_4, pH 2.0–2.3, in the presence of 1–5 mM EDTA; (c) dissociation in urea at room temperature, by means of 1-h incubation in 8 M urea in the presence of 1 mM EDTA under nitrogen; (d) dissociation in guanidine hydrochloride at room temperature, by means of 0.1–1-h incubation in 6 M guanidine hydrochloride plus 1 mM EDTA under nitrogen.

Reconstitution of the Complex. For reconstitution experiments after resolution by KI treatment, stoichiometric amounts of the separated subunits E1α, E1β, E2, and E3 were incubated at various temperatures and assayed for overall pyruvate dehydrogenase complex activity. For reconstitution experiments after preceding inactivation and dissociation at pH ~2 or in the presence of urea or guanidine, the denaturation mixtures were diluted 1:50 or 1:100 into 0.2 M potassium phosphate buffer, pH 7.6, containing 5 mM EDTA and 0.5–5 mM dithiothreitol (or 20 mM 2-mercaptoethanol). In cases where SH-protecting agents were omitted, the renaturation buffer was degassed and saturated with N_2 at 0 °C. In the case of long-term reactivation experiments at low enzyme concentrations, all procedures were performed under nitrogen. For investigation of the effect of cofactors on the yield and rate of reconstitution, NAD^+, CoA, pyruvate, and TPP were added in concentrations equal to those in the standard assay mixture. The FAD concentration used was 70–100 μM (exceeding the estimated affinity constant of E3 at least 100-fold).

The kinetics of reconstitution were measured by transferring samples of the above mixtures at defined times to the buffer used for the enzyme assay held at various temperatures. Final values of reactivation were determined after up to 72 h. In order to correct for possible alterations to the native enzyme during the reconstitution experiments, the untreated native enzyme was incubated under identical conditions of concentration, temperature, and time. In general the enzyme was found to be stable enough for no corrections to be required.

Characterization of the Reconstituted/Reactivated Enzyme. Solutions were concentrated by ultrafiltration (Amicon Diaflo with PM 10 filters), and high molecular weight aggregates were removed by centrifugation (15 min at 5000*g*). Characterization included sedimentation analysis (analytical and sucrose density gradient centrifugation), electron microscopy, and $NaDodSO_4$–polyacrylamide gel electrophoresis. For electron microscopy, negatively stained samples (1% phosphotungstic acid, pH 7) were photographed in a Phillips EM 301 electron microscope (nominal magnification 43 000×), as described by Henderson et al. (1979).

Results

In order to investigate the reconstitution of the pyruvate dehydrogenase complex after dissociation, it is desirable to

Table I: Subunit and Protomer Molecular Weights of the Components of the Pyruvate Dehydrogenase Complex from *B. stearothermophilus*

enzyme	EC	band [a]	$M_{subunit}$ [a]	$s^0_{20,w}$ (S)	$D^0_{20,w}$ (F)	$(M_{s,D})_{protomer}$ [b]
pyruvate decarboxylase	1.2.4.1	E1α 3	42 000	(15) [c]		
		E1β 4	36 000			
lipoate acetyltransferase	2.3.1.12	E2 1	57 000	35	2.3 [d]	(1 400 000)
lipoamide dehydrogenase	1.6.4.3	E3 2	54 000	6.4	5.5	110 000 [d]

[a] From $NaDodSO_4$–polyacrylamide gel electrophoresis [cf. Henderson et al. (1979) and Henderson & Perham (1980)]; bands numbered according to their position from the cathode to the anode. [b] From ultracentrifugation (synthetic boundary sedimentation velocity runs), with peak fractions of the KI-treated enzyme complex (cf. Figure 1) after equilibrium dialysis against 20 mM sodium phosphate, pH 7.0, containing 2 mM EDTA, 5 mM 2-mercaptoethanol, and 0.02% NaN_3 at 4 °C. [c] Partially aggregated and contaminated by E2 and E3 (cf. Figure 1). [d] Not corrected for anomalous broadening due to contamination with E1α.

separate the constituent protein subunits before attempting to reassemble them again to form the native quaternary structure [cf. Koike & Koike (1976) and Koike et al. (1963)]. Two different approaches were tried: (i) preparative chromatographic separation of the subunits after treatment of the complex with 2 M potassium iodide (Henderson et al., 1979) and recombination of the components in stoichiometric amounts and (ii) dissociation of the native complex to the subunit mixture under denaturing conditions and subsequent renaturation by changing the solvent to a nondenaturing one. Both approaches require the characterization of the enzyme in its native and dissociated states.

