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Purification and some properties of a novel microbial lactate oxidase

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Abstract *Geotrichum candidum* was found to produce a lactate oxidase. The enzyme was purified by gel filtration and ion-exchange chromatography. The purified lactate oxidase showed a molecular mass of 50 kDa under denaturing and about 400 kDa under non-denaturing conditions. Transmission electron microscopy analysis confirmed an octameric structure. FMN was found to be a cofactor for this enzyme. Polarographic studies confirmed an oxygen uptake by the lactate oxidase. The enzyme showed specificity towards the L isomer of lactate and did not oxidise pyruvate, fumarate, succinate, maleate and ascorbate. It was stable at alkaline pH and also for 15 min at 45°C. The addition of glycerol and dextran 500 000 to the enzyme sample enhanced storage stability.

Introduction

The enzymic oxidation of lactate has been reported with several microbial enzymes. Lactate oxidase from *Pediococcus* sp. oxidises L-lactate to pyruvate producing hydrogen peroxide. Some Mycobacteria also produce lactate oxidase (Mascini et al. 1984; Massey et al. 1980; Lockridge et al. 1972; Sullivan PA 1968). Lactate oxidase derived from *Mycobacterium phlei* is a riboflavin-5′-phosphate-containing enzyme. It has a dual function, i.e. oxidation and decarboxylation of lactate to acetate and carbon dioxide. Lactate oxidase from *Mycobacterium smegmatis* is very similar to the enzyme from *M. phlei*. The molecular mass of the native enzyme from both species suggests an octameric structure and is within the range 345–350 kDa. The molecular mass of one subunit is in the range 43655–47000 Da. The lactate oxidase from *Acetobacter peroxidans* converts L-lactate to pyruvate via pyruvate decarboxylase. It does not require the addition of an external cofactor to initiate activity (Cannon et al. 1984). Another lactate oxidase, L-lactate cytochrome *c* or flavocytochrome b_2 oxidoreductase, is produced by aerobic yeasts (Matsumoto et al. 1990). It catalyses conversion of L-lactate to pyruvate, but other L-hydroxy acids are also substrates for this enzyme.

There is a strong demand for lactate determination in dairy and fermentation products and in clinical analysis, because of the association of lactate with several diseases (coronary artery disease, pneumonia, epileptic attacks and diabetic coma) (Xia et al. 1987; Makovos and Liu 1984). Until now, an enzymatic method with soluble lactate dehydrogenase (LDH, EC 1.1.1.27) associated with NADH detection has been used. Bi-enzyme systems such as lactate oxidase/LDH and cytochrome b_2/LDH have also been proposed (Bardeletti et al. 1986). Most of the systems described are generally slow and laborious to set up and exhibit performances far from what is required in clinical and food analysis. Very often pretreatment of the sample is compulsory in order to obtain accurate results. Even when electrochemical sensors are available and easy to set up, preparations of the associated enzymatic systems appear generally time-consuming and dependent on the local availability of material, and thus can be difficult to carry out outside the laboratory where they were originally conceived. Lactate oxidase is an enzyme that could be used for such analyses. It could be rapidly and simply immobilized on a commercially available

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pre-activated membrane fixed to an amperometric probe detecting hydrogen peroxide. Among the lactate oxidases, only the enzyme from *Pediococcus* sp. has been commercialized, but unfortunately it is not stable. We have screened a new lactate oxidase producer, and purified and characterised this enzyme.

Materials and methods

Materials

Natural samples: soil samples, fermented corn, fermented bread, fermented cabbage and dairy waste water, were used as sources of microorganisms. Some microorganisms from the German culture collection (DSM) were also tested for lactate oxidase production.

