Biochem. J. (1977) **165**, 375–383
Printed in Great Britain

The Structure of L-Lactate Oxidase from *Mycobacterium smegmatis*

By PATRICK A. SULLIVAN, CHOONG YEE SOON, WILHELMUS J. SCHREURS, JOHN F. CUTFIELD and MAXWELL G. SHEPHERD
Department of Biochemistry, University of Otago, Dunedin, New Zealand

(*Received* 10 *January* 1977)

1. An improved purification was developed for L-lactate oxidase from *Mycobacterium smegmatis*. 2. The mol.wt. of the native enzyme by a sedimentation-equilibrium analysis was 345000, and other ultracentrifuge methods gave values in the range 345000–350000. 3. An amino acid analysis, determinations of protein and flavin, a sedimentation-velocity analysis and an approach to equilibrium analysis gave values for the subunit mol.wt. in the range 43500–47000. 4. It was concluded that L-lactate oxidase contains eight subunits of mol.wt. 43500. 5. Cross-linking of the subunits with dimethyl suberimidate and electron-microscopy studies were consistent with an octameric structure.

The reaction mechanism of the flavoenzyme L-lactate oxidase (decarboxylating) (EC 1.13.12.4) from *Mycobacterium smegmatis* has been the subject of several recent studies (Lockridge *et al.*, 1972; Walsh *et al.*, 1972, 1973; Ghisla *et al.*, 1976; Schonbrunn *et al.*, 1976).

The structure of lactate oxidase has not been studied in the same detail as the reaction mechanism. Sutton (1957) obtained highly purified preparations of the enzyme from *Mycobacterium phlei*: the reported mol.wt. of 260000 and the flavin content of 2mol of FMN/mol were shown to be incorrect (see below). Takemori *et al.* (1968) found that the enzyme from the same source has a mol.wt. in the range 340000–390000 and a minimum mol. wt. of 56000g of protein/mol of FMN.

Takemori *et al.* (1974) revised the mol.wt. estimate to 350000: it was reported that the native enzyme was resistant to dissociation in 8M-urea, but was completely dissociated by treatment with 5M-guanidine hydrochloride or 0.2% (w/v) sodium dodecyl sulphate, yielding six identical subunits of mol.wt. 54000–57000. Quantitative *N*- and *C*-terminal analyses were reported to be consistent with this minimum molecular weight: the *N*- and *C*-terminal residues detected were serine and arginine respectively. This analysis, together with electron-microscopy studies, has been used to propose a hexameric model of the native enzyme.

As expected, lactate oxidase from *M. smegmatis* is very similar in structure to the enzyme from *M. phlei* in that the enzyme has a mol.wt. in the range 300000–400000 and contains 6–8mol of FMN/mol (Sullivan, 1968). However, we have described studies on the preparation of the apoenzyme of lactate oxidase from *M. smegmatis* and reconstitution of this enzyme with FMN that indicated that the native enzyme is composed of eight identical subunits and contains 8mol of FMN/mol (Choong *et al.*, 1975). In the present paper we describe studies which show that lactate oxidase from *M. smegmatis* consists of eight protomers of mol.wt. 43500. In addition we summarize a purification method that gives a substantially higher yield of enzyme.

Materials and Methods

Aldolase, dansyl*-amino acid standards, 5,5′-dithiobis-(2-nitrobenzoic acid), Coomassie Blue R and guanidine hydrochloride were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Sodium dodecyl sulphate (especially pure) was from BDH Ltd., Poole, Dorset, U.K., and suberonitrile (dicyanohexane) was the product of Aldrich Chemical Co., Milwaukee, WI, U.S.A. Polyamide chromatography sheets were supplied by the Cheng Chin Trading Co., Taipei, Taiwan. All other chemicals were of analytical grade.

Buffers, protein determinations, the enzyme assay, enzyme units and specific activity were as described previously (Choong *et al.*, 1975).

Ultracentrifuge studies

These were carried out with a Spinco model E ultracentrifuge. Sedimentation and diffusion measurements were performed as described in the manual compiled by Chervenka (1969). Lactate oxidase was dissolved in either 0.1M-sodium phosphate buffer, pH7.0, or 0.2M-NaCl/0.01M-sodium phosphate buffer, pH7.0. The temperature was maintained at 20°C. Schlieren optics were used and photographic

* Abbreviation: dansyl, 5-dimethylaminonaphthalene-1-sulphonyl.

plates were analysed for boundary sedimentation with either a travelling microscope or a micro-comparator. Diffusion coefficients were computed from synthetic-boundary analyses at 3600 rev./min. Areas under the curves were determined with a planimeter. The sedimentation-equilibrium analysis of native lactate oxidase, kindly performed by the late Dr. J. Lyttleton, was carried out at 15°C and 10000 rev./min. by using the long-column technique of Chervenka (1970) and interference optics. The protein concentration was 0.3 mg/ml in 0.1 M-sodium phosphate buffer, pH 6.8.

Amino acid analysis

A Beckman model 120B analyser equipped with a long-path-length cell was used by the methods of Spackman (1967). Enzyme solutions of measured flavin (see the Results section and Table 2) content in 15 mM-sodium phosphate buffer, pH 7.0, were dried, then hydrolysed in 6.27 M-HCl under vacuum for 24, 48 and 72 h. Values of threonine and serine were computed by extrapolation to zero time of hydrolysis. A separate sample was hydrolysed in the presence of dimethyl sulphoxide (Spencer & Wold, 1969) for determination of half-cystine as cysteic acid and proline. Tryptophan was measured by the method of Spies & Chambers (1949) as modified by Harrison & Hofmann (1961). These analyses were kindly done by Dr. C. H. Williams, Jr., Veterans Hospital, Ann Arbor, MI, U.S.A. Thiol groups were measured with 5,5′-dithiobis-(2-nitrobenzoic acid) and *p*-chloromercuribenzoate as described by Ellman (1959) and Riordan & Vallee (1972). Disulphide groups were measured with 5,5′-dithiobis-(2-nitrobenzoic acid) as described by Robyt *et al.* (1971).

