Quaternary organization of subunits in the L-leucine dehydrogenase from *Bacillus cereus*

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L-Leucine dehydrogenase from *Bacillus cereus* was examined in the electron microscope. The quaternary structure reveals a molecule that is built up from 8 subunits, identical in mass, arranged in 2 layers which are oriented mainly in a staggered form. In each layer subunits are positioned at the vertices of a square, leaving free a central protein-deficient region of 2.6 nm in diameter. The enzyme measures 11.1 nm in diagonal and 9.0 nm in edge length. Mean subunit diameter is 4.0 nm. The overall shape is a cube, slightly compressed, with 90% edge length in height.

Leucine dehydrogenase *Electron microscopy* *Subunit stoichiometry* *Quaternary structure* (Bacillus cereus)

1. INTRODUCTION

L-Leucine dehydrogenase [L-leucine: NAD^+ oxidoreductase (deaminating), EC 1.4.1.9] was isolated from *Bacillus cereus* according to Schütte et al. [1]. The enzyme catalyzes reversible deamination of L-leucine and other straight- and branched-chain amino acids. Under equilibrium conditions reductive amination of the corresponding keto acid to form L-leucine is favored [1].

Most amino acid dehydrogenases, derived from eucaryotic and procaryotic organisms, are composed of 6 subunits, identical in mass. For many such enzymes models have been elaborated, based on light- and X-ray scattering data [2–6] as well as on electron microscopical images [7–9], the overall shape was found to be a hollow cylinder (reviews [10–12]).

From biochemical studies it has recently been shown that L-leucine dehydrogenase from *B. cereus* is unique in the sense that it is an octameric enzyme composed of 8 identical subunits [1]. To obtain some insight into the quaternary structure of this enzyme, the present work was initiated.

2. MATERIALS AND METHODS

Samples for electron microscopy were made from the NAD^+ form of the enzyme (60 μg/ml) in 10 mM potassium phosphate buffer, pH 7.0. The enzyme molecules were negatively stained for 5 s with 3% (w/v) uranyl acetate (pH 4.5) following the procedure of Valentine et al. [13], and mounted onto a reticulo grid that had been prepared according to [14].

Electron microscopy was done with a Zeiss EM 10 B at primary magnification of 80000 × and 80 kV acceleration voltage. Tilting series were done at 50000 × and 80 kV in the range from 45 to −42°.

3. RESULTS

All of the electron microscopical studies reported here were carried out with the NAD^+ holoenzyme. Under the staining conditions used the enzyme molecules were equally engulfed with the contrasting solution, while the enzyme-free area showed a homogeneous background (fig.1).

