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Molecular Structure of the Pyruvate Dehydrogenase Complex from *Escherichia coli* K-12

(multienzyme complex/three different polypeptide chains/1:1:1 molar ratio/48 total chains)

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ABSTRACT **The pyruvate dehydrogenase core complex from *E. coli* K-12, defined as the multienzyme complex that can be obtained with a unique polypeptide chain composition, has a molecular weight of 3.75×10^6. All results obtained agree with the following numerology. The core complex consists of 48 polypeptide chains. There are 16 chains (molecular weight = 100,000) of the pyruvate dehydrogenase component, 16 chains (molecular weight = 80,000) of the dihydrolipoamide dehydrogenase component, and 16 chains (molecular weight = 56,000) of the dihydrolipoamide dehydrogenase component. Usually, but not always, pyruvate dehydrogenase complex is produced *in vivo* containing at least 2–3 mol more of dimers of the pyruvate dehydrogenase component than the stoichiometric ratio with respect to the core complex. This "excess" component is bound differently than are the eight dimers in the core complex.**

Since the pioneering experiments of Gunsalus and Hager (1) and of Koike, Reed, and Carroll (2), α-ketoacid dehydrogenase complexes from various sources have been extensively studied. Several years ago, we began to study the *Escherichia coli* pyruvate dehydrogenase complex from a genetic point of view (3), because it appeared that the molar ratio of the constituent polypeptide chains was far from unity. We therefore began to study the regulation of the synthesis of the enzyme complex, and it became clear during these studies (4–6) that one would be hampered in further such experiments without a precise knowledge about the composition of this multienzyme complex; thus, we turned our attention toward an elucidation of its structure (7, 8,‡).

The complex contains three different polypeptide chains: those of the pyruvate dehydrogenase component (molecular weight = 100,000), those of the dihydrolipoamide transacetylase component (80,000), and those of the dihydrolipoamide dehydrogenase component (56,000). When separated from the complex (2), the pyruvate dehydrogenase and dihydrolipoamide dehydrogenase components exist as dimers (7, 2). The amount of pyruvate dehydrogenase present in the complex can vary (probably depending on growth conditions). A complex can be isolated that reproducibly has a unique polypeptide chain composition. From this complex, which we have called core complex, "excess" pyruvate dehydrogenase has been removed by chromatography on calcium phosphate gel. The molecular weight of the core complex is 3.75×10^6.

In this communication we present data on the molecular structure of core complex and of complexes containing more pyruvate dehydrogenase component than does the core complex.

MATERIALS AND METHODS

Cells and Reagents. Pyruvate dehydrogenase complex was prepared from K-12 strain YMel and from the regulatory mutants K1-1 LR8-13 and K1-1 LR8-16, which synthesize the enzyme complex constitutively (6). Growth conditions, purification procedures, enzyme assays, and sources of reagents were described (8).

Determination of Flavin-Adenine Dinucleotide (FAD). Our instrumentation was checked with FAD purchased from Boehringer Mannheim GmbH. The expected extinction coefficient (450 nm) of 1.13 cm^2/mmol (9) was found after the substance was dried for 3 days under reduced pressure at 60° over P_2O_5. All spectra were measured in 0.05 M potassium phosphate, pH 7.5, with a Zeiss PMQII spectrophotometer. Reduction and reoxidation of enzyme-bound FAD in the presence of 6.5 M urea was performed exactly as described by Massey, Hofmann, and Palmer (10). All protein concentrations for these spectral analyses were determined by the biuret reaction (11); the relation of dry weight to biuret assay has been reported‡.

Determination of Polypeptide Chain Ratio. Pure polypeptide chains were isolated by preparative dodecylsulfate–polyacrylamide gel electrophoresis (8). They were subjected to analytical electrophoresis under conditions essentially the same as those of Weber and Osborn (12). The gels contained 10% acrylamide, 0.135% methylene bisacrylamide, 0.05% sodium dodecylsulfate, and 0.05 M phosphate, pH 7.0. The gel columns were 60-mm high and had a diameter of 5 mm. Electrophoresis was for about 2.5 hr at 6 mA per column. The gels were stained for 2 hr with Commassie Brilliant Blue (12), and were immediately destained electrophoretically. The gels were then kept for 8 days in 7.5% acetic acid–5% methanol to remove all background stain. Stain intensity was measured with a Joyce–Loebl microdensitometer equipped with a red filter. Each column was measured twice; after the first tracing the column was rotated around its longitudinal axis by 90° and a second tracing was taken. The difference between the two measurements did not exceed 5% per band. Each point recorded in Fig. 1 is an average value of these two measurements. The areas below the tracings were cut out and weighed with a microbalance. (The density of the recording paper used was perfectly uniform.) Protein concentrations were determined by amino-acid analysis (8).