Sephadex G-200 gel filtration

A column (2.4 cm × 52 cm) of Sephadex G-200 was calibrated for molecular-weight determination as described by Andrews (1964). The column was equilibrated with 50 mM-sodium phosphate buffer, pH 7.0, containing 0.1 mM-EDTA. Samples were applied in 1 ml of buffer containing 3% (w/v) sucrose. The protein concentration of samples was in the range 2–20 mg/ml. The flow rate was 5 ml/h and the eluate was collected in 1.3 ml fractions. The following proteins were used to construct the standard curve; horse haemoglobin (68000), hexokinase (92500), fumarase (204400), catalase (247500) and urease (483000). The molecular weights given in parentheses were from the tables compiled by Smith (1968). The elution of Blue Dextran and haemoglobin from the column was monitored at 280 nm. The enzymes were assayed as follows: hexokinase, fumarase and catalase (Bergmeyer *et al.*, 1974) and urease by the colorimetric method of Van Slyke & Archibald (1944).

Polyacrylamide-gel electrophoresis

The gels had the following final composition (%, w/v): 5% acrylamide, 0.135% *NN′*-methylenebisacrylamide, 0.075% ammonium persulphate and 0.1% sodium dodecyl sulphate. All solutions were prepared in 0.1 M-sodium borate/0.1 M-sodium acetate, pH 8.5, and this was also the electrophoresis buffer. In some cases the gels contained 3.5% acrylamide, 0.185% *NN′*-methylenebisacrylamide and the other components as listed above. Samples (20–50 μg) of protein were denatured for 2 h at 37°C in 1% (w/v) sodium dodecyl sulphate/1% 2-mercaptoethanol, then mixed with 0.05 ml of 1% (w/v) Bromophenol Blue in 50% (v/v) glycerol and layered on the gels. Electrophoresis at 6 mA/gel was carried out in a cold-room. Gels were stained with Coomassie Blue R and destained as described by Weber & Osborn (1969).

Cross-linking of subunits

Cross-linking with dimethyl suberimidate was as described by Davies & Stark (1970). Dimethyl suberimidate, synthesized from suberonitrile (Davies & Stark, 1970), was kindly supplied by Dr. G. S. Bailey, Department of Biochemistry, University of Otago, Dunedin, New Zealand.

Electron microscopy

Lactate oxidase (8 mg/ml) in 0.1 M-potassium phosphate buffer, pH 7.0, was applied to a carbon-coated collodion film on a copper grid (200 mesh) and stained with several drops of 2% (w/v) potassium phosphotungstate solution, pH 7.0. Micrographs were taken in a Siemens 102 electron microscope at an electron optical magnification of ×33000. The assistance of Mr. B. Smirk and Dr. D. Rayns in operating the electron microscope is acknowledged.

Enzyme preparation

Cell-free extracts of *M. smegmatis* were prepared as described previously (Sullivan, 1968; Lockridge *et al.*, 1972). The modified procedure described below gives substantially higher yields of pure enzyme. Unless otherwise stated all steps were performed at 0–4°C.

Cell-free extracts were fractionated with $(NH_4)_2SO_4$ and heat treatment as described previously (Sullivan, 1968). The enzyme solution obtained (fraction 2) was adjusted to pH 4.5 with 1.0 M-acetic acid, stirred for 10 min, then centrifuged at 10000*g* for 10 min. Solid $(NH_4)_2SO_4$ was added to the supernatant to give 20% saturation (114 g/l), stirring was continued for 10 min, then the precipitate was removed by centrifuging at 10000*g* for 10 min. Further $(NH_4)_2SO_4$ (189 g/l) was added to the supernatant to give 50% saturation, and stirring was

continued for a further 20min before centrifuging at 20000*g* for 30min. The pellet was dissolved in 1.0M-sodium acetate buffer, pH5.4, to give a total volume of 0.05vol. of the original extract. This solution, fraction 3, was dialysed for 12h against 2×2 litres of the same buffer. After dialysis the preparation was centrifuged at 10000*g* for 10min. Lactate oxidase was extracted from the pellet into approx. 0.05vol. of 1.0M-sodium acetate buffer, pH5.4 at 30°C (i.e. 5% of the original volume of cell-free extract) (fraction 4). Crystalline enzyme was recovered by chilling the solution on ice for approx. 30min, then centrifuging at 10000*g* for 10min. The pellet was dissolved in 0.05vol. of 1.0M-sodium acetate buffer, pH5.4 at 30°C (fraction 5), and solid $(NH_4)_2SO_4$ was added until a precipitate was visible. White inactive material was removed as a pellet by centrifuging (10000*g* for 10min at 25°C) and further $(NH_4)_2SO_4$ was added to salt-out the white material. This procedure was continued up to the stage at which lactate oxidase began to be precipitated. The extent of contamination with this white material was variable, and ranged from nearly zero to 50% on a weight basis. The enzyme was recovered by increasing the $(NH_4)_2SO_4$ concentrations to 470 g/l and centrifuging at 20000*g* for 10min. The enzyme was dissolved in 1.0M-sodium acetate buffer, pH5.4, dialysed against the same buffer and crystallized as above (fraction 6).