Native and Dissociated States. Examination of the native enzyme complex in the ultracentrifuge at neutral pH and 0.1–0.2 M ionic strength (phosphate) unveiled a heterogeneous system characterized by a major component (85–90%) of $s^0_{20,w}$ = 70 ● 4 S, and an aggregation product (10–15%) of $s^0_{20,w}$ = 104 ± 4 S, which may be assumed to be the dimer (Henderson et al., 1979). The main component shows anomalous boundary spreading (Gilbert & Gilbert, 1980). Determinations of the diffusion coefficient from synthetic boundary runs ($D_{20,w}$ = (1.0 ± 0.2) × 10^{-7} cm^2 s^{-1}) gave a value roughly twice that from intensity fluctuation spectroscopy ($D_{20,w}$ = (0.6 ● 0.2) × 10^{-7} cm^2 s^{-1}). The synthetic boundary experiments may be affected by subunit stripping whereas anomalously low values of $D_{20,w}$ from light scattering may be attributed to the presence of high molecular weight material that has not been completely removed by Millipore filtration and of aggregates. However, combining the ultracentrifugation data in the Svedberg equation indicated the relative molecular mass of the pyruvate dehydrogenase complex to be of the order of 1 × 10^7, in agreement with earlier estimates (Henderson et al., 1979).

The pattern obtained after $NaDodSO_4$–polyacrylamide gel electrophoresis of the complex reflects the four-component system reported previously (Henderson et al., 1979; Henderson & Perham, 1980). The corresponding subunit molecular weights are summarized in Table I, which also contains sedimentation coefficients of the respective protomers, after dissociation of the components in 2 M potassium iodide and separation by gel filtration (see Figure 1). This treatment strips E1 and E3 from the E2 core. In order to obtain the components in their native state without significant irreversible denaturation, the gel filtration on Sephadex CL-6B was performed as rapidly as possibly [~10 mL/h flow rate [cf. Koike & Koike (1976) and Koike et al. (1963)]]. While E2 and E3 appeared to retain their native structure (as indicated by their high specific activity), E1α and E1β underwent irreversible denaturation during the separation procedure. The residual complex activity in the resolved E1 peak (Figure 1) could be ascribed to the presence of insufficiently separated native or reconstituted pyruvate dehydrogenase complex rather than to intrinsic E1 activity (see below). The respective $s_{20,w}$ values were also perturbed by aggregation, apart from insufficient

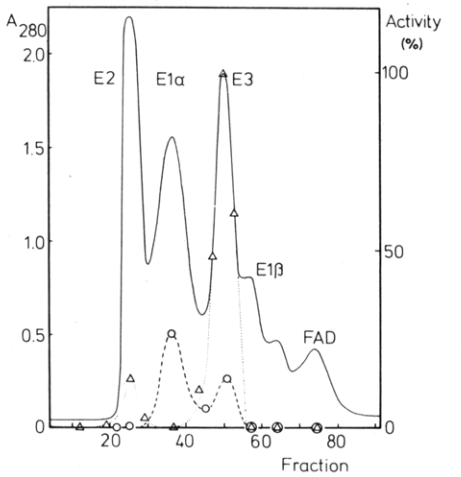

FIGURE 1: Gel chromatography of pyruvate dehydrogenase complex on Sephadex CL-6B in the presence of 2 M KI. 4 mL, c_{PDH} = 10 mg/mL in 50 mM sodium phosphate, pH 7.0, containing 2 M KI, 2 mM EDTA, 10 mM 2-mercaptoethanol, and 0.02% NaN_3; column 1.5 × 93 cm, 8 mL/h; 20 °C. Fractions were monitored for A_{280} (—), overall enzyme activity (○), and specific E3 activity (△). Peak fractions were pooled and dialyzed immediately againt 20 mM sodium phosphate, pH 7.0, 2 mM 2-mercaptoethanol, and 0.02% NaN_3 at 4 °C. Further identification of the respective enzyme components was by $NaDodSO_4$–polyacrylamide gel electrophoresis.

separation of both enzymes from E2. The large decrease in molecular weight upon dissociation of the native enzyme complex into its constituent components suggests a number of methods for monitoring the dissociation of the native quaternary structure: turbidity (A_{320nm}), (quasi-elastic) light scattering, ultracentrifugation, and gel filtration.

In the "stripping" of pyruvate dehydrogenase complex with 2 M KI the reaction is found to be slow (half-time at 20 °C ~15 min). Visual inspection of the change in the opalescence upon mixing native complex with 1 M glycine/H_3PO_4, pH 2.3 (1:1), or 8.8 M urea and 6.6 M guanidine hydrochloride (1:11), showed immediately that the multienzyme complex undergoes rapid dissociation. As indicated by ultracentrifugation and quasi-elastic light scattering measurements, the final products under these denaturing conditions are the monomeric subunits characterized by an average sedimentation coefficient $s^c_{20,w}$ = 2.0 ± 0.7 S and a scattering intensity decreased by a factor of ~20 compared with that of the native complex, the average diffusion coefficient being $\bar{D}$ = 7.5 × 10^{-7} cm^2 s^{-1} (corrected for viscosity).[2]

Treatment with KI leads to at least partly active components of the pyruvate dehydrogenase complex. On the other hand, the enzymatic activity after acid dissociation, as well as after incubation in 8 M urea or 6 M guanidine hydrochloride, is

[2] The presence of aggregates does not allow a quantitative evaluation of the scattering data.

