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Preparation of the Lactate Oxidase Apoenzyme and Studies on the Binding of Flavin Mononucleotide to the Apoenzyme

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1. Lactate oxidase from *Mycobacterium smegmatis* is completely resolved into free flavin and apoenzyme by treatment with acid $(NH_4)_2SO_4$. 2. Reconstitution involves rapid binding of FMN, but the recovery of enzyme activity was slower and appeared to be biphasic. 3. The preparation of the holoenzyme obtained differs from the native enzyme in specific activity, extinction coefficients and mobility on disc-gel electrophoresis. 4. Dialysis of this reconstituted enzyme in 0.1M-sodium phosphate buffer, pH7.0, at 0°C for 1 week yields a preparation which closely resembles the native enzyme.

Although it is difficult to prepare the apoenzymes of flavoproteins to which the flavin group are bound covalently (Singer *et al.*, 1971; Friesel & McKenzie, 1970; Möhler *et al.*, 1972) reversible dissociation has been described for many flavoproteins in which flavin binding involves multiple non-covalent linkages. Examples of the latter class include D-amino acid oxidase (Walaas & Walaas, 1956), glucose oxidase (Swoboda, 1969), flavodoxin (Mayhew, 1971), cytochrome b_5 reductase (Strittmatter, 1961), xanthine oxidase (Komai *et al.*, 1969), and the Shethna flavoprotein (Hinkson, 1968).

Sutton (1955) reported the reversible dissociation of the FMN-containing flavoprotein L-lactate oxidase (EC 1.13.12.4) from *Mycobacterium phlei*, but the preparations of apoenzyme were only partially resolved as judged from the high residual activity. More recently Takemori *et al.* (1968) completely resolved the enzyme from the same source, but the specific activity of the reconstituted enzyme was only 60–80% that of the native enzyme.

Detailed studies on flavin binding have been undertaken with several flavoproteins which have molecular weights in the range of 50000–150000 and contain 1 or 2mol of flavin/mol. Lactate oxidase, however, has a molecular weight of 348000 and is composed of eight identical subunits (P. A. Sullivan, M. G. Shepherd and Y. S. Choong, unpublished work). We have found that the octamer readily dissociates when the flavin is removed, resulting in the formation of a mixture of different states of aggregation of the monomer unit, and this complicates the reconstitution process. The present paper describes the preparation of the apoenzyme of lactate oxidase from *Mycobacterium smegmatis* and the reconstitution of the holoenzyme.

Materials and Methods

FAD (type III) and FMN (commercial grade), sodium salts, were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A.; riboflavin was obtained from Koch–Light, Colnbrook, Bucks., U.K. Stock solutions of the flavins were prepared in glass-distilled water and stored at −5°C wrapped in aluminium foil. Concentrations of stock flavin solutions were measured in 0.1M-sodium phosphate buffer, pH7.0, from the absorbance at 450nm [$\varepsilon = 11.3 \times 10^3$ litre·mol^{-1}·cm^{-1} for FAD and 12.2×10^3 litre·mol^{-1}·cm^{-1} for both FMN and riboflavin (Whitby, 1953)]. Pure FMN was prepared by heat-denaturation of native lactate oxidase (Sullivan, 1968). Phenazine methosulphate, *p*-iodonitrotetrazolium violet (grade I) and 2,6-dichlorophenol-indophenol (grade I) were from Sigma. Protein molecular-weight markers used in polyacrylamide disc-gel electrophoresis were from Schwarz/Mann, Orangeburg, N.Y., U.S.A. L-Lactate solutions were prepared as described previously (Sullivan, 1968).

Buffers

Buffers were prepared from tables compiled by Dawson & Elliott (1959) except those listed below. Disc-gel-electrophoresis buffers were as described by Davis (1964) and Hendrick & Smith (1968). Tris–acetate buffers contained Tris at the concentration given and were adjusted to the final pH with 0.1M-acetic acid. Piperazine-*NN'*-bis-(2-ethanesulphonic acid) was adjusted to the final pH with 0.1M-NaOH and then to the final concentration.

Determination of protein

Protein was determined by a modification (Eggstein & Kreutz, 1967) of the method of Lowry *et al.* (1951),

with sodium citrate being used instead of sodium tartrate. Samples used were in the range 5–50 μg of protein. Crystalline bovine serum albumin (Mann Research Laboratories, New York, N.Y., U.S.A.) were used as a standard.

Spectrophotometry

Absorption spectra were recorded with a Unicam SP. 1800 spectrophotometer.

Fluorimetry

Fluorescence measurements for flavin-binding studies were carried out with a Locarte fluorimeter and a Fairchild model 7050 digital voltmeter. The filters selected were Locarte IF/3 (primary) and Chance OY3 (secondary).

Ultracentrifuge studies

Sedimentation-velocity measurements were done in a Spinco model E ultracentrifuge in a standard 12.0mm cell with quartz windows. Sedimentation coefficients were determined by the method of Schachman (1957).