Lactate oxidase may be stored as a crystalline suspension at 2–10mg/ml at 0°C for at least 6 months without loss of activity. Preparations were stored as a routine in foil-wrapped containers to exclude light. This method gives enzyme preparations with a specific activity of 1200–1400 units/mg and an overall yield of 40–60%. A typical purification is summarized in Table 1.

Results

Crystalline forms of lactate oxidase

As reported previously (Sullivan, 1968), lactate oxidase from *M. smegmatis* crystallized readily from 1.0M-sodium acetate buffer, pH5.4, as fine needles. The enzyme also crystallized from buffered $(NH_4)_2SO_4$ solutions as rhombic plates (Plate 1). Sutton (1957) and Takemori *et al.* (1974) crystallized the enzyme from *M. phlei* as square plates. The *M. smegmatis* enzyme has on a few occasions crystallized in this form, but the rhombic plates were more frequently encountered.

Extinction coefficients of lactate oxidase

The details of the flavin absorption spectrum of lactate oxidase, including absorption maxima, molar absorption coefficients and the presence of shoulders on the spectrum around 480 and 420nm, are affected by the composition, concentration and pH of the solvent buffer. For example, the molar absorption coefficient at 450nm increased by 15% when a solution of the enzyme in 10mM-imidazole/HCl buffer, pH7.0, was titrated with sodium phosphate, pH7.0, to a final concentration of 0.2M (Lockridge *et al.*, 1972). This could, if overlooked, introduce significant errors into calculations of the enzyme concentration, flavin content and minimum molecular weight. Molar absorption coefficients for lactate oxidase in a range of buffers are summarized in Table 2. Previously reported values in 0.1M-sodium phosphate, pH7.0 (Sullivan, 1968), and in 10mM-imidazole/HCl, pH7.0 (Lockridge *et al.*, 1972), have been included for comparison.

Table 1. *Purification of* L-*lactate oxidase*

Fraction	Volume (ml)	Total enzyme (units)	Protein (mg/ml)	Sp. activity (units/mg of protein)
Crude extract	1250	175000	13.4	10.5
1 Solution of fraction precipitated by $(NH_4)_2SO_4$ (35–65% satd.)	250	130000	46	11.0
2 Supernatant after heat treatment	226	118000	32	16.0
3 Solution of fraction precipitated by $(NH_4)_2SO_4$ (20–50% satd.)	75	120000	21	76.0
4 Solution of extract after dialysis against acetate buffer, pH5.4	75	120000	2.8	571.5
5 Crystalline L-lactate oxidase	63	114000	1.8	1005
6 $(NH_4)_2SO_4$ fractionation and recrystallization	16	100000	5.2	1202

Table 2. *Molar absorption coefficients of lactate oxidase in various solvents*

Molar absorption coefficients were obtained as described previously (Sullivan, 1968) or by computation from the A_{450} with respect to buffered solutions of the enzyme for which the absorption coefficients had been established by heat liberation of the FMN.

Solvent buffer	ε_{450} (litre·mol^{-1}·cm^{-1})
0.1 M-Sodium phosphate, pH 7.0	12500
0.02 M-Sodium phosphate, pH 7.0	11400
0.1 M-Sodium phosphate, pH 6.0	12800
0.02 M-Mes (4-morpholine-ethanesulphonic acid)/NaOH, pH 6.0	9000
0.1 M-Tris/acetate, pH 7.8	10700
0.01 M-Imidazole/HCl, pH 7.0	10600

Table 3. *Amino acid analysis of M. smegmatis* L-*lactate oxidase*

Time of hydrolysis (h) ...	24	48	72	Proposed composition (mol of residue/mol of FMN)
Amino acid				
Aspartic acid	38.0	38.1	38.0	38
Threonine	18.8	18.2	17.4	20
Serine	20.5	19.0	17.4	23
Glutamic acid	30.7	30.7	30.5	31
Proline	—	21.5	—	22
Glycine	42.7	41.2	41.5	42
Alanine	48.7	49.3	49.0	49
Valine	25.0	26.0	26.6	27
Methionine	8.1	8.0	7.9	8
Isoleucine	18.0	18.7	18.5	19
Leucine	37.0	37.1	36.6	37
Tyrosine	13.2	13.5	13.1	13
Phenylalanine	13.4	13.5	13.7	14
Lysine	12.6	12.2	12.3	12
Histidine	11.3	10.7	10.7	11
Arginine	24.2	22.5	22.4	23
Ammonia		27.7		28
Half-cystine		4.7		
		3.8		4–5
Tryptophan		10.1*		10
Total				432
Mol.wt. (mol/mol of FMN)				43655

* Determined separately as described in the Materials and Methods section.

Amino acid analysis

Table 3 summarizes the results of an amino acid analysis of lactate oxidase of known FMN content. The enzyme has an unusually high arginine/lysine ratio and a relatively high aromatic amino acid content. The total amino acid composition proposed is 432 residues/molecule of FMN, which gives a minimum mol.wt. of 43655g of protein/mol of FMN. This analysis was based directly on the flavin content of the sample. In an analysis of the enzyme from *M. phlei*, Takemori *et al.* (1974) calculated the number of residues per 56000g of protein, this being the proposed minimum molecular weight on the basis of flavin and protein determinations. The sum of the amino acids listed was 471.5. It is apparent that this is incorrect, because the proposed minimum mol. wt. of 56000 is only obtained if the molecular weights of the constituent amino acids rather than the residue weights (i.e. molecular weight minus 18) are used in the summation.