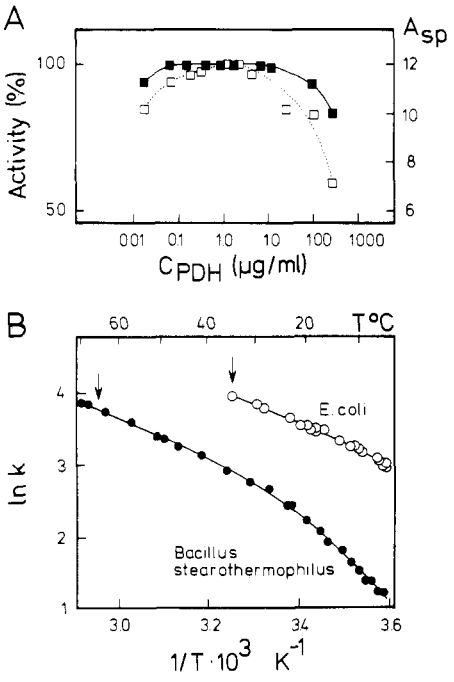

FIGURE 2: Concentration and temperature dependence of pyruvate dehydrogenase complex activity. (A) Concentration dependence of specific activity of enzyme complex from *B. stearothermophilus*. (Dotted line) Standard test: 52 mM potassium phosphate, pH 8.0, 2.6 mM NAD^+, 0.2 mM TPP, 1.0 mM $MgCl_2$, 0.13 mM CoA, 3 mM Cys, and 2 mM pyruvate plus ~0.05 IU (~2 µg) of the pyruvate dehydrogenase complex, 50 °C. (Solid line) Modified test with 5-fold excess of CoA and pyruvate. (B) Temperature dependence (Arrhenius diagram) of overall activity for the pyruvate dehydrogenase complex from *E. coli* (○) and *B. stearothermophilus* (●). Note that under their normal in vivo conditions both enzymes show similar temperature dependence and specific activity.

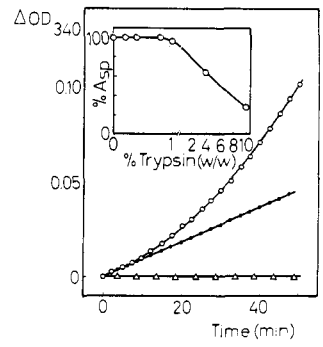

FIGURE 3: Effect of trypsin on reconstitution and overall activity of pyruvate dehydrogenase complex from *B. stearothermophilus*. Standard assay of 45 µg of enzyme complex after 2-min denaturation in 1 M glycine/H_3PO_4, pH 2.3 (0 °C), and 1:50 dilution with 0.2 M potassium phosphate, pH 7.0, containing 5 mM EDTA and 2 mM dithiothreitol in the presence and absence of trypsin: (△) plus 0.4% (w/w) trypsin upon dilution (no reconstitution); (●) plus 0.4% (w/w) trypsin in the enzymatic assay, after 32-min reconstitution in the absence of trypsin; (○) in the absence of trypsin (sigmoidal trace reflects further reconstitution during the enzymatic assay). (Inset) Effect of trypsin on overall activity of the native pyruvate dehydrogenase complex from *B. stearothermophilus*. Standard assay in the presence of increasing amounts of trypsin (30 °C).

zero. Reconstitution from these denaturants may therefore be considered to start from the fully dissociated inactive polypeptide chains.

Reconstitution. (a) Experimental Premises. Previous reconstitution studies on a variety of oligomeric enzymes have shown that the yield of reconstitution depends on a number of variables. Most important is the long-term stability of the given enzyme and constant enzyme activity in a wide range of enzyme concentrations. For the pyruvate dehydrogenase complex the low specific activity of the starting enzyme (2.9 IU/mg at 30 °C) represents an additional problem since the small changes of absorbance at low enzyme concentrations do not allow accurate kinetic measurements.

The standard assay for the overall complex reaction showed an unpromising concentration dependence under standard assay conditions: the rate was not linearly dependent on enzyme concentration, with decreasing activity at very low and high enzyme concentrations. However, raising the CoA and pyruvate concentrations by a factor of 5 improved the linearity sufficiently over the range of $0.1 < c_{PDH} < 120$ µg/mL (Figure 2A). Much higher enzyme concentrations are not feasible because of the formation of "wrong aggregates" upon reconstitution (Zettlmeissl et al., 1979, 1982). Errors due to the leveling off at low enzyme concentrations can be eliminated by assaying at a fixed enzyme concentration. Since reactivation does not exceed ~40% (see below), solutions subjected to reconstitution may contain up to ~200 µg/mL enzyme without a significant decrease of specific activity. The restrictions caused by the low specific activity could be overcome by applying elevated temperatures in the assay. As shown in Figure 2B, the Arrhenius plots gave nonlinear profiles; thermal denaturation of the thermophilic enzyme did not occur below ~70 °C.[3] The reconstitution experiments that follow were performed at 50 °C for two reasons: (i) difficulties in maintaining constant temperature upon starting the assay at higher temperatures caused severe errors in the kinetics; (ii) preliminary experiments proved intermediates of reshuffling to be unstable at 60 °C.