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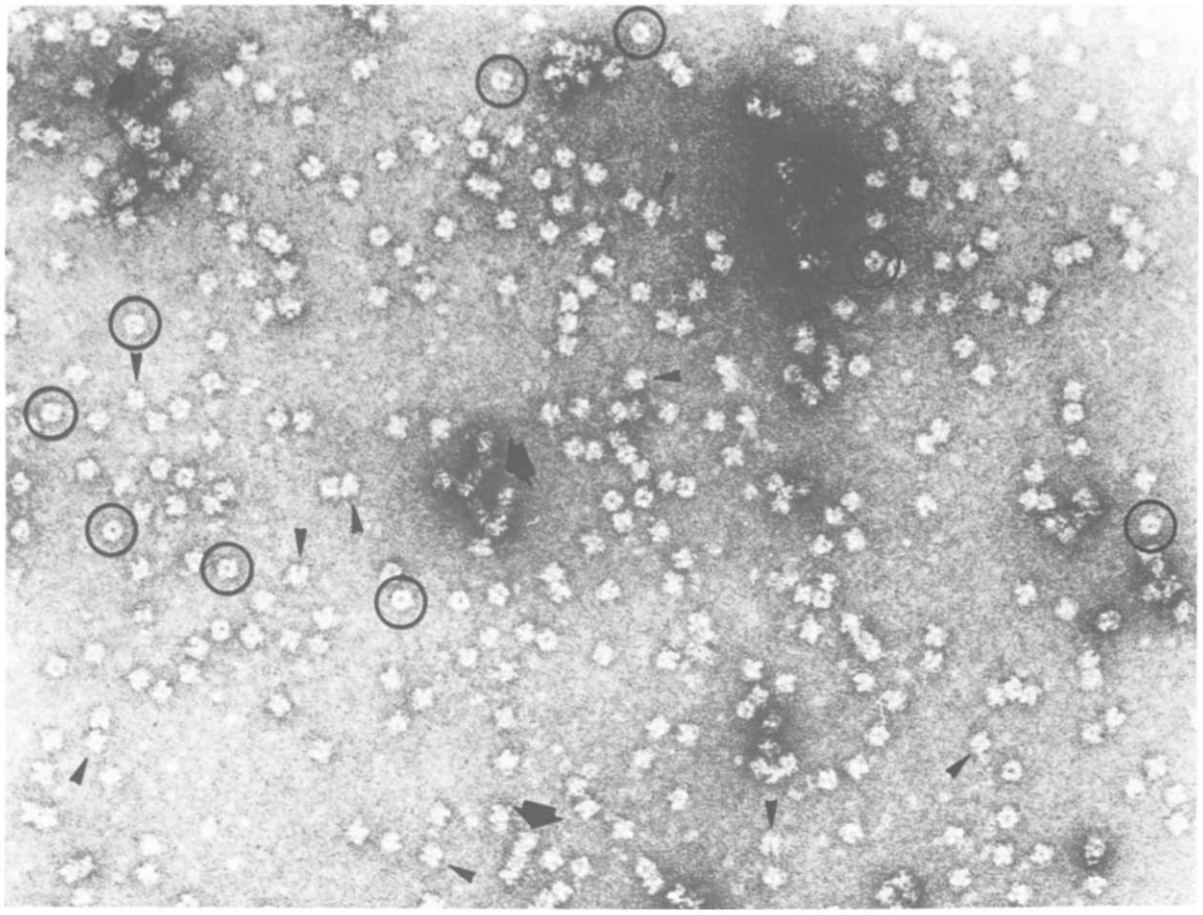

Fig.1. Negatively stained L-leucine dehydrogenase from *B. cereus*. Tetragonal top view projections of the molecule are encircled, side view projections are marked by arrowheads; linearly aggregated molecules are indicated by solid arrows.

By this process all hydrophilic parts of the enzyme were negatively stained, giving a distinct picture of individual protein masses.

In most cases the enzyme is shown in a top view projection (fig.1, encircled structures; fig.3a). Here, 4 single protein masses equivalent to the 4 subunits are positioned in a planar tetragonal arrangement at the vertices of a square. The mean length of the edge is 9.0 ± 0.9 nm (n = 31), whereas the mean length of the diagonal is 11.1 ± 0.6 nm (n = 44). A characteristic appearance of this top view projection is the central hole, recognized as a stain-filled region, measuring 2.6 ± 0.3 nm (n = 38) in diameter (figs 1 and 3). In addition, the 4 subunits are separated by fine stain lines and the shape of individual subunits is globular, but somewhat deformed with diameters ranging from 3.8 to 4.2 nm (figs 1 and 3a).

Doing Markham rotation analysis on the quadrangular projection of a single molecule, it was possible to enhance the signal-to-noise ratio, thus reinforcing structural details [15,16]. As can be seen in fig.2A, there is a certain contrast enhancement at 2-fold rotational operations as compared to the unprocessed molecule (fig.2A, top). Maximal enhancement could be achieved by rotating the molecule at 90°, indicating a radial symmetry of the molecule with a 4-fold symmetry axis. No contrast enhancement was obtained by rotational operations at 120° (fig.2A, 3×) and 60° (fig.2A, 6×).