Molecular weight of the native enzyme

In the previous reported estimation of the molecular weight (Sullivan, 1968), the apparent diffusion coefficient was obtained by analysing boundary spreading during a high-speed-sedimentation analysis. A more reliable value has now been obtained by analysing boundary spreading in a synthetic-boundary cell at 3600 rev./min; sedimentation effects are negligible at this speed. The results were analysed as described by Swoboda & Massey (1965). As shown in Fig. 1 a plot of $(A/H)^2$ against time, where

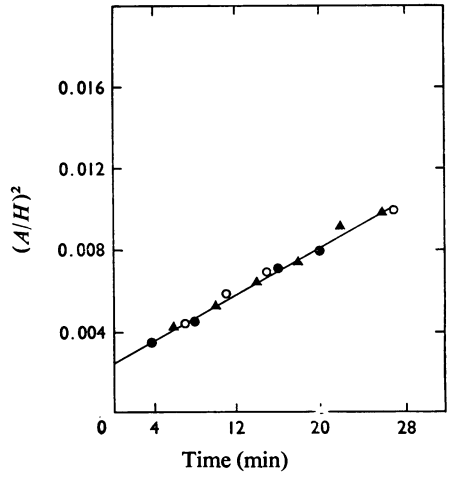
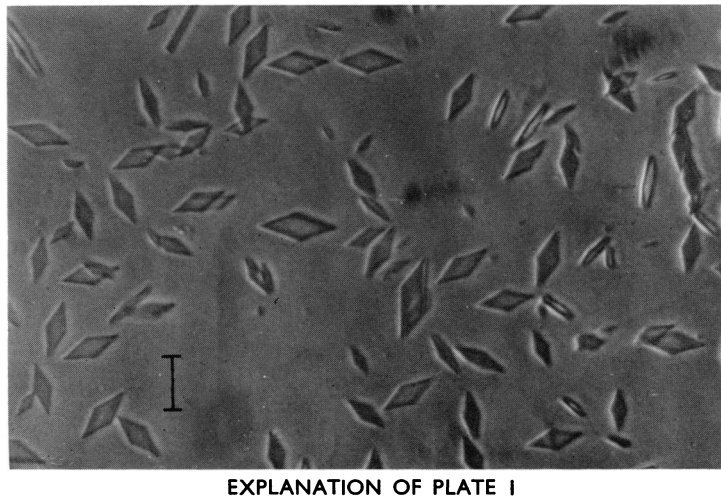


Fig. 1. *Determination of the diffusion coefficient*

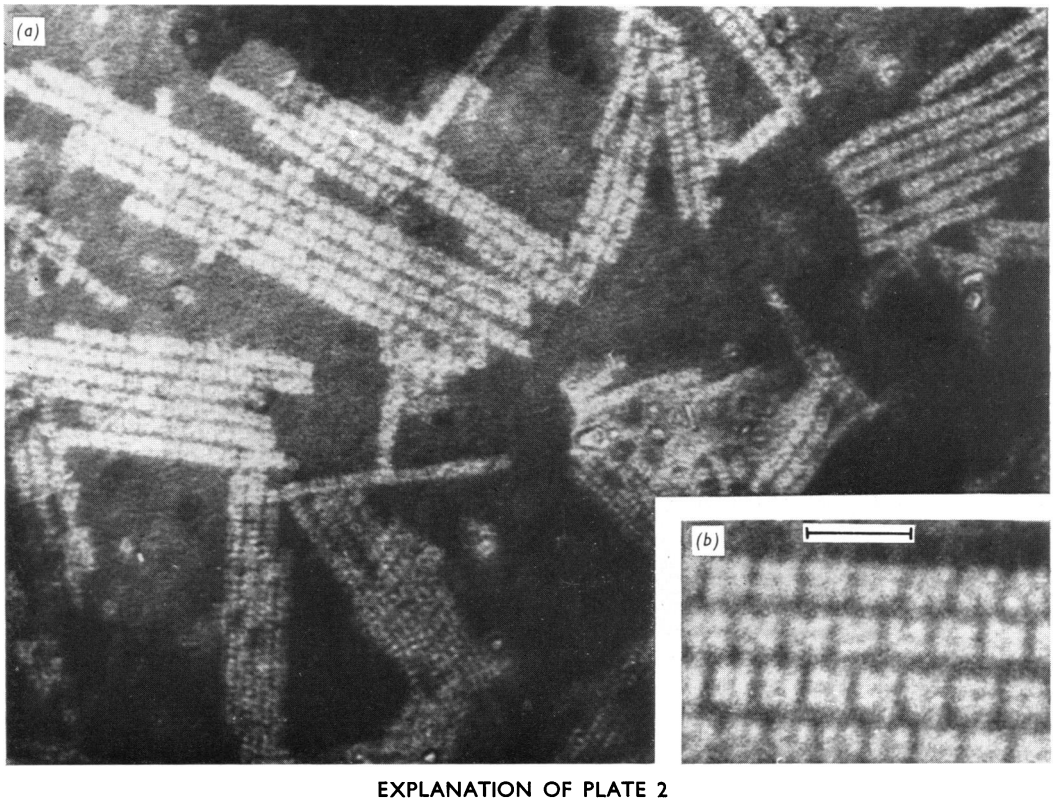
Plots of $(A/H)^2$ versus time were constructed at three protein concentrations: 6.64mg/ml (○), 4.42mg/ml (●) and 1.77mg/ml (▲). The enzyme was dissolved in 0.1 M-sodium phosphate buffer, pH 6.8. Photographs were taken at various times after the formation of the enzyme/solvent boundary in a double-sector synthetic-boundary cell. Measurements were made as described in the Materials and Methods section, and $D_{20,w}$ was calculated from the slope of the plot.