In order to eliminate reconstitution during the enzymatic assay, tests were performed in the presence of trypsin (Chan et al., 1973). As demonstrated in Figure 3 (inset), addition of <1.0% (w/w) trypsin to the standard assay did not affect the specific activity of the native enzyme complex. For the denatured enzyme (e.g., after incubation at pH 2.3 and subsequent dilution with standard buffer) no residual activity was observed as long as reconstitution was excluded by the addition of trypsin to the assay mixture. This is in agreement with the general observation that dissociated and (partially) unfolded oligomers are catalytically inactive. Enzyme activity observed after readjusting to quasi-physiological conditions must therefore reflect reconstitution rather than residual catalytic function.

(b) Reconstitution from Separated Enzyme Components. For reconstitution of the activity of the pyruvate dehydrogenase complex from its components after KI fractionation, the purified components were mixed at various concentrations and temperatures. The separate fractions were assayed for complex activity and then mixed and incubated at 0, 25, and 40 °C. The concentrations of the separate components (0.6–1.2 mg/mL) were normally adjusted to an equimolar ratio of E1, E2, and E3, but in some experiments excess E1α and E1β were added in order to compensate for the relative instability of these components. Only the exact sum of the residual complex activities of the purified components was obtained, without any further time-dependent regain of enzymatic activity reflecting reconstitution. Similarly, joint dialysis of the components immediately after KI fractionation in the presence of 5 mM EDTA, 1 mM dithiothreitol, and 0.2 mM PMSF did

[3] It is worthwhile mentioning that the enzymes from *E. coli* and *B. stearothermophilus* show closely similar specific activities and activation energies in the temperature range of their respective natural environments (Figure 2B).

not regenerate any catalytic activity in the pyruvate dehydrogenase complex reaction. Since electron microscopy and activity measurements clearly indicated that the KI treatment did not significantly alter the structure of E2 and E3, the unsuccessful attempts to reconstitute the enzyme complex from its separate components are probably due to irreversible denaturation of the pyruvate decarboxylase components E1α and E1β. Attempts to achieve reconstitution by lowering the incubation temperature failed.

With the enzymes from *E. coli* or from pig heart, it has been demonstrated (Koike & Koike, 1976; Koike et al., 1963) that incubation in ≥0.3 M KI leads to rapid irreversible loss of activity. Owing to the gel-filtration step needed to separate the components after incubating with potassium iodide, this approach does not allow much shortening of the incubation time, and it was not pursued further.

(*c*) *Reconstitution after Joint Dissociation–Reassociation.* Dissociation–reassociation of the enzyme after incubation at pH ~2 or in the presence of 8 M urea or 6 M guanidine hydrochloride was investigated next. Preliminary experiments showed that immediately after readjustment to solvent conditions favoring the native state of the enzyme by transfer of the dissociated enzyme to the reconstitution buffer, a significant recovery of the overall activity occurred (Figure 3). The effect was abolished completely if trypsin was present in the assay mixture. After longer periods of reconstitution, the effect of trypsin became insignificant so that for long-term kinetics, and for the determination of the final yield of reactivation, the proteinase was omitted.

The yield and the kinetics of reconstitution depended on the incubation time under dissociating conditions, as well as on temperature and enzyme concentration. After short incubation at pH 2.3, the yield of reconstitution amounted to ~20%. With increasing incubation time (t_i > 2 min) the yield (and rate) of reactivation decreased. A similar pattern has been observed for the reconstitution of lactic dehydrogenase after dissociation and denaturation at acid pH (Zettlmeissl et al., 1982). Different denaturants showed different effects. Thus, maximum yields of reactivation were achieved after denaturation in 8 M urea (~45%), whereas 6 M guanidine hydrochloride led to relatively low yields of reconstitution (probably because of insufficient purity of the denaturant).

With 2-min incubation in 1 M glycine/H_3PO_4, pH 2.3, as standard conditions, reconstitution was found to be negligible at low temperatures (≤10 °C). Beyond 60 °C biphasic kinetics, characterized by a rapid initial reactivation (with low yield), followed by denaturation, were observed, suggesting that intermediates of reconstitution are thermally unstable. With constant amounts of protein in the enzymatic assay, optimum conditions for reconstitution experiments were found to be t_i = 2 min, temperature ≤ 50 °C, and 10 < c_{PDH} < 170 μg/mL. The various cofactors had no significant effect. Since FAD is easily dissociated from the enzyme, 1 mM dithiothreitol plus 5 mM EDTA, 0.2 mM TPP, and 2.6 mM NAD^+ were tested in the presence and absence of up to 1 mM FAD. Neither the yield nor the rate of reconstitution was increased upon addition of the cofactor. The same holds for CoA (1 mM) and pyruvate (100 mM).