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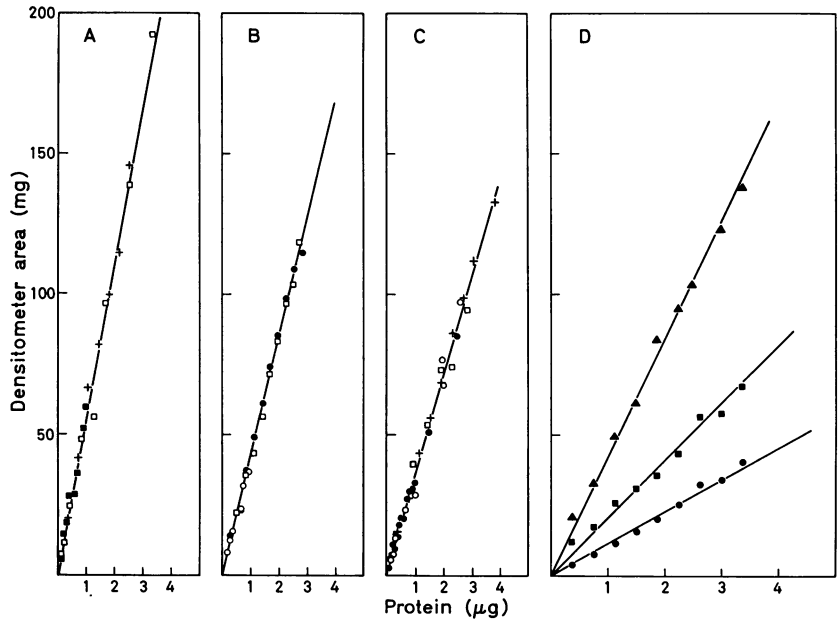

FIG. 1. Staining intensities of isolated subunits and of core complex. (*A*) pyruvate dehydrogenase; (*B*) transacetylase; (*C*) dihydrolipoamide dehydrogenase; (*D*) core complex. In *A–C*, different symbols represent different preparations of the polypeptide chains. In *D*: ▲, pyruvate dehydrogenase; ■, transacetylase; ●, dihydrolipoamide dehydrogenase. *A–C* are the standard curves for which the slopes are given in Table 1. *D* is an example of the determination of the polypeptide chain ratio in a core complex preparation. The data shown in Table 2 were derived from such sets of analyses; for each enzyme complex preparation, 5–10 different quantities of protein were electrophoresed and analyzed as in *D*.

Crystallization of the Enzyme Complex. Structures such as those shown in Fig. 2 were first found in an enzyme solution (4%) that had been stored at 4° for 8 months in 0.01 M potassium phosphate, pH 7, and to which ammonium sulfate had been added until a slight turbidity appeared. These "crystals" were enzymatically inactive in the oxidation of pyruvate to acetyl-CoA. They were used as seed crystals in 4% enzyme solutions to which a 50% (w/v) ammonium sulfate solution in 0.01 M phosphate, pH 7 (adjusted with ammonia), had been added (48 ml/100 ml). Dialysis at 4° against a 25% ammonium sulfate solution (same buffer and pH) in a desiccator, in the presence of silica gel, caused an almost quantitative conversion of dissolved protein to crystals. Suspensions do not show silkiness; although microscopically the overal appearance of the crystals are very uniform, it remains questionable whether each particle is a true crystal as clear crystal shape is not always evident (see Fig. 2). These crystalline preparations are fully active enzymatically.

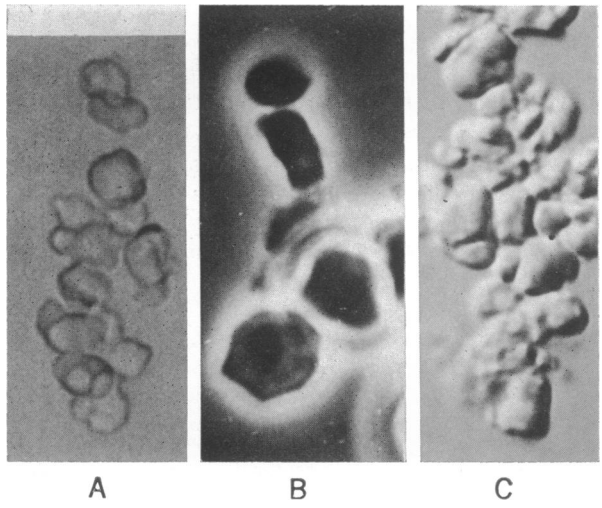

FIG. 2. Crystalline structures of pyruvate dehydrogenase complex. (*A*) bright-field; (*B*) phase-contrast; (*C*) Nomarski interference-contrast. Magnifications: *A*, ×1750; *B*, ×3200; *C*, ×1800.