EXPLANATION OF PLATE 1

Crystalline L-*lactate oxidase*

Crystals were prepared in 0.1 M-sodium phosphate buffer, pH 6.0, to which solid $(NH_4)_2SO_4$ had been slowly added to induce crystallization. The preparation was stored at 0°C. Magnification ×375. The bar represents 0.02 mm.



EXPLANATION OF PLATE 2

Electron micrographs of negatively stained lactate oxidase

(*a*) General view showing the aggregation of lactate oxidase molecules into linear arrays (magnification ×245000); (*b*) superposition of four successive molecular units (magnification ×740000) indicating a square planar or cubic configuration of subunits. The bar represents 20nm.

A is the area under the Schlieren peak and H is the maximum ordinate, was linear and the slope was independent of protein concentration. The diffusion coefficient ($D_{20,w}$) obtained from this plot was 3.67×10^{-7}cm$^2\cdot$s^{-1}.

Values for the partial specific volume $\bar{v}$ obtained by the pycnometric method (Schachman, 1957) and from the amino acid composition (Cohn & Edsall, 1943; Lee & Timasheff, 1974) were 0.746 (at 20°C) and 0.734ml/g respectively.

When the following data [s°_{20} 14.35×10^{-13}cm$^{-1}\cdot$s^{-1} (Sullivan, 1968); D_{20} 3.84×10^{-7}cm$^2\cdot$s^{-1}; $\bar{v}$ 0.734ml/g] were used in the Svedberg equation (Svedberg & Pedersen, 1940) the mol.wt. obtained was 349000.

Estimates of the molecular weight obtained by several different methods are summarized in Table 4. Values obtained by sedimentation-equilibrium analysis and an approach-to-equilibrium analysis are consistent with the above analysis. Values obtained in this study by gel chromatography and previously by non-dissociating gel electrophoresis (Choong *et al.*, 1975) are higher, but, allowing for the accuracy of these techniques, are within experimental error.

Dissociation of native lactate oxidase

When native lactate oxidase was treated with sodium dodecyl sulphate the native enzyme dissociated into subunits. Polyacrylamide-gel electrophoresis revealed a single band of material which migrated considerably faster than that for the native enzyme (Fig. 2). The mobility was the same with or without sodium dodecyl sulphate in the gel system, and it was subsequently found that 0.1% sodium dodecyl sulphate without mercaptoethanol caused dissociation. As expected, the FMN was dissociated from the enzyme by this treatment, as judged by flavin fluorescence and dialysis.

Table 4. *Summary of mol.wt. analyses for native lactate oxidase and the subunits*

Values summarized were obtained as described in the text.

Method	Mol.wt.
Native enzyme	
$s_{20,w}$, $D_{20,w}$ and ρ	349000
Sedimentation equilibrium	345000
Approach to equilibrium	349000
Gel chromatography	355000
Gel electrophoresis	360000
Subunit	
Amino acid analysis	43655
Protein and flavin contents of native and reconstituted enzyme	44000
$s_{20,w}$, $D_{20,w}$ and ρ	47000
Approach to equilibrium	44800

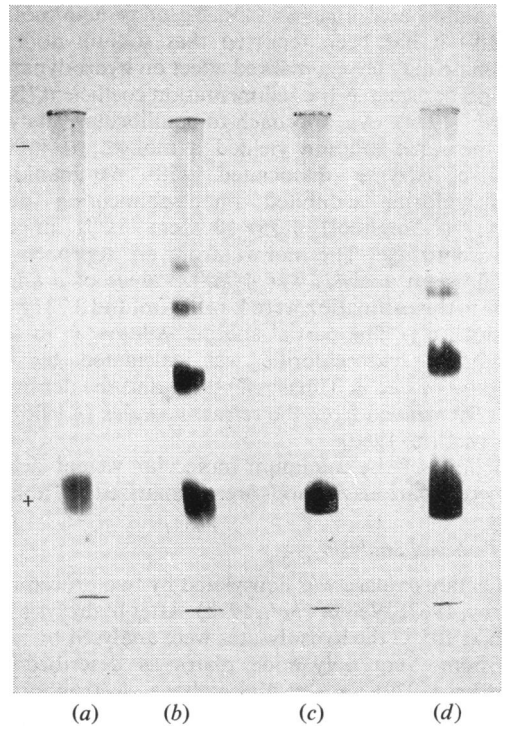


Fig. 2. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of native and cross-linked lactate oxidase*
Solutions of aldolase (2mg/ml) and lactate oxidase (1.3mg/ml) were incubated with dimethyl suberimidate (3mg/ml) for 24h at room temperature (25°C) and 1 week at 4°C respectively. The incubation buffer was 0.2M-triethanolamine/HCl, pH8.5. Samples of the native and cross-linked enzymes were denatured with sodium dodecyl sulphate and analysed by gel electrophoresis (5% gels) as described in the Materials and Methods section. The stained gels contained: (*a*) 20μg of denatured native aldolase; (*b*) 40μg of denatured cross-linked aldolase; (*c*) 20μg of denatured native lactate oxidase; (*d*) 40μg of denatured cross-linked lactate oxidase. The bar at the bottom of each gel is the Bromophenol Blue tracking dye.