(*d*) *Kinetics of Reactivation.* After acid or urea denaturation of the enzyme complex, the initial rate of regain of pyruvate dehydrogenase complex activity was found to be independent of enzyme concentration at concentrations above ~30 μg/mL (Figure 4). Obviously, the reconstitution of overall activity obeys first-order kinetics with respect to the enzyme complex. Since catalysis of the overall reaction requires the cooperation of all three component activities, this result suggests that reshuffling of the reassembled subunits is rate determining in the given concentration range. For enzyme concentrations below 30 μg/mL (the temperature of the enzymatic assay was raised in order to increase the sensitivity), the rate of the reaction showed a slight concentration dependence. However, the accessible concentration range was insufficient to allow an accurate evaluation of the corresponding rate constant (inset, Figure 4).

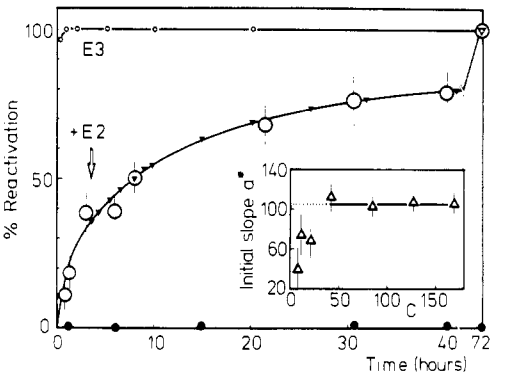

FIGURE 4: Kinetics of reactivation of the pyruvate dehydrogenase complex from *B. stearothermophilus*. Inactivation at acid pH (1:10 dilution with 1 M glycine/H_3PO_4, pH 2.3, 2-min incubation, 0 °C) and subsequent dilution (1:50) with 0.2 M potassium phosphate, pH 7.0, containing 5 mM EDTA and 2 mM dithiothreitol. Overall complex activity after reactivation at 0 (●) and 53 °C (○), calculated relative to final values determined after 72 h of reactivation. Open circles refer to concentration-dependent measurements in the range 20 < c_{PDH} < 170 μg/mL. At ↓E2, 8 μg of E2 core was added to the assay mixture ["seedling" (▼)]. E3: Aliquots of the reconstituting enzyme solution (8 μg) were assayed for E3 activity. (Inset) Contribution of higher than first-order kinetics to reactivation at low enzyme concentration. *c* (micrograms per milliliter) represents the concentration during reconstitution; a* represents the initial slope in enzyme assays containing a constant amount (7 μg) of enzyme in 1 mL of the standard test mixture (50 °C).

The reconstitution of E3 was observed to precede substantially the recovery of overall catalytic activity from denatured complex (Figure 4, curve E3). It appeared that the recovery of overall activity is slower by at least 2 orders of magnitude. In a further experiment, it was found that "seeding" with the E2 core (obtained from KI fractionation) did not alter the kinetics of reconstitution (Figure 4, ↓E2). These observations are consistent with reshuffling of the complex being rate determining in the process of reconstitution.

(*e*) *Product of Reconstitution.* The foregoing kinetic experiments have been based on the recovery of catalytic activity. In order to characterize the reconstitution product by structural means, analytical ultracentrifugation, sucrose gradient centrifugation (with the native enzyme as the standard), NaDodSO$_4$–polyacrylamide gel electrophoresis, and electron microscopy were undertaken.

In the analytical ultracentrifuge, schlieren pictures revealed three major components in varying proportions, a typical example being 20% 4S "subunits", 70% 40S E2 core plus 70S enzyme complex, and 10% >80S aggregates. Sucrose gradient centrifugation yielded a similar result (Figure 5). Apart from ~30% inactive subunits and <10% inactive high molecular weight particles, about 60% of the reconstituted material was found to band in the region of the native enzyme complex. These three fractions from the sucrose gradient were dialyzed against 20 mM sodium phosphate buffer, pH 7.6, and examined by NaDodSO$_4$–polyacrylamide gel electrophoresis. Densitometric analysis of the relative proportions of the bands showed that the enzymatically active species had a composition similar to that of the native complex. The values given in

Table II: Relative Amount of Subunits in the Native Pyruvate Dehydrogenase Complex and after Denaturation–Renaturation and Subsequent Sucrose Gradient Centrifugation[a]

state of enzyme	rel amount of subunits			
	E1α	E1β	E2	E3
native enzyme complex	0.99 ± 0.04	0.74 ± 0.08	1.00 ± 0.09	0.76 ± 0.08
aggregates (band I)	0.88	0.54	1.00	0.53
reconstituted enzyme complex (band II)	0.92	0.68	1.00	0.91
subunits (band III)	0.38	0.35	1.00	0.48

[a] $NaDodSO_4$–polyacrylamide gels were run of fractions in bands I–III (Figure 5) and examined in a densitometer. Peak areas are expressed relative to that of the E2 chain. Centrifugation conditions: 5 → 25% sucrose: 5 h at 24 000 rpm; SW 27 rotor.