RESULTS

Molar proportions in the complex

The size of all of its component polypeptide chains is known, and the complex can be dissociated into the component enzymes (2). Thus, at first sight it would appear a simple matter to determine the polypeptide chain proportions in the complex by mere dissociation of a known quantity of complex and measurement of the quantities of component enzymes liberated. However, there are intolerable sources of error in this procedure. First, the classical dissociation procedure (2) is not sufficiently quantitative for such a purpose (see ref. 8). Second, separation of the components cannot, of course, be achieved without losses of protein, and these losses cannot be

TABLE 1. *Slopes of standard curves from Fig. 1*

Component	Slope of standard curve (mg area/ μg protein)	Mean deviation of the average (%)
Pyruvate dehydrogenase	55.6	±2.4
Dihydrolipoamide transacetylase	42.0	±1.2
Dihydrolipoamide dehydrogenase	35.7	±1.8

TABLE 2. *Molar ratios of polypeptide chains in various pyruvate dehydrogenase complexes*

Type of enzyme complex	Strain	Preparation no.	Subunits: Pyruvate dehydrogenase	Subunits: Dihydrolipoamide transacetylase	Subunits: Dihydrolipoamide dehydrogenase	Average ratio
Core complex	LR8-13	1	1.10	0.94	1.00	1.07:0.95:1.00
	LR8-13	2	1.06	0.96	1.00	
	LR8-13	3	1.05	0.93	1.00	
	LR8-13	4	1.06	0.99	1.00	
	YMel	1	1.08	0.98	1.00	
	YMel	2	1.10	0.90	1.00	
"Complete" complex	YMel		1.35	1.00	1.00	1.34:1.01:1.00
	LR8-13		1.35	0.95	1.00	
	LR8-16		1.33	1.07	1.00	
"Native" core complex	YMel		1.03	1.07	1.00	1.00:1.02:1.00
	"K-12"		0.97	0.98	1.00	

All values were calculated by normalizing the value for the flavoprotein subunit to 1.00. *Core complex* is enzyme complex from which excess pyruvate dehydrogenase component had been separated by chromatography on calcium phosphate gel. It is the complex that upon rechromatography on this gel loses no further components. *"Complete complex"* from strains LR8-13 and LR8-16 has been obtained in only these two instances; it is unknown why in these cases excess pyruvate dehydrogenase was not removed by the preceding gel chromatography. "Complete" complex from strain YMel had been reconstituted; i.e., separated pyruvate dehydrogenase component had been added back to core complex, and the complex had been reisolated from the mixture by two centrifugations (2 hr 144,000 × *g*). *"Native" core complex* was found in two cases. In one ("K-12"), it was complex that had been purified from commercially available cells (7). These cells (*E. coli* K12) were grown by the manufacturer in an enriched medium (not specified) 67% of the way through the logarithmic phase. In the other case, it was complex we purified from strain YMel grown to about 2×10^8 cells/ml in complete medium. Chromatography of these preparations on calcium phosphate gel did not result in separation of any pyruvate dehydrogenase component.

measured with sufficient reliability. Therefore, we chose another strategy. The component polypeptide chains can easily be separated by dodecylsulfate–polyacrylamide gel electrophoresis (7, 8, 13). The intensity of staining of the resolved bands can be measured with high accuracy and, thus, should directly yield the desired information. The great advantage of the procedure is that the three chains are separated in one gel, i.e., for each amount of complex applied to a gel, identical experimental conditions occur for each individual protein component.

It has been shown (14) with starch gel electrophoresis and Commassie Brilliant Blue stain that the same amounts of different proteins can stain to different degrees. Standard curves for each polypeptide chain were prepared, as shown in Fig. 1. A linear increase of staining intensity was found for up to 5 μg of protein per band, and replicate intensity values deviated by $\leqslant \pm 10\%$. It was possible to greatly reduce the mean error of the slopes of the standard curves by measurement of a sufficiently large number of individual values. Statistical treatment of the data then showed this error to fall below that (about 3%) introduced by determination of the protein concentration (Table 1).