A single symmetrical peak, $s_{20,w}$ (7.25mg/ml) 2.35×10^{-13}cm$^{-1}\cdot$s^{-1}, was obtained in the ultracentrifuge with a solution of enzyme containing 1% (w/v) sodium dodecyl sulphate and 1% (w/v) 2-mercaptoethanol. An apparent diffusion coefficient obtained from a low-speed synthetic-boundary analysis of the same sample was $D_{20,w}$ 4.8×10^{-7}cm$^2\cdot$s^{-1}. These values and an assumed partial specific volume of 0.74ml/g applied to the Svedberg equation gave a mol.wt. of 47000. Although this value is consistent with the minimum molecular weight from

the amino acid analysis (43655g of protein/mol of FMN) it has been reported that sodium dodecyl sulphate may have a marked effect on hydrodynamic properties such as the sedimentation coefficient (Bais *et al.*, 1974). An approach-to-equilibrium analysis of the same solution yielded a mol.wt. of 46000. Native enzyme dissociated with 6M-guanidine hydrochloride exhibited one sedimenting peak [$s_{20,w}$ (7.2mg/mol) $1.35\times10^{-13}cm^{-1}\cdot s^{-1}$] in the ultracentrifuge. The mol.wt. from an approach-to-equilibrium analysis was 44800. Values of ρ and $\bar{v}$ used in this estimation were 1.1418g/ml and 0.731 ml/g respectively. The partial specific volume, $\bar{v}$, in 6M-guanidine hydrochloride was calculated by the method of Lee & Timasheff (1974) and the density ρ was determined from the refractive index (Kielley & Harrington, 1960).

Values of the minimum molecular weight determined by various methods are summarized in Table 4.

N-Terminal analysis

Lactate oxidase was dansylated by two procedures (Gray, 1967; Weiner *et al.*, 1972). After hydrolysis for 16h at 105°C the hydrolysates were analysed by t.l.c. on 5cm×5cm polyamide plates as described by Hartley (1970) and by high-voltage electrophoresis at pH1.9 (Gray, 1967). The only fluorescent spots detected were identified as dansylic acid, dansylamide, *O*-dansyltyrosine, ε-dansyl-lysine and α-dansyl-arginine. Takemori *et al.* (1974) identified the *N*-terminal residue of *M. phlei* lactate oxidase as serine by dinitrophenylation.

Cross-linking of subunits

Solutions of the enzyme in dimethyl suberimidate were incubated for 1 week at 4°C. After this incubation, electrophoresis in the presence of sodium dodecyl sulphate revealed four bands (Fig. 2), indicating at least a tetramer. A densitometric tracing suggested that the relative concentrations of the bands in the cross-linked enzyme were 1:2:4:16. The fastest-migrating band had the same mobility as the single band derived from sodium dodecyl sulphate/2-mercaptoethanol treatment of native lactate oxidase. When this band was assigned the monomer mol.wt. of 44000, a plot of logarithm of molecular weight versus mobility of the bands (Weber & Osborn, 1969) gave a straight line, indicating putative mol.wts. of 44000, 88000, 132000 and 176000, i.e. monomer, dimer, trimer and tetramer (Fig. 3). Aldolase, a tetrameric protein of subunit mol.wt. 42400 (Castellino & Barker, 1968), was cross-linked with dimethyl suberimidate at 20°C for 24h (Davies & Stark, 1970). This preparation, analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis in parallel with the cross-linked lactate oxidase (Fig. 2), separated as four bands with

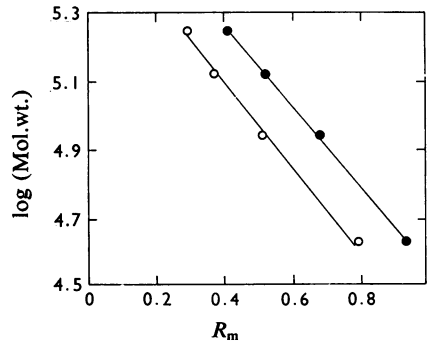


Fig. 3. *Cross-linked lactate oxidase*
Plots of log(molecular weight) versus relative mobility of the oligomers of denatured cross-linked lactate oxidase are shown. Conditions were as described in Fig. 2; ○, 5% gels; ●, 3.5% gels.

virtually the same mobilities as those obtained from lactate oxidase. These findings do not contradict other results in the present paper, which indicate that lactate oxidase has an octameric structure.

Electron microscopy

A typical electron micrograph of negatively stained lactate oxidase is shown in Plate 2(*a*). The molecules have a squarish appearance, with a centrally located hole, and show a tendency to aggregate in linear arrays. When four successive molecular units in one of these rows were superimposed photographically the result (Plate 2*b*) showed four subunits arranged in a square, suggesting a tetrameric structure or an octamer of cubic configuration made up of two such square planar tetramers face to face. The dimensions of the molecule are approx. 8.7nm×9.2nm in cross section.

Thiol and disulphide groups in lactate oxidase

Amino acid analyses (Table 3) indicated the presence of 4–5 half-cystine residues/molecule in lactate oxidase from *M. smegmatis*. This estimate was refined by reaction of the enzyme with 5,5′-dithiobis-(2-nitrobenzoic acid) and *p*-chloromercuribenzoate (Table 5). Lactate oxidase treated with 0.1% (w/v) sodium dodecyl sulphate showed a mean content of 3.0mol of thiol groups/mol of enzyme-bound FMN. Native enzyme contained less accessible thiol groups: values of 2.1 and 2.4mol/mol of enzyme-bound FMN were obtained with 5,5′-dithiobis-(2-nitrobenzoic acid) and *p*-chloromercuribenzoate respectively. Reaction of the enzyme with 5,5′-dithiobis-(2-nitrobenzoic acid) and incubation at pH10.5 as described by Robyt *et al.* (1971) indicated the presence of 0.8mol of disulphide groups/mol of enzyme-bound FMN.