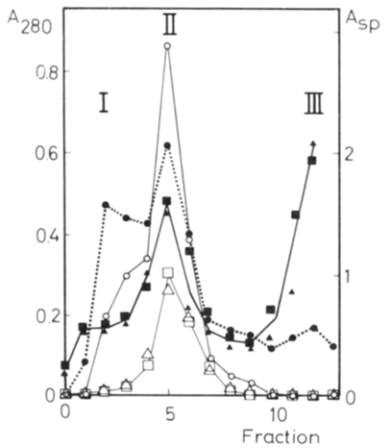

FIGURE 5: Sucrose gradient centrifugation of native and reconstituted pyruvate dehydrogenase complex from the *B. stearothermophilus* (○, ●) native enzyme complex (as reference), (△, ▲) reconstituted complex after acid dissociation and reactivation in the presence of 0.15 mM PMSF, concentrated by Amicon ultrafiltration (PM 10), and (□, ■) reconstituted complex after dissociation in 8 M urea and reactivation by 1:100 dilution (0.2 M potassium phosphate, pH 7.0, plus 5 mM EDTA), concentrated by Amicon ultrafiltration (PM 10). 5 → 25% sucrose gradient in 20 mM potassium phosphate, pH 7.0, containing 5 mM EDTA. Centrifugation was for 5 h at 24000 rev/min, 20 °C, SW 27 rotor. Fractions were monitored for A_{280} (closed symbols) and pyruvate dehydrogenase complex activity (open symbols). I, aggregates; II, pyruvate dehydrogenase complex; III, unreconstituted subunits.

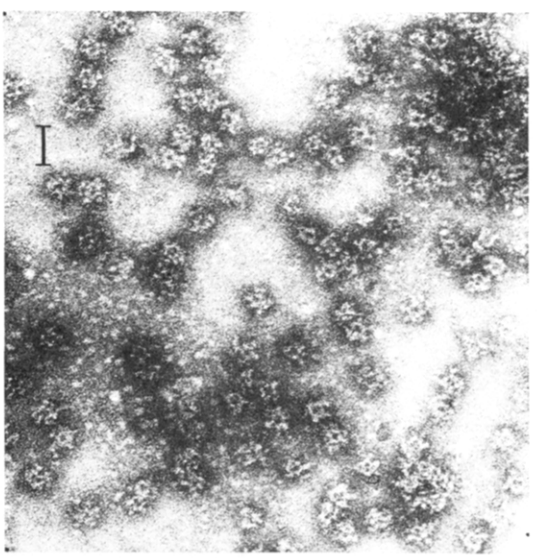

FIGURE 6: Electron microscopy of reconstituted pyruvate dehydrogenase complex after joint dissociation–reassociation. The sample was taken from fraction II (▲) of the sucrose gradient (Figure 5). The enzyme complex was fixed with glutaraldehyde, negatively stained with 1% phosphotungstic acid (pH 7). Philips EM 301 electron microscope, nominal magnification 43000×. Bar = 50 nm.

Table II represent the relative Coomassie stain of the four bands (expressed relative to that of the E2 chain), not the molar polypeptide chain stoichiometry. These results were supported by electron micrographs of fraction II from Figure 5, which demonstrated the existence of the E2 core (with both 2- and 5-fold rotational axes) and of the assembled pyruvate dehydrogenase complex as structural entities (Figure 6).

Discussion

It is a well-established fact that enzymes related to a metabolic reaction sequence may be specifically organized to form functional assemblies of noncovalently bound components. Owing to inherent structural instability, some multienzyme complexes may fall apart upon fractionation and purification, and the natural occurrence of multienzyme systems might therefore be more frequent than commonly assumed.

Among the multienzyme complexes that have been best studied in the past are the 2-oxo acid dehydrogenase complexes from various sources. They play a key role at the junction of the metabolic pathways of carbohydrates, lipids, and certain amino acids, with the tricarboxylic acid cycle. Their close relationship is clearly established by the observation that different enzyme complexes are found to share certain components, e.g., the E3 component of the pyruvate and 2-oxoglutarate dehydrogenase complexes of *E. coli* (Perham et al., 1978). This suggests that assembly in vivo proceeds by nascent cores of the two complexes being complemented from a single pool of E3 component common to both systems. Attempts to accomplish this process in vitro after separating the component enzymes in an active form were successful for a number of 2-oxo acid dehydrogenase complexes (Koike & Koike, 1976). With the pyruvate dehydrogenase complex from *B. stearothermophilus*, however, similar reconstitution in vitro was unsuccessful owing to irreversible denaturation and loss of activity of component enzymes (probably E1) of the complex during purification (Henderson, 1979). Comparable experiments described briefly above were equally unsuccessful.