Furthermore, some projections exist that show

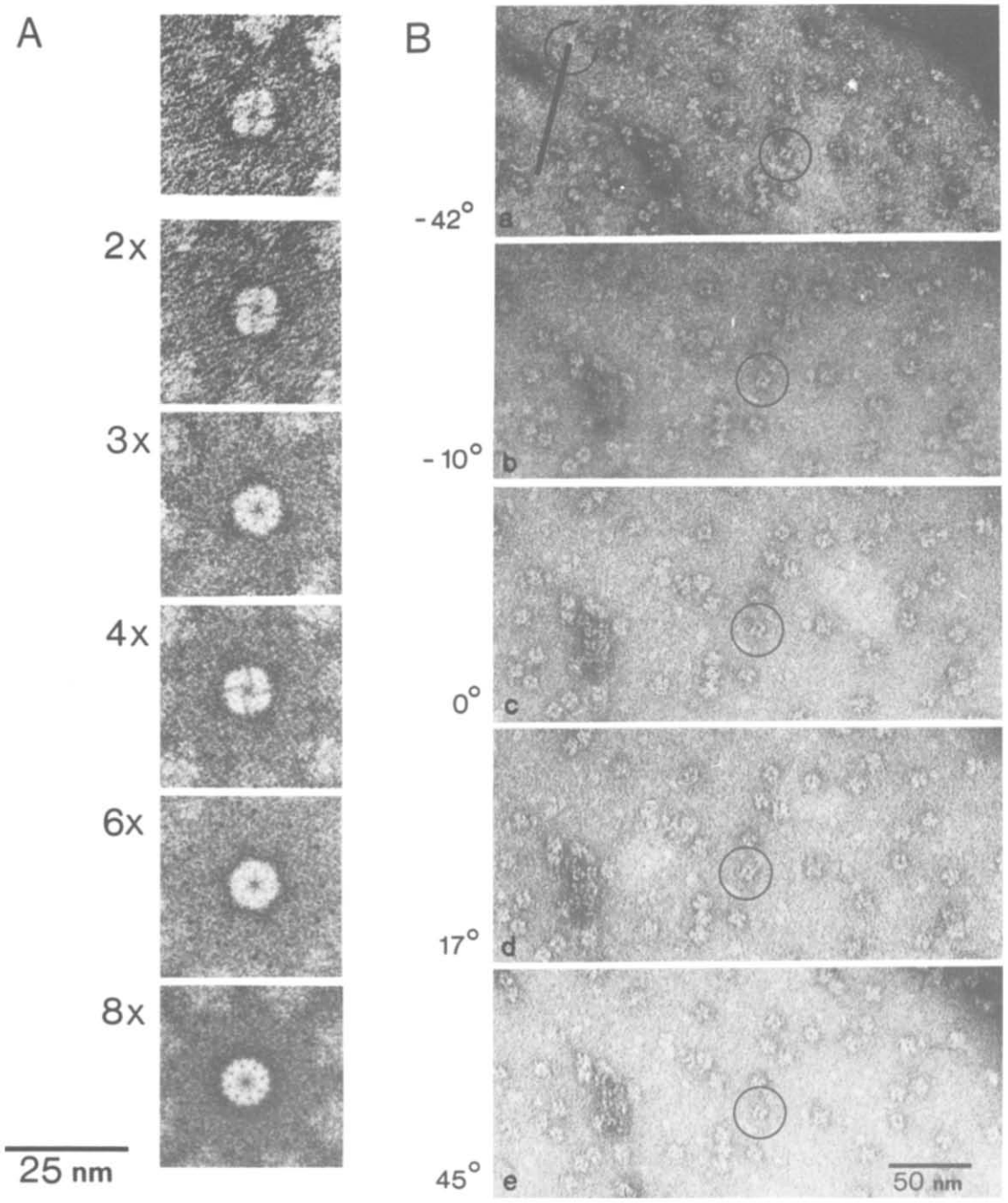

Fig.2. (A) Markham rotation analysis of a single L-leucine dehydrogenase molecule. Top, normal exposure of the molecule; 2×, exposing twice with 180° rotation each; 3×, exposing 3 times with 120° rotation; 4×, 4 times exposure with 90° rotation; 6×, 6 times exposure with 60° rotation; 8×, 8 times exposure with 45° rotation. (B) Tilting series of L-leucine dehydrogenase. Encircled molecule shows the change from a quadrangular top view (a–c) to a bilayered side view projection (d,e); bar in (a) represents tilting axis.