Media

Medium 1 consisted of 7.68 g sodium DL-lactate, 1.13 g $K_2HPO_4 \cdot 3H_2O$, 0.88 g KH_2PO_4, 1 g NH_4Cl, 0.5 g $MgSO_4 \cdot 7H_2O$, 1 g yeast extract, 5 mg, $CaCl_2 \cdot 2H_2O$, 1.2 ml trace elements, 1000 ml distilled H_2O; pH 7 adjusted with 1 M KOH or 1 M HCl. Trace elements solution consisted of: 50 g Na_2EDTA, 22 g $ZnSO_4 \cdot 7H_2O$, 5.54 g $CaCl_2$, 5.06 g $MnCl_2 \cdot 4H_2O$, 1.1 g $(NH_4)_2MO_7O_{24} \cdot 4H_2O$, 5.0 g $FeSO_4 \cdot 7H_2O$, 1.57 g $CuSO_4 \cdot 5H_2O$, 1.61 g $CoCl_2 \cdot 6H_2O$, 1000 ml distilled H_2O. Medium 2 consisted of 7.68 g sodium DL-lactate, 0.88 g K_2HPO_4, 1.5 g NH_4Cl, 0.5 g $MgSO_4 \cdot 7H_2O$, 5 mg $CaCl_2 \cdot 2H_2O$, 1.2 ml trace elements solution, 1000 ml distilled water, pH 7.0. For plates we developed medium 1 supplemented with 1 mg/ml ABTS [2.2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt], 2 U/ml peroxidase and 2% (w/v) agar. ABTS and horseradish peroxidase sterilized by filtration were added after the medium had been autoclaved and cooled to below 50°C.

Screening procedure

Natural samples were cultivated in 100 ml medium 1. Cultivation was carried out at 30°C with shaking at 150 rpm. After 24 h and 72 h, the culture was used to inoculate plates containing ABTS and peroxidase, which subsequently were used for the isolation of single colonies with positive oxidase reaction. Single colonies were cultured for 48 h or 72 h on the plates without ABTS and peroxidase, and used to inoculate liquid medium 1 and medium 2 (50 ml in 250-ml conical flasks). After 24, 48, 72, 96 h of cultivation, the cells were harvested by centrifugation (12 000 rpm, 30 min, 2°C) and then washed twice with Millipore-quality water. Following washing, the cells were resuspended in 0.01 M imidazole/HCl buffer pH 7.0 or 0.1 M glycine/NaOH buffer pH 10. The next step involved disruption of the cells with glass beads, using a swing mill (Retsch Co., Germany) for 45 min at 4°C and a maximum frequency of 1800 swings/min. After disruption the cells were further centrifuged (12 000 rpm, 10 min at 2°C); the supernatant was collected and used as an enzyme source.

Cultivation

The inoculum (200 ml in 500-ml conical flask) was grown for 14 h. Batch fermentations were carried out in a 5-l fermenter (Setric Genie Industriel) with 2.5 l medium 2. The agitation speed was set at 160 rpm, and the sparge air control was set at 75 l/h. The fermentation was carried out for 24 h.

Assay of lactate oxidase activity

Spectrophotometric assay

A reaction mixture (1 ml), consisting of 49 mM dimethylglutarate buffer pH 6.5, 49 mM DL-lactate, or L-lactate, 1.5 mM 4-aminoantipyrine, 3.1 mM *N*,*N*-dimethylaniline and 2.5 U/ml peroxidase, was preheated at 30°C for 5 min. Then 0.030 ml enzyme solution was added and well mixed. The appearance of quinoneimine dye was measured at 565 nm by spectrophotometry. The absorbance at 565 nm was determined during 1 min reaction time in a 1-ml cuvette.

One unit was the amount of enzyme that caused the formation of one micromole of hydrogen peroxide per minute under test conditions.

A quick test on microtitre plates was used for qualitative measurement. To 0.090 ml reaction mixture, 0.01 ml enzyme solution was added and incubated at room temperature for 10–20 min. The resulting pink colour showed the active samples.

Polarographic measurement of lactate oxidase activity by using flow-injection analysis

Polarographic detection of the dependence of O_2 consumption on lactate oxidase was carried out using a Clark-type oxygen electrode (Schott-Geräte GmbH) connected to a potentiostat (Bank Elektronik GmbH) set at 0.8 V. The output was registered using a strip-chart potentiometric recorder (Hipp and Lannen, model 4 no. BD41). The electrode was placed in a chamber with a capillary inlet suitable for microsyringes. Measurements were made in the dark in order to avoid the photochemical consumption of O_2 by FMN. The reaction mixture consisted of 50 mM sodium DL-lactate in 50 mM imidazole buffer pH 7.0. For the blank, only 50 mM imidazole buffer was used as the reaction mixture. Basal O_2 uptake was measured after the injection of the enzyme sample into the buffer without lactate. The reaction was carried out at 30°C.