On the other hand, reconstitution of the component enzymes after joint dissociation and inactivation of the *B. stearothermophilus* complex at acid pH or in 8 M urea led to partial recovery of overall enzymatic activity (Figures 3 and 4). Under these conditions the presence of all the component enzymes of the complex during the folding and self-assembly process may protect E1 from irreversible denaturation by incorporating metastable forms into the "growing" complex. As indicated by ultracentrifugation, sucrose gradient centrifugation (Figure 5), $NaDodSO_4$–polyacrylamide gel electrophoresis, and electron microscopy (Figure 6), the final product of reconstitution that shows pyruvate dehydrogenase complex activity was an assembled structure akin to the native enzyme. Its quaternary structure resembled the starting material in terms of its hydrodynamic and topological properties, especially having in mind the variation of polypeptide chain stoichiometries observed for different preparations of the native complex

(Henderson & Perham, 1980; R. Jaenicke, unpublished results).

The dissociated forms of the enzyme used for these experiments represent two widely differing states of the disassembled complex. Treatment with KI brings about a separation of the components of the complex (Figure 1 and Table I), the final products being the E2 core and E3 in their catalytically active form and the destabilized or even irreversibly denatured E1 components. In contrast, acid pH, 8 M urea, and 6 M guanidine hydrochloride led to complete dissociation of all four enzyme components, generating the separate polypeptide chains in their denatured and inactivated form. The heterogeneity of the respective systems was clearly shown by the asymmetry of the sedimentation boundary, as well as the heterogeneity factor calculated from quasi-elastic laser light scattering.

Two properties of the separate polypeptide chains need to be considered: (i) under the dissociating conditions we used (pH 2.3, 8 M urea) the separated subunits were enzymatically inactive when assayed for all component activities of the complex; (ii) the chain configuration of the enzyme components in the different denaturants differed widely. Both observations are important in connection with the quantitative interpretation of the reconstitution data (Jaenicke & Rudolph, 1980; Zettlmeissl et al., 1982).

Attempts to reconstitute the pyruvate dehydrogenase complex of *B. stearothermophilus* from the separated components have failed so far because of the instability of the pyruvate decarboxylase (E1), which tends to be irreversibly denatured in the presence of high molar concentrations of KI [cf. Henderson (1979) and Koike et al. (1963)]. As judged from the relatively low yields of reactivation after treatment at acid pH or with guanidine, similar side reactions also affected the "joint dissociation and reconstitution" experiments, which produced aggregates and unreconstituted material of low molecular weight apart from the active enzyme complex. Obviously the mode of denaturation, i.e., the incubation time and the chemical nature of the denaturant, strongly affects the kinetics and the yield of reconstitution. The effects are similar to those observed with less complex oligomeric systems (Jaenicke & Rudolph, 1980; Zettlmeissl et al., 1979, 1982; Jaenicke, 1982), and similar kinetic mechanisms can be used to describe them. For example, the transition from hyperbolic to sigmoidal profiles in the course of increasing incubation time at acid pH may be taken to reflect conformational rearrangements within the partially unfolded enzyme in its dissociated state. The mechanism

$$\begin{array}{ccccc} M_2 & \rightleftharpoons & M_1 & \rightleftharpoons & N \\ \downarrow & & \downarrow & & \\ D & & D & & \end{array} \qquad (1)$$

with M_1 and M_2 as different conformers of the monomeric species, D as (irreversibly) denatured protein, and N as native enzyme has been shown quantitatively to fit the data (Zettlmeissl et al., 1982).

With respect to the overall mechanism of reconstitution, comparison with simple oligomeric systems leads us tentatively to propose a kinetic scheme comprising fast unimolecular and bimolecular transconformation and association steps as precursor reactions, followed by a rate-determining reshuffling process that generates the native pyruvate dehydrogenase complex:

$$n\mathrm{M}_i^* \xrightarrow[\text{fast}]{\text{folding}} n\mathrm{M}_i \xrightarrow[\text{fast}]{\text{association}} (\mathrm{M}_i)_n \xrightarrow[\text{slow}]{\text{reshuffling}} \mathrm{N} \qquad (2)$$

where M_i represents the various enzyme components in their monomeric state and n the number of monomers assembled in the native complex (N). In the given scheme, the transition from the (partially) denatured (M_i^*) to the quasi-native format (M_i) of the monomeric species is the prerequisite of quaternary structure formation.