Table 5. *Thiol and disulphide groups in lactate oxidase*

These were determined as described in the Materials and Methods section. For measurements with 5,5′-dithiobis-(2-nitrobenzoic acid) the incubation contained 0.270mg of protein and 1 μmol of reagent in 2.5ml of 0.1M-sodium phosphate buffer, pH8.1. Reactions were followed to completion at 412nm. The measurement was carried out with and without 0.1% (w/v) sodium dodecyl sulphate. Titrations with *p*-chloromercuribenzoate were carried out with 0.44 and 1.1mg of enzyme in 0.1M-sodium phosphate buffer, pH7.0. The initial volume was 0.8ml: 5μl portions of 2.5mM-reagent were added to the enzyme and the reaction was followed at 250nm. Solutions of 5,5′-dithiobis-(2-nitrobenzoic acid) and *p*-chloromercuribenzoate were standardized with reduced glutathione and 2-mercaptoethanol. The values given in parentheses are the means of the determinations.

	Content (mol/mol of FMN)	
	Thiol	Disulphide
Reagents		
5,5′-Dithiobis-(2-benzoic acid)	2.2, 2.0, 2.2 (2.1)	0.9, 0.7, 0.7 (0.8)
+0.1% sodium dodecyl sulphate	2.9, 2.9, 3.2, 3.0 (3.0)	
p-Chloromercuribenzoate	2.3, 2.5 (2.4)	

It is unlikely that thiol groups are implicated in catalysis. When lactate oxidase was treated with a 6-fold molar excess of *p*-chloromercuribenzoate under conditions outlined in Table 5 the change in A_{250} was biphasic. The rapid phase was complete in less than 5min and, on the basis of the extinction coefficient for the mercaptide of monothiols, could account for the reaction of three thiol groups. This was followed by a slow reaction which continued for 3h. No attempt was made to measure the extent of thiol-group modification, because it was judged that protein unfolding and flavin dissociation contributed significantly to the change in absorption. During the incubation enzyme activity decreased in an exponential manner: after 5min the activity was 90% of that at zero time; at 1h there was 50% residual activity but at 3h 90% of the activity was lost. Thus the initial rate of inactivation was much slower than the rate of modification of thiol groups.

Discussion

The results summarized in the present paper show that L-lactate oxidase from *M. smegmatis* is very similar to the enzyme from *M. phlei* (Takemori *et al.*, 1974). This is perhaps not surprising and it might be expected that species variation would only account for minor deletions or substitutions in the amino acid sequence. It is clear that the mol.wt. of the enzyme from both species is within the range 345000–350000. The most precise method used in this work, sedimentation equilibrium, gave the lowest value, 345000 (Table 4).

Analyses of the subunit and minimum mol.wt. by a number of methods gave values in the range 43655–47000: the most accurate method is probably amino acid analysis, which gave a value of 43655. Dissociation of the native enzyme with either guanidine hydrochloride or sodium dodecyl sulphate gave only one type of subunit, as judged by gel electrophoresis and sedimentation-velocity analyses. The enzyme treated with 8M-urea showed many bands in gel electrophoresis, similar to the preparation obtained when apo-(lactate oxidase) was treated with acid $(NH_4)_2SO_4$ to dissociate the FMN (Choong *et al.*, 1975). The pattern of protein bands was consistent with different states of aggregation of the monomer. Takemori *et al.* (1974) did not detect dissociation when the *M. phlei* enzyme was treated with 8M-urea, as judged by a sedimentation-velocity analysis.

The homology between lactate oxidase from the two species *M. phlei* and *M. smegmatis* is striking: the analysis of the *M. phlei* enzyme, based incorrectly on molecular weights rather than residue weights of the constituent amino acids, indicates 1–2 more residues of most amino acids/molecule of FMN. Proposed values for aspartate, alanine, isoleucine, tyrosine, arginine and half-cystine are greater by 3–6 residues/molecule of FMN.

It is difficult to reconcile this homology and the close agreement between the molecular-weight values of the native enzymes with a suggestion that the *M. phlei* enzyme consists of six subunits of mol.wt. 56000 and the *M. smegmatis* enzyme contains eight subunits of mol.wt. 43500. Two possible sources of error which could account for the differing analyses are the variation of the molar absorption coefficient of the enzyme-bound FMN with different solvents (Table 2) and the measurement of protein concentration by the biuret method relative to albumin as a standard. It is not clear whether the variation in the molar absorption coefficient of the enzyme–FMN was taken into account by Takemori *et al.* (1974), but this could account for up to a 25% error. We have based the amino acid analysis of the *M. smegmatis* enzyme directly on the FMN content of the sample, whereas the analysis of the *M. phlei* enzyme appears

to be based on protein concentration determined by the biuret method. It is well documented that the specific absorption coefficient $A^{1\%}_{1cm}$ varies from one protein to another by as much as 25% (Massey & Williams, 1965). The specific absorption coefficient for lactate oxidase in the biuret reaction has not been reported. We have, however, measured protein concentration as a routine by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard. From numerous such determinations the minimum mol.wt. obtained by this method has been revised from the previously reported value of 47000 (Sullivan, 1968) to 44000. Thus the enzyme concentration may be accurately determined by either flavin or protein estimations by the method of Lowry *et al.* (1951).

Further support for an octameric subunit structure for lactate oxidase was obtained from the previous studies on the reconstitution of lactate oxidase (Choong *et al.*, 1975) and the cross-linking studies described in the present paper. During reconstitution of apoenzyme with FMN the preparation revealed three bands on gel electrophoresis, with mol.wts. of 40000, 175000 and 360000. These values are consistent with a monomer, tetramer, octamer relationship. Unless the value for the monomer is too low by almost 50% these results would not support a monomer–trimer–hexamer system. Further, the cross-linking studies rule out the possibility of a hexameric structure. The protein band with the lowest molecular weight seen in the sodium dodecyl sulphate/polyacrylamide gels of both the native and the cross-linked enzyme had a relative mobility almost identical with that of the aldolase subunit (mol.wt. 42400). The other bands of cross-linked subunits were consistent with the dimer, trimer and tetramer of a monomer of mol. wt. 44000 (Fig. 2). The fact that eight bands were not observed does not contradict the proposed octameric model. A similar structural model was proposed for the octameric alcohol oxidase (Kato *et al.*, 1976).