Influence of FAD, FMN and 3*-amino-*1,2,4*-triazole on lactate oxidase activity*

The influence of FMN, FAD or 3-amino-1,2,4-triazole on enzyme activity was checked by applying various concentrations of these chemicals in the test assay system.

Purification

Following incubation, the cells were washed twice with Millipore-quality water and then, after centrifugation, resuspended in 0.01 M glycine/NaOH buffer, pH 10. The next step involved disrupting the cells and centrifugation. The resulting supernatant was collected and used as an enzyme source. Ammonium sulphate precipitation at a final concentration of 40% and 80% (w/v) was applied as the first step of purification. The resulting pellet (after 80% w/v precipitation) was dissolved in 100 mM glycine buffer, 100 mM NaCl, 5% (w/v) glycerol, 1 μM FMN, pH 10. Then the Sephacryl S-300 column (2.5 cm × 90 cm) equilibrated with 100 mM glycine buffer, pH 10, 100 mM NaCl, 1 μM FMN and 5% (w/v) glycerol was used for gel filtration. Active fractions were pooled and precipitated with ammonium sulphate at a final concentration of 50% and 80% (w/v).

After being dissolved in 100 mM glycine buffer pH 10, 100 mM NaCl, 1 μM FMN, 5% (w/v) glycerol, the sample was loaded on Sephacryl S-300. Fractions showing the highest specific activity were loaded onto a Q-Sepharose fast-flow column equilibrated with buffer A: 50 mM TRIS/HCl buffer pH 8.0, 5% (w/v) glycerol, 1 μM FMN. The 50 mM TRIS/HCl buffer pH 8.0, 5% (w/v) glycerol, 1 μM FMN, 1 M $(NH_4)_2SO_4$ was applied to the column as buffer B in a five-bed-volume gradient. The eluted fractions were checked for purity and activity.

Gel electrophoresis

The purity of the enzyme was analysed using the Phast System according to the manufacturer's instructions (Pharmacia). Native polyacrylamide gel electrophoresis (PAGE), SDS-PAGE (8%–25% polyacrylamide with sodium dodecyl sulphate) and isoelectric focusing gels (3–9, 2.5–6.5) were used. Staining was performed with the silver staining method according to Butcher and Tomkins (1985). The low-molecular-mass standard and high-molecular-mass standard supplied by Pharmacia Co. were used as standard proteins for PAGE.

For activity staining of lactate oxidase, the native gels were incubated in an enzyme assay reaction mixture at 30°C.

Protein determination

The protein concentration of the cell extract and of the chromatography fractions was determined using a bicinchoninic acid protein assay kit (Sigma procedure TRPO-562). Bovine serum albumin was used as standard.

N-terminal sequence analysis

N-terminal sequencing was routinely performed using both a gas-phase sequencer 470 A, Applied Biosystems, Germany (five analyses) and a pulsed liquid sequencer 473 A, Applied Biosystem (five analyses).

Transmission electron microscopy

An aliquot of a highly purified sample of the enzyme (70 μg/ml final concentration) was prepared for electron microscopy as described by Valentine et al. (1968). Aqueous uranyl acetate, pH 4.5 (3% final concentration w/v), was used as the staining solution. Negatively stained molecules, adhering to a 30-nm carbon foil, were picked up with a perforated grid (Lünsdorf and Spiess 1986). The probes were analysed in a Zeiss CEM 902 transmission electron microscope equipped with an integrated electron energy-loss spectrometer. Two images were made from each aspect of the sample, one at an electron energy loss of 0 eV and the other at 115 eV, optimal for the $O_{4,5}$ edge of uranium. The energy resolution was 15 eV; a 90-μm objective aperture was used and exposures were taken at 50 000 × –85 000 × primary magnification.

Kinetic studies

Steady-state kinetic studies were carried out by the spectrophotometric measurement of the enzyme activity present with various concentrations of lactate. The Michaelis constant (K_m) and maximum reaction velocity (V_{max}) were calculated by using the computer programme Enzfitter (Perrella 1988).