Knowledge of these slopes allowed us to determine the relative proportions of the polypeptide chains in the complex. In Fig. 1, the results of one set of such experiments are shown. The slopes of these curves divided by the slopes of the standard curves give the weight proportions of the chains in the complex; division of these by the molecular weights of the polypeptide chains gives the molar proportions of the chains in the complex. Table 2 shows the results. Quite clearly, there is a 1:1:1 ratio of the three different polypeptide chains in the core complex.

The molar ratios shown in Table 2 have been normalized by setting the flavoprotein data at unity. The values for the pyruvate dehydrogenase chain in most cases are too high by 5–10% for a strict 1:1:1 ratio, and the values for the transacetylase chains in several cases are too low by about the same extent. The explanation for the deficiency of transacetylase is clear; we have not found conditions that can be used reliably to prevent fragmentation (8, 13) of the transacetylase chain. In those cases where the transacetylase values are too low, this fragmentation had already begun. The apparently high values for the pyruvate dehydrogenase polypeptide chains may be explainable by the presence of residual "excess" pyruvate dehydrogenase in the core complex.

It is evident (Table 2) that in pyruvate dehydrogenase complex from which "excess" pyruvate dehydrogenase has not been removed, the polypeptide chain ratio is 1.35:1:1. We have shown‡ that after it is removed, "excess" pyruvate dehydrogenase can bind again to core complex; these data show that such reconstitution yields a complex with the same polypeptide chain ratio, 1.35:1:1. Finally, core complex apparently can also be produced *in vivo* ("native" core complex, Table 2).

Number of chains in core complex

We determined‡ the molecular weight of core complex to be $3.75 \pm 0.2 \times 10^6$. With a 1:1:1 molar ratio of the different polypeptide chains in the core complex, an excellent fit for the molecular weight is reached when 16 copies of each chain are present in the complex:

$16 \times 100{,}000$ (pyruvate dehydrogenase subunit)
$+ 16 \times 80{,}000$ (dihydrolipoamide transacetylase subunit)
$+ 16 \times 56{,}000$ (dihydrolipoamide dehydrogenase subunit)
$= 3.78 \times 10^6$.

TABLE 3. *FAD content*

	Protein (mg/ml)	Extinction (455 nm)	nmol FAD/ml	Minimum chemical molecular weight	Mol FAD per mol of complex
Complex 1	6.4	0.318	28.2	227,000	16.5
Complex 2	3.5	0.170	15.4	227,000	16.5
Dihydrolipoamide dehydrogenase	4.2	0.780	69	58,000	—

Complex 1 and complex 2 (molecular weight 3.75×10^6) are two different core complex preparations from the same strain (LR8-16). Dihydrolipoamide dehydrogenase, shown for comparison, was prepared from complex 2. In each case a complete spectrum between 350 and 540 nm has been measured.

FAD content of the core complex

From the data presented, the core complex should contain 16 dihydrolipoamide dehydrogenase polypeptide chains. In order to obtain independent evidence for this number of chains, we determined the FAD content of the enzyme complex.

The extinction coefficient of FAD bound to dihydrolipoamide dehydrogenase at 455 nm is not different from that of free FAD at 450 nm (10, 15); thus, it was likely that the same would be true for the coenzyme bound in the core complex.

In order to standardize the assay, native dihydrolipoamide dehydrogenase was prepared from core complex (2). As judged by dodecylsulfate–polyacrylamide gel electrophoresis, the protein was more than 95% pure upon ammonium sulfate fractionation (2). Using an extinction coefficient of 1.13 cm^2/mmol (455 nm), we found the minimum chemical molecular weight of the dehydrogenase to be 58,000; i.e., there is 1 mol of FAD per polypeptide chain (Table 3), in confirmation of several reports (2, 15, 16).

Next, we ascertained that the extinction coefficient does not change when the flavoprotein is bound in the core complex. To this end, the classical experiment of Massey, Hofmann, and Palmer (10) was repeated with the core complex. In brief, reduction with dithionite of the FAD bound to the dehydrogenase in the presence of 6.5 M urea leads to liberation of the coenzyme, upon reoxidation by air, the spectrum of enzyme-bound FAD is replaced by that of free FAD. The outcome of the experiment was exactly as had been described by Massey *et al.* (10) for the dihydrolipoamide dehydrogenase from pig heart (see their Fig. 3). In other words, free FAD at 450 nm has the same extinction coefficient as FAD present in the core complex at 455 nm.