As noted before, long incubation at acid pH leads to a decrease in the yield and rate of reconstitution. The corresponding preequilibrium (eq 1) is not observed and may even be reverted (Zettlmeissl et al., 1982) by "complete denaturation", e.g., by urea, that is found to lead to an increased yield and rate of reactivation.

With respect to the rate-limiting step in the overall reconstitution, our experiments clearly indicate that the formation of neither the E2 core nor E3 is crucial. The various cofactors participating in the catalytic reactions did not affect the kinetics or the yield of reconstitution significantly. Instead intramolecular (first-order) transconformation reactions are found to govern the overall reconstitution. This is readily understood if we assume that the heterologous interactions between the components of the multienzyme complex are involved in mutally induced conformational changes, which finally generate enzymatic activity. Since the overall reaction requires all components, their assembly would be expected to precede the occurrence of enzyme activity. Only at very low enzyme concentrations did rate-determining second-order association processes become detectable.

The fact that active enzyme complex can be reconstituted from the fully dissociated protein subunits proves that in vitro assembly is a spontaneous process that is determined neither by the vectorial folding process of the nascent polypeptide chain nor by obligatory proteolytic processing steps.

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Enzymatic Condensation of Nonassociated Peptide Fragments Using a Molecular Trap†

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ABSTRACT: We have tested the feasibility of achieving protease-catalyzed condensation between nonassociating peptide fragments through mediation of a molecular trap. In this study, two subfragments of bovine pancreatic ribonuclease S-peptide, containing residues 1–10 and 11–15, were rejoined by clostripain catalysis to form the 1–15 peptide. The extent of this stereospecific condensation was enhanced by adding ribonuclease S-protein (residues 21–124), which acts as a trap in binding 1–15 but not 1–10 or 11–15 and which thus shifts the equilibrium to favor 1–15 formation. The resultant (1–15)·(21–124) noncovalent complex, defined as [des-16–20]-ribonuclease S, was detected by the enzymatic activity characteristic of the naturally derived ribonuclease S complex. Reaction of 1 mM S-protein and 20 mM fragments leads to 80% of the ribonuclease activity expected from the amount of 21–124 present. This indicates that 4% of the fragments 1–10 and 11–15 were condensed, compared to a maximal condensation of 5% based on the amount of trap. The less than theoretical yield is due largely to slow proteolytic degradation of 21–124 to a form which is no longer able to bind the condensation product 1–15. Yields were increased to 15% by addition of further trap. The successful synthesis of 1–15 emphasizes the usefulness of molecular traps to promote stereospecific fragment condensation between nonassociating peptide fragments for the synthesis and semisynthesis of polypeptides.

It is now recognized that by perturbing the equilibrium constant for peptide bond hydrolysis, peptide bonds can be synthesized between peptide fragments by using "proteolytic" enzymes. For example, inclusion of 90% glycerol in solutions of the noncovalently associated protein fragment complexes RNases S[1] (Homandberg & Laskowski, 1979; Homandberg et al., 1980), staphylococcal nuclease T (Homandberg & Chaiken, 1980; Komoriya et al., 1980) and the cytochrome *c* complex [(1–38)·(39–104)] (Homandberg et al., 1980; Juillerat & Homandberg, 1981) has been shown to promote resynthesis of the interfragment peptide bonds by the same proteolytic enzyme used initially under aqueous conditions to hydrolyze those bonds. The glycerol cosolvent decreases the free energy change associated with peptide bond hydrolysis principally by suppressing the ionization of the carboxylate liberated by the hydrolysis (Homandberg et al., 1978). For fragment condensation, this method has many advantages over chemical condensation, since it is stereospecific, has a high yield (25–50%/equilibration), does not require blocking of side chain groups, and proceeds under gentle conditions. However, an important limitation of the above procedure is its reliance on the existence of a noncovalently associated fragment complex. This fragment association must orient the carboxyl and amino groups to be attached so that they are close to one another as well as accessible to the proteolytic enzyme. Further, the complex must be stable in the presence of the organic cosolvent used to shift the equilibrium toward peptide bond synthesis.

A more general need in peptide fragment condensation is to join fragments that are nonassociating. In such a case, the method of enzymatic peptide fragment condensation suffers from a considerably unfavorable equilibrium in the synthesis direction. To increase yields for condensing nonassociating fragments, one can employ mass action as well as solvent-induced perturbation of equilibrium constants. Unfortunately, for many peptides, the high concentrations required are difficult to achieve. A more likely way to achieve such enzymatic fragment condensation would be to couple the unfavorable reaction of a synthesis

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[1] Abbreviations: RNase, RNase A, RNase S, and SRNase S, bovine pancreatic ribonuclease, ribonuclease A, ribonuclease S, and semisynthetic ribonuclease S, respectively; RNase-(1–15), the sequence of residues 1–15 of native RNase; RNase-S-(1–20) and -(21–124), the fragments of RNase containing residues 1–20 and 21–124, respectively.

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