Substrate specificity

The specificity of lactate oxidase was determined by using various substrates in a standard assay system.

Cofactor analysis

To remove excess flavin, the enzyme sample for cofactor analysis was applied onto a gel-filtration column (Sephadex G-25) or a Centricon microconcentrator. The flavin content was determined by HPLC [13] after denaturation of the enzyme sample according to Sullivan (1968). Analytical HPLC was performed with columns of Nucleosil 120–5 C_{18} from VDS Optilab (4 × 125 mm). Optimum separation was achieved with isocratic eluent containing 20% (v/v) methanol in 0.1 M ammonium formate at a flow rate of 1.0 ml/min. Injection volumes did not exceed 60 μl and the quantity of flavin phosphates did not exceed 1 nmol. The effluent was monitored by fluorometry and photometry. With fluorometric detection, sensitivity higher than 1 pmol (excitation 456 nm; emission 520 nm) was achieved.

Determination of temperature and pH effects on the lactate oxidase activity

Aliquots of the enzyme solution were heated at various temperatures in a water bath for 15 min. The remaining activity was measured using the standard assay system. The influence of FMN and FAD on enzyme activity was checked by applying them at various concentrations. The effect of pH on the enzyme stability was analysed after incubation of the enzyme in buffered solutions for 60 min at 30°C. The remaining activity was measured in a standard assay system. The pH optimum was determined by incubation of the enzyme/substrate mixture at various pH values: 6.0, 7.0, 7.5, 8.0, 8.5, 9.5, 10.5, 11.5.

The effect of various inhibitors

The enzyme solution was incubated with various compounds at 30°C for 30 min; the remaining activity was measured using the standard assay system.

The effect of various components on storage stability of lactate oxidase

To enhance enzyme stability, various chemicals were added to the solution of the purified enzyme. The following chemicals were used: glycerol, sorbitol, lactitol, heparin, *n*-octyl *β*-D-glucopyranoside and dextran 10 000, 70 000, 500 000. The samples were stored for 28 days at 4°C and, during this period, activity was measured in the standard assay system. The chemicals analysed were always present in the blank sample.

Results

Purification

A total of 54 different strains of microorganisms were capable of growing in the medium containing lactate as the only carbon source, but only one strain showed remarkable oxygen uptake and hydrogen peroxide

production. This strain was identified at the German Culture Collection Laboratory as *Geotrichum candidum* Link:Fr. (DSM 10452). The polarographic analysis of cell extracts confirmed that oxygen uptake was indeed involved in the enzyme reaction. An oxygen uptake (3.9 nmol/min) was observed when the cell extract of *G. candidum* was injected into the reaction mixture containing L-lactate. A higher oxygen uptake (31.4 nmol/min) was observed when partially purified lactate oxidase from this strain was injected into the reaction mixture. The activity of the cell extract in both the fermenter and the shaking culture was about 19 U/l and reached a maximum after 24 h of incubation time. The addition of 3-amino-1,2,4-triazole to the enzyme assay had no influence on the enzyme activity. Following harvesting, the cells were disrupted with glass beads and centrifuged. The resulting cell extract was used for further purification. After ammonium sulphate precipitation, the enzyme exhibited some sixfold higher specific activity in comparison to the cell extract. Following dissolution in a 0.1 M glycine buffer containing 0.1 M NaCl, 5% (w/v) glycerol and 1 μM FMN, pH 10, the enzyme sample was loaded onto a Sephacryl S-300 column. This purification step was very efficient. The highest lactate oxidase activity was found in fractions with lower protein contents. Following ammonium sulphate precipitation, active fractions were loaded onto a Sephacryl S-300 column. After these two steps an increase in specific activity (40.4 U/mg) was observed. Fractions containing lactate oxidase were loaded onto a Q-Sepharose fast-flow column. After washing with buffer A, an ammonium sulphate gradient (0–1 M) was applied to the column, and the enzyme was eluted with 0.2–0.3 M ammonium sulphate. Use of NaCl instead of ammonium sulphate resulted in the loss of enzyme activity. A summary of the purification is shown in Table 1.