Finally, additional evidence that lactate oxidase from *M. smegmatis* consists of eight subunits was furnished by the electron-microscopy studies, which in the light of the subunit and cross-linking studies are interpretable in terms of an octameric aggregate arranged in a cubic (or possibly square antiprism) configuration (Plate 2). The observed symmetry rules out the possibility of a hexameric structure. The size of the images seen is also consistent with the subunit mol.-wt. estimate quoted above. Assuming a spherical shape for the monomer and a partial specific volume of 0.746ml/g (as determined in the present paper) the observed diameter of 4.4–4.5nm is close to the 'ideal' diameter of 4.7nm required to yield a mol.wt. of exactly 44000.

The symmetry of the subunit packing in this molecule is of course best determined by X-ray diffraction.

References

Andrews, P. (1964) *Biochem. J.* **91**, 222–233

Bais, R., Greenwell, P., Wallace, J. C. & Keech, D. B. (1974) *FEBS Lett.* **41**, 53–57

Bergmeyer, H. U., Gawehn, K. & Grassl, M. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.), vol. 1, pp. 425–522 Academic Press, London

Castellino, F. J. & Barker, R. (1968) *Biochemistry* **7**, 2207–2217

Chervenka, C. H. (1969) *A Manual of Methods for the Analytical Ultracentrifuge*, Spinco Division of Beckman Instruments Inc., Palo Alto

Chervenka, C. H. (1970) *Anal. Biochem.* **34**, 24–29

Choong, Y. S., Shepherd, M. G. & Sullivan, P. A. (1975) *Biochem. J.* **145**, 37–45

Cohn, E. J. & Edsall, J. T. (1943) *Proteins, Amino Acids and Peptides*, pp. 370–381, Reinhold, New York

Davies, G. E. & Stark, G. R. (1970) *Proc. Natl. Acad. Sci. U.S.A.* **66**, 651–656

Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82**, 70–77

Ghisla, S., Ogata, H., Massey, V., Schonbrunn, A., Abeles, R. H. & Walsh, C. T. (1976) *Biochemistry* **15**, 1791–1795

Gray, W. R. (1967) *Methods Enzymol.* **11**, 139–151

Harrison, P. M. & Hofmann, T. (1961) *Biochem. J.* **80**, 38P

Hartley, B. S. (1970) *Biochem. J.* **119**, 805–822

Kato, N., Omori, Y., Tani, Y. & Ogata, K. (1976) *Eur. J. Biochem.* **64**, 341–350

Kielley, W. W. & Harrington, W. F. (1960) *Biochem. Biophys. Acta* **41**, 401–421

Lee, J. C. & Timasheff, S. N. (1974) *Arch. Biochem. Biophys.* **165**, 268–273

Lockridge, O., Massey, V. & Sullivan, P. A. (1972) *J. Biol. Chem.* **247**, 8097–8106

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Massey, V. & Williams, C. H., Jr. (1965) *J. Biol. Chem.* **240**, 4470–4480

Riordan, J. F. & Vallee, B. C. (1972) *Methods Enzymol.* **25**, 449–456

Robyt, J. F., Ackerman, R. J. & Chittenden, C. G. (1971) *Arch. Biochem. Biophys.* **147**, 262–269

Schachman, H. S. (1957) *Methods Enzymol.* **4**, 32–103

Schonbrunn, A., Abeles, R. H., Walsh, C. T., Ghisla, S., Ogata, H. & Massey, V. (1976) *Biochemistry* **15**, 1798–1807

Smith, M. H. (1968) in *Handbook of Biochemistry* (Sober, H. A., ed.), pp. C-3–47, The Chemical Rubber Co., Cleveland

Spackman, D. H. (1967) *Methods Enzymol.* **11**, 3–15

Spencer, R. L. & Wold, F. (1969) *Anal. Biochem.* **32**, 185–189

Spies, J. R. & Chambers, D. C. (1949) *Anal. Chem.* **21**, 1249–1266

Sullivan, P. A. (1968) *Biochem. J.* **110**, 363–371

Sutton, W. B. (1957) *J. Biol. Chem.* **240**, 2209–2215

Svedberg, T. & Pedersen, K. O. (1940) *The Ultracentrifuge*, Oxford University Press, London

Swoboda, B. E. P. & Massey, V. (1965) *J. Biol. Chem.* **240**, 2209–2215

Takemori, S., Nakazawa, K., Nakai, K., Suzuki, K. & Katagiri, M. (1968) *J. Biol. Chem.* **243**, 313–319

Takemori, S., Tajima, H., Kawahara, F., Nakai, Y. & Katagiri, M. (1974) *Arch. Biochem. Biophys.* **160**, 289–303

Van Slyke, D. D. & Archibald, R. M. (1944) *J. Biol. Chem.* **154**, 623–642

Walsh, C. T., Schonbrunn, A., Lockridge, O., Massey, V. & Abeles, R. H. (1972) *J. Biol. Chem.* **247**, 6004–6006

Walsh, C. T., Lockridge, O., Massey, V. & Abeles, R. H. (1973) *J. Biol. Chem.* **248**, 7049–7054

Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412

Weiner, A. M., Platt, T. & Weber, K. (1972) *J. Biol. Chem.* **247**, 3242–3251