For a 1:1:1 molar ratio of the three different polypeptide chains in the core complex, a minimum chemical molecular weight of 236,000 is to be expected: 100,000 (pyruvate dehydrogenase) + 80,000 (transacetylase) + 56,000 (flavoprotein). Table 3 shows that the minimum chemical molecular weight for the core complex based on the FAD content was 227,000 for two different preparations, i.e., 96% of the expected value. It can be calculated that for the molecular weight of 3.75×10^6 of the core complex, we find 16.5 mol of FAD per mol of enzyme complex. The agreement with the number of polypepetide chains seems reasonable.

DISCUSSION

Models

Space-filling models for the *E. coli* Crookes pyruvate dehydrogenase complex have been devised, on the basis of electron microscope studies (17–20), by Reed and colleagues; a modified model for the transacetylase has been drawn by Perham and Thomas (13). Unquestionably, excellent evidence exists for the transacetylase having cubic shape (*see* micrographs in ref. 18). Unless the assumption is made that *E. coli* Crookes produces a different enzyme complex than *E. coli* K-12, the arrangement of polypeptide chains in the transacetylase cube and the enzyme complex must be other than that proposed. The easiest way to construct a cube from 16 chains would, of course, be to use dimers as morphological subunits. The question as to how the pyruvate dehydrogenase and dihydrolipoamide dehydrogenase molecules are arranged around the transacetylase may have a relatively simple answer. A cube consisting of 16 identical polypeptide chains (or 8 dimers) naturally possesses 16 equivalent binding sites (or 8 when dimers are looked at), and the transacetylase *does* bind, in the core complex, eight dimers (each) of the other two components. A more intriguing question is how, and why, additional pyruvate dehydrogenase can be bound to the core complex. A polypeptide chain ratio of 1.35:1:1 (*see* Table 2) implies the presence of about three additional pyruvate dehydrogenase dimers per enzyme complex. It is entirely unclear how they may be bound. The very fact, however, that the "excess" pyruvate dehydrogenase is removed rather easily, while none of the remaining dimers are lost, shows that the "excess" is bound differently; this finding constitutes indirect evidence for the 8 equivalent positions mentioned above.

We have obtained preparations of the core complex that appear to be crystalline (Fig. 2). It may be that x-ray diffraction studies such as those reported for the transsuccinylase of the α-ketoglutarate dehydrogenase complex (21), will yield more information on the structure of the core complex.

Function of the enzyme complex

Considering a 1:1:1 molar ratio of polypeptide chains, one could expect the same ratio of active centers in the core complex. The pyruvate dehydrogenase dimer has two binding sites for pyruvate (22); we have confirmed this (unpublished data). There are two molecules of FAD in the dihydrolipoamide dehydrogenase dimer (2, 15, 16). If there were one lipoic acid residue per transacetylase chain, then the ratio of active sites in the core complex would be unity. However, Reed and associates, most probably working with the main fragment of 38,000 daltons (23) of the 80,000-dalton transacetylase chain, have found that there is one lipoic acid residue per about 36,000 daltons of transacetylase chain. The intact transacetylase should then have two lipoic acid residues per chain, which would bring the ratio of active sites to 1:2:1; this would not be impossible, but would be somewhat strange.

We have presented evidence‡ that the "excess" pyruvate dehydrogenase present in the enzyme complex may not participate in enzymatic catalysis; at least there is no significant loss in overall activity when this excess is removed. Therefore, it remains rather puzzling what purpose this excess enzyme serves, if indeed it does have any physiological significance. It should perhaps be kept in mind that bacterial behavior does not always exhibit recognizable teleological relevance.

Size of the enzyme complex

The pyruvate dehydrogenase complex appears to be a uniquely large soluble enzyme. When crude extracts are chromatographed on molecular sieves, the enzyme complex is the first material to be eluted after the void volume; it is then closely followed by α-ketoglutarate dehydrogenase (8). The bulk of cellular proteins are eluted only after these two enzymes. Why are the two ketoacid dehydrogenase complexes so big? A complete complex is not necessary for enzymatic catalysis (ref. 24, and unpublished data). It appears that the size of an enzyme and the complexity of its function are generally not correlated; e.g., lysozyme has a molecular weight of 14,500 (25) and β-galactosidase is 540,000 (26, 27). It may well be that, at least to a certain degree, the size of an enzyme molecule is the consequence of evolutionary chance events that do *not* follow selective pressure.

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