Enzyme characterization

The purified enzyme showed an apparent molecular mass of 50 kDa in SDS-PAGE (Fig. 1). A non-denaturing PAGE showed a molecular mass for the enzyme of 400 kDa (Fig. 2). The molecule of this configuration showed activity; this was confirmed by activity staining of the native gel. The molecular mass of 360–450 kDa was determined by gel filtration, and the isoelectric point was 5.0, determined by means of isoelectrofocusing. The enzyme was stable in the pH range 8–11 after 15 min of incubation at 30°C and was stable for 15 min at 45°C. The addition of FMN slightly (11%) increased enzyme activity, while FAD showed no effect. The addition of FMN at a concentration of 30 μM increased the enzyme stability (30% after 10 days storage at 10°C). The enzyme specificity was checked by using various substrates at a concentration of 47 mM. Lactate oxidase from *G. candidum* is specific towards L-lactate and does not oxidise D-lactate, fumarate, pyruvate, succinate, maleate, ascorbate, dihydroxyacetone, DL-alanine or D-serine. The stimulating effect of $MnCl_2$ (94.6% remaining activity), dextran 500 000 (90% remaining activity), glycerol (90% remaining activity) and octyl glucopyranoside (99% remaining activity) was observed after 10 days of incubation at 4°C. The loss of activity was very low in comparison to the

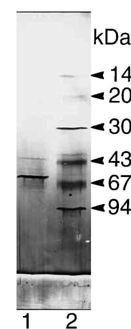

Fig. 1 Polyacrylamide gel electrophoresis under denaturing conditions. *Lane 1* purified lactate oxidase from *G. candidum*; *lane 2* standard protein

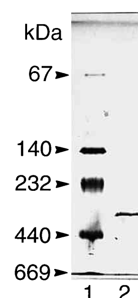

Fig. 2 Polyacrylamide gel electrophoresis under non-denaturing conditions. *Lane 1* standard protein; *lane 2* purified lactate oxidase from *G. candidum*

Table 1 Summary of purification of lactate oxidase from *Geotrichum candidum*

Step	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification factor	Yield (%)
Cell extract	530	143	2213	0.065	1	100
80% $(NH_4)_2SO_4$	17	115	306	0.379	5.7	80.4
Sephacryl S-300	134	90	20.1	4.5	69.2	62.9
80% $(NH_4)_2SO_4$	8	70	6.2	11.3	173.8	48.9
Sephacryl S-300	16	64.6	1.6	40.4	621.5	45.2
Q-Sepharose	7.0	48.6	0.8	60.7	934	34.0

control (65.2% remaining activity). A decrease of enzyme activity was found in the presence of $HgCl_2$ and SDS (0.0% and 3.6% remaining activity). To enhance storage stability, the enzyme was also incubated with lactitol, inositol, heparin, dextran 10 000, and dextran 70 000, but none of these chemicals was better than those mentioned above. For future storage, 5% (w/v) glycerol was applied.

Apparent K_m and V_{max} values were determined for L-lactate by using the computer programme Enzfitter, and were as follows: K_m 3.6×10^3 μM, V_{max} 1.26 μmol $min^{-1} mg^{-1}$. When a sample of denatured lactate oxidase was applied to HPLC, one peak corresponding to 5′FMN (Fig. 3) was found. The area of this peak was increased by coinjecting commercial FMN together with denatured lactate oxidase. One can deduce unequivocally from this experiment that lactate oxidase contains 0.125 mol pure 5′FMN per monomer, which is not covalently bound.