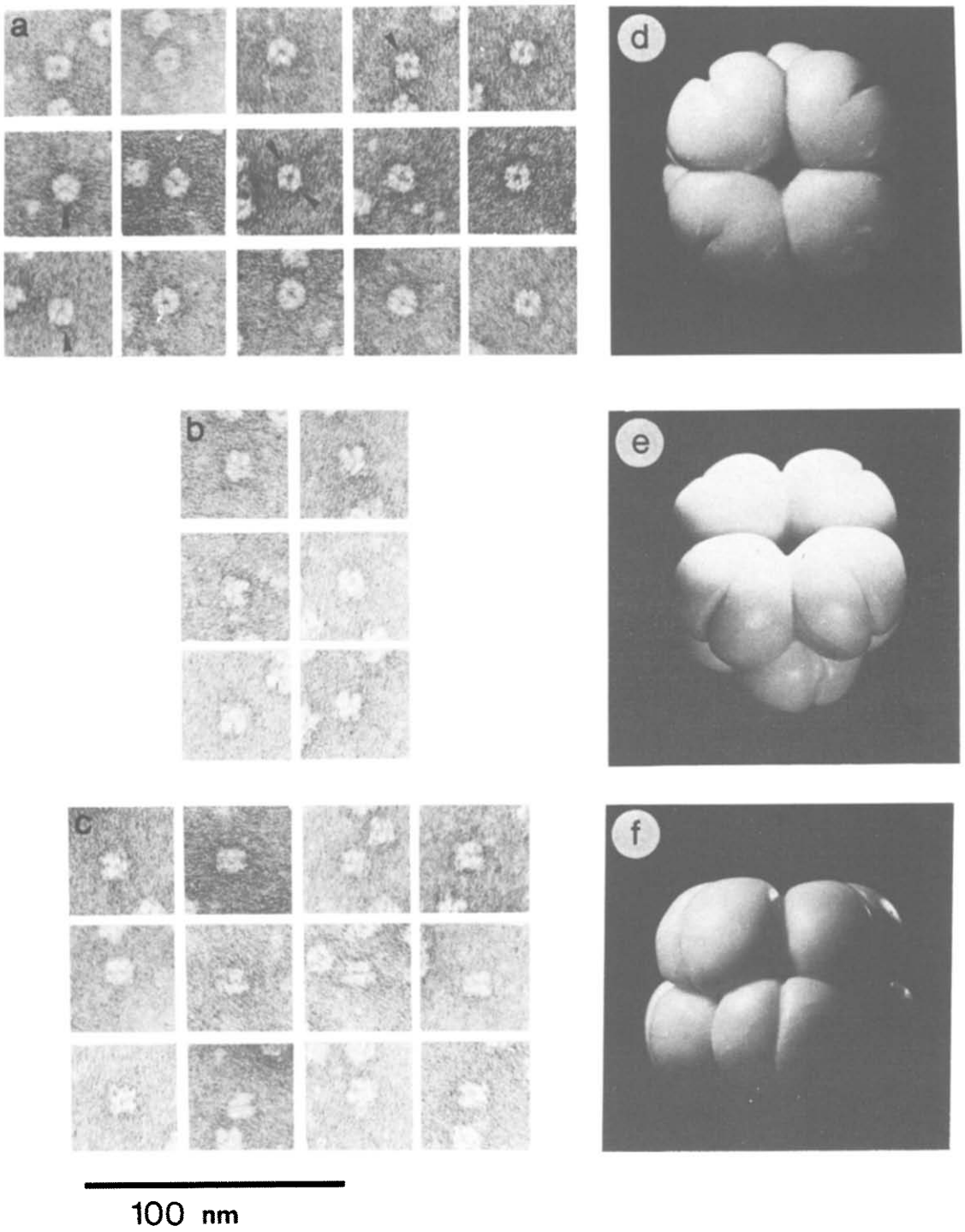

Fig.3. Outstanding projections of L-leucine dehydrogenase molecules and physical model. (a) Top view projection of tetragonal subunit arrangement; arrowheads indicate details of subunit structure. (b) Projection of the molecule tilted by 45°. (c) Side view projection, showing 2 subunit layers. (d–f) Physical models corresponding to (a–c).

the enzyme molecules from the side view (fig.1, arrowheads; fig.3c). Here, the molecule has a bipartite construction made of 2 protein layers that are equal in thickness (4.1–4.4 nm) but often differ in length: the shorter is 9.1–9.4 nm and the longer 10.9–12.2 nm.

These findings were further confirmed by looking at one typical projection of a single molecule and changing its view by tilting the molecule in the electron microscope. Such a tilting series is shown in fig.2B (a–e), ranging from 45 to −42°. The encircled molecule in fig.2B(a) shows a distinct change in projection from a 4-centered protein layer with a dark central spot (−42°, −10°, 0°) to a bipartite projection, showing two layers of protein mass separated by a dark line of stain (45°).

In certain top view projections it is also possible to recognize some fine substructures of individual subunits that are indicated by arrowheads in fig.3a. Here it is seen that the subunits in the intact enzyme are more or less U-shaped as we have exemplified in the model (fig.3d–f).

Combining all of these structural measurements, it appears that the overall shape of the enzyme molecule is a cube, though slightly compressed with about 90% of its edge length in height.

4. DISCUSSION

L-Leucine dehydrogenase from *B. cereus* has an M_r of 310000 and is composed of 8 identical subunits [1]. In contrast to the electron microscopical images of bovine liver L-glutamate dehydrogenase, which shows a triangular arrangement of subunits by its top view projection [7,9], this enzyme molecule is characterized by a tetragonal arrangement of