The N-terminal sequence of lactate oxidase was determined to be Tyr-Tyr-Ser-Ala-Ser-Ser-Ala-Trp-Pro-Gln-Ile-Ala-Gly-Tyr-Lys-Leu-Pro-Glu-Val-Asp-Asn-Glu-Pro-Val-Gly-. The sequence has been compared with the sequences deposited in the Swissprot database, where no homologue was detected. The lactate oxidase probe was of pure and homogeneous appearance (Fig. 4a) when viewed in the electron microscope at an electron energy loss of 0 eV, i.e. the elastic bright-field mode. The most prominent view of the enzyme molecule is a top-view projection with four distinct protein masses in a planar-tetragonal arrangement of square-like contour (Fig. 4a, b: large circles). Characteristically a protein-mass-deficient aspect of the molecule is presented, which indicates that four subunits are positioned at the vertices of a square, thus outlining a central hole, as shown in detail in the gallery subset (Fig. 4d). A second characteristic feature of the enzyme is represented by its being composed of two elongated bars of mass; this is indicated by a twin arrowhead in Fig. 4a, b, and in greater detail in the gallery subset shown in Fig. 4e. These two layers represent two planar quartets of subunits, seen from the side, and they appear to be twisted against each other in a staggered manner. Ultrastructural morphologies, which are supposed to represent such individual planar quartets because of rather weak and thus flat-staining characteristics, are indicated by an open arrow (Fig. 4a, b). The above-mentioned molecular aspects of the subunit arrangement indicate that the lactate oxidase is an octameric enzyme of identical 52-kDa subunits. This octameric organisation of the quaternary structure is corroborated by native gel electrophoresis data (see above). The overall edge length of the top-view projection ranges from 8.4 nm to 14.5 nm with a mean value of 11.3 + 1.2 nm ($n = 170$), when seen in the elastic bright field at 0 eV.

A further characteristic detail is seen in many top-view projections, which show some knob-like features in the central area of the molecules, when seen near Scherzer focus in the elastic bright field at 0 eV electron energy loss (see Fig. 4a: small circles). When seen at an electron energy loss of 115 eV at the Gaussian focus, these additional features appear more linear or spoke-like in shape (Fig. 4b: small circles) and seem to connect opposing subunits, as is shown in detail in the gallery subset of Fig. 4c. Since the enzyme is composed of identical subunits of only one polypeptide, these features imply a distinct deviation of the subunit's tertiary structure from a simple sphere.

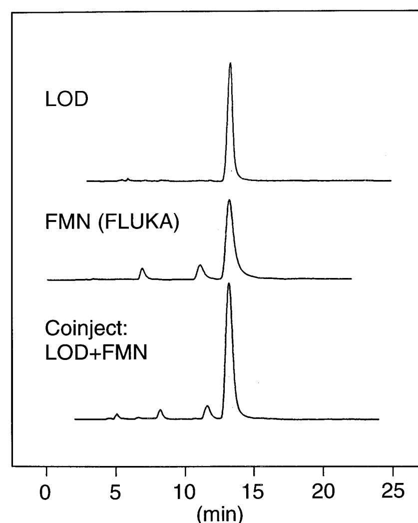

Fig. 3 HPLC analysis of cofactor of lactate oxidase (*LOD*) from *G. candidum*

Discussion

The results presented in this paper show that L-lactate oxidase from *G. candidum* is structurally similar to the enzyme from *M. smegmatis* and *M. phlei*. As electron microscopy analysis and PAGE show, the enzyme consists of eight subunits and is an FMN-containing flavoprotein. However, the octameric organisation of the quaternary structure is quite different in both enzymes (Sullivan et al. 1977). *G. candidum* lactate oxidase does not form linear aggregates under the conditions described; spoke like-appendices in the central area of the molecules were observed. Such structures have been observed in a related octameric enzyme, alcohol oxidase from *Hansenula polymorpha*, by image analysis of two-dimensional crystals (Vonck and Bruggen 1990). Here, however, the appendices or protuberances do not traverse the central stain-filled hole, when seen in the planar-tetragonal top-view projection as above, but are oriented to the outer periphery and the contact area of both planar-tetragonal halves. These differences in the enzyme's quaternary organization, i.e. the presence and absence of spoke-like appendices, may reflect some

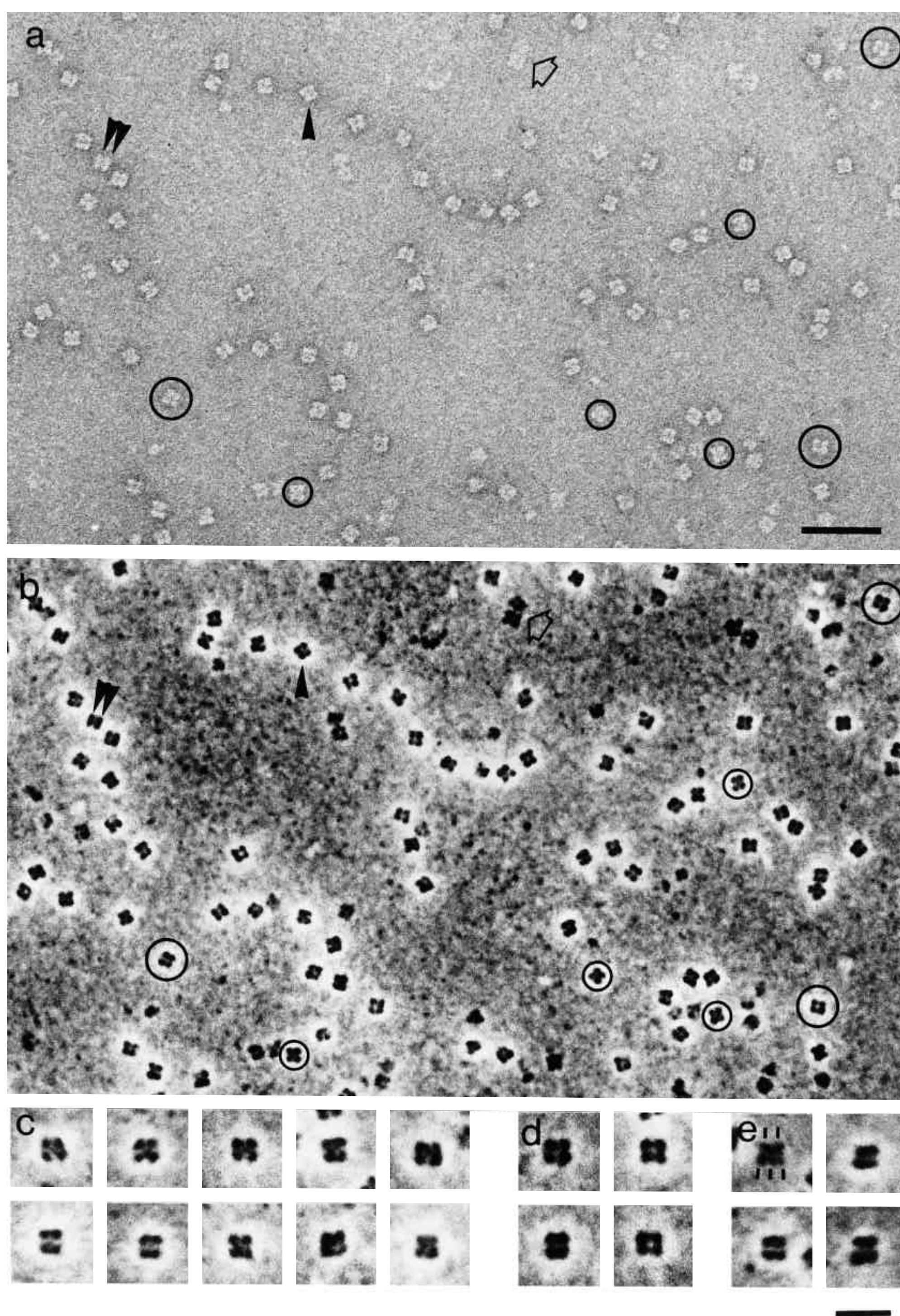

Fig. 4a–e Transmission electron microscopic presentation of negatively stained lactate oxidase molecules of *G. candidum*. Survey of enzyme particles in (**a**) the elastic bright field at an electron energy loss of 0 eV and (**b**) the inelastic dark field at 115 eV, specific for uranium edge $O_{4,5}$. Top-view projections with a centre free of protein mass are indicated (*large circles*), those with centrally positioned protein masses are shown within *small circles*. *Single arrowhead* particles of non-planar tetragonal morphology; *twin arrowheads* molecules that represent the side-view projection; *open arrow* individual half-sized molecules. *Bar* in (**a**) is also valid for (**b**): 50 nm. **c–e** Typical projection contours are shown in detail in the gallery subsets in the inelastic dark field at an electron energy loss of 115 eV. **c, d** Top-view projections show centrally positioned spoke-like protuberances (**c**), which are absent in **d**. **e** Side-view projections of the two staggered layer halves. Individual 52-kDa protein masses are indicated. *Bar* for all gallery subsets: 20 nm