subunits, leaving a central dark stained region. This dark stained region predicts a low protein mass area in the central part of the molecule. Based on the staining conditions it cannot be strictly ruled out that this indicates peripheral depressions, i.e., cavities on both top and bottom sides of the molecule, or existence of a hollow core as it has been proposed for L-leucine dehydrogenase from *B. sphaericus* on the basis of small-angle X-ray scattering data [6].

Upon changing the top view projection (which is indistinguishable from the bottom view) by tilting about 90°, it was possible to convert the 4-centered planar subunit arrangement into a projection of 2 elongated protein layers of bipartite construction which is a characteristic feature of the molecule's side view (fig.1, arrowheads). The difference in length of the 2 protein layers may be attributed to the relative orientation of both layers. As can be seen in the model (fig.3d,f) the 2 layers are twisted against each other leading to a staggered orientation. In this way the longer dimensions appear to result from looking diagonally, having the projection of 3 subunits in register. The shorter dimensions are the consequence of looking parallel to the edge of the quadrangular subunit layer. The situation that both of the 2 layers are in the same orientation without being twisted against each other, i.e., the eclipsed form of the molecule, could be observed less frequently. In the eclipsed form each subunit is in contact with 3 subunits, whilst in the staggered form each subunit is in contact with 4 neighboring subunits which may be favored for energetical reasons. This finding is compatible with the data derived from studies of L-glutamate dehydrogenase [9]. In that case small-angle X-ray scattering curves of the model representing the eclipsed form of the enzyme did not fit with experimental data, but that of the staggered form fitted nicely.

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