distinct conformational states of still unknown biochemical or physiological significance. Lactate oxidase from *G. candidum* catalyses a different enzyme reaction from the reaction of *M.* lactate oxidase. The *G. candidum* enzyme produces hydrogen peroxide and pyruvate from L-lactate, while *M. smegmatis* produces carbon dioxide, acetate and water. The enzyme we have isolated acts in similar manner to the *Pediococcus* sp. lactate oxidase. Pyruvate is not a substrate for these enzymes, and both enzymes show similar pH, thermo- and storage stability. The crystal structure of microbial lactate oxidase is not known. Further crystallisation may permit an analysis of the structure of the enzyme in detail, as well as providing more information about cofactor binding and the reaction mechanism.

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References

Bardeletti G, Sechaud F, Coulet PR (1986) A reliable L-lactate electrode with a new membrane for enzyme immobilization for amperometric assay of lactate. Anal Chim Acta 187:47–54

Butcher LA, Tomkins JK (1985) A comparison of silver staining methods for detecting proteins in ultrathin polyacrylamide gel on support film after isoelectric focusing. Anal Biochem 148: 348–388

Cannon JJ, Chen L-F, Flickinger MC, Tsao GT (1984) The development of an immobilized lactate oxidase system for lactic acid analysis. Biotechnol Bioeng 26:167–173

Lockridge O, Massey V, Sullivan PA (1972) Mechanism of action of the flavoenzyme lactate oxidase. J Biol Chem 247:8097–8106

Lünsdorf H, Spiess E (1986) A rapid method of preparing perforated upporting foils for the thin carbon films used in high resolution transmission electron microscopy. J Microsc 144:211–213

Makovos EB, Liu CC (1984) Measurement of lactate concentration using lactate oxidase and an electrochemical oxygen sensor. Biotechnol Bioeng 25:167–170

Mascini M, Moscone D, Palleschi A (1984) Lactate electrode with lactate oxidase immobilized on nylon net for blood serum samples in flow system. Anal Clin Acta 157:45–51

Massey V, Ghisla S, Kieschke K (1980) Studies on the reaction mechanism of lactate oxidase. J Biol Chem 255:2796–2806

Matsumoto K, Matsubara H, Hamada M, Ukeda H, Osajima Y (1990) Simultaneous determination of glucose, ethanol and lactate in alcoholic beverages and serum by amperometric flow injection analysis with immobilized enzyme reactors. J Biotechnol 14:115–126

Nielsen P, Rauschenbach P, Bacher A (1986) Preparation, properties and separation by high-performance liquid chromatography of riboflavin phosphates. Methods Enzymol 122:209–220

Perrella FW (1988) EZ-FIT: a practical curve-fitting microcomputer program for the analysis of enzyme kinetic data on IBM-PC compatible computers. Anal Biochem 174:437–447

Sullivan PA (1968) Crystallization and properties of L-lactate oxidase from *Mycobacterium smegmatis*. Biochem J 110:363–371

Sullivan PA, Soon CY, Schreurs WJ, Cutfield JF, Shepherd G (1977) The structure of L-lactate oxidase from *Mycobacterium phlei*. Biochem J 165:375–383

Valentine RC, Shapiro BM, Stadtman ER (1968) Regulation of glutamine synthetase; Electron microscopy of the enzyme from *Escherichia coli*. Biochemistry 7:3266–3273

Vonck J. Bruggen EFJ (1990) Electron microscopy and image analysis of two-dimensional crystals and single molecules of alcohol oxidase from *Hansenula polymorpha*. Biochim Biophys Acta 1038:74–79

Xia Z-X, Shamala N, Bethge PH, Lim LW, Bellamy HD, Xuong NH, Lederer F, Mathews SF (1987) Three-dimensional structure of flavocytochrome b_2 from bakers yeast at 3.0-A resolution. Proc Natl Acad Sci USA 84:2629–2633