Assay of L-lactate oxidase activity

Oxygen consumption was measured with a Clark-type oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio, U.S.A.; electrode 5331), an amplifier and a Kipp and Zonen model BD1 micrograph pen recorder. The reaction chamber was enclosed in a constant-temperature water jacket and the reaction mixture was stirred by means of a magnetic bar.

The standard assay mixture consisted of 100 μmol of sodium citrate–citric acid buffer, pH 5.7, 100 μmol of L-lactate, pH 5.7, and 0.5–2.0 units of enzyme in a total volume of 3.0ml, at 25°C. When necessary a portion of the enzyme solution was diluted with water immediately before the assay. Enzyme was added last and the rate of oxygen consumption was measured for 2–3 min.

The oxygen electrode was standardized each day with N_2-saturated and air-saturated solutions. The rate of oxygen consumption (μl of O_2/min) was determined by using the formula given by Peel (1963). An enzyme unit is defined as the amount of enzyme that catalyses the uptake of 1 μl of oxygen/min in the assay system. Specific activity is defined as the number of enzyme units/mg of protein. The assay system described previously (Sullivan, 1968) contained D-lactate and phosphate anions, which are competitive inhibitors of lactate oxidase (Lockridge *et al.*, 1972). The components of the assay system described in the present paper do not inhibit enzyme activity, and the specific activities quoted are 1.35 times those quoted for the previous system (Sullivan, 1968).

Polyacrylamide disc-gel electrophoresis

The methods of Davis (1964) and Hendrick & Smith (1968) were used. Gels were stained for protein by immersion in 1% (w/v) Amido Black in 7% (v/v) acetic acid for 15min. Destaining was carried out by washing with 7% (v/v) acetic acid. Results were expressed as R_m values, i.e. the ratio of the protein band migration to the Bromothymol Blue tracking-dye migration.

Densitometric tracings of 7.5% (w/v) polyacrylamide disc gels were carried out with a Joyce–Loebl u.v. scanner (type D8 MK 2).

Bands of active lactate oxidase were located by incubating gels in a solution containing 300 μmol of sodium phosphate buffer, pH 7.0, 0.3mg of phenazine methosulphate and 0.4mg of *p*-iodonitrotetrazolium violet in an anaerobic cuvette. L-Lactate, pH 7.0 (100 μmol), was placed in the side arm. The total volume was 4.5ml. The system was evacuated and flushed with O_2-free N_2 three times. The lactate was tipped from the side arm and the red formazan stain was allowed to develop in the dark at room temperature for 20min. After staining the gels were washed and suspended in water.

Least-squares analysis

Results required for graphical analysis were computed from a least-squares analysis by using an Olivetti Programma 101 Desk-Top Computer.

Enzyme preparation

L-Lactate oxidase from *M. smegmatis* was prepared as described previously (Sullivan, 1968).

Preparation of apo-(lactate oxidase)

It was necessary to maintain a temperature of −5–0°C at all stages of the preparation to avoid protein denaturation. A solution of $(NH_4)_2SO_4$, saturated at room temperature and adjusted to pH 1.5 with 7% (v/v) H_2SO_4, was added with gentle stirring to a solution (3–10mg/ml) of lactate oxidase in 0.1M-sodium acetate buffer, pH 5.7, containing 1mM-EDTA to give 90% saturation (9ml/ml). The mixture was centrifuged immediately at 40000*g* for 10min. The yellow supernatant was decanted and the last traces of the supernatant were removed from the centrifuge tube with absorbent paper. The precipitate was dissolved in 1.0ml of 0.1M-acetate buffer, pH 5.7, and entrapped acid was neutralized with 1.0ml of 2.5M-sodium acetate. The protein solution was subjected to two more cycles of acid-$(NH_4)_2SO_4$

precipitation as described above. The protein was finally precipitated by the addition of saturated $(NH_4)_2SO_4$, pH 7.0, to give 80% saturation (4ml/ml) and the white precipitate was dissolved in 0.1M-Tris–acetate buffer, pH 7.8, to give a protein concentration in the range 3–10mg/ml. Dust and any denatured protein were removed by centrifugation at 40000*g*.

It was found that when the neutral-$(NH_4)_2SO_4$-precipitation step was omitted, the apoprotein was not soluble in neutral or alkaline buffers.

Enzyme concentration

The concentration of enzyme-bound FMN in native lactate oxidase was calculated from the absorbance of enzyme solutions at 450nm [ε at 450nm in 0.1M-sodium phosphate, pH 7.0, is 12.2×10^3 litre·mol^{-1}·cm^{-1} (Sullivan, 1968)]. When appropriate, the molar concentration of apoenzyme solutions were calculated in terms of FMN-binding sites, since a minimum molecular weight of 44000g of protein per mol of FMN has been determined for the native enzyme by several methods (P. A. Sullivan, M. G. Shepherd and Y. S. Choong, unpublished work). The titration of apoenzyme with pure FMN (see the Results section) shows that the apoenzyme retained full binding capacity with respect to original active sites.

Results

Preparation of the apoenzyme and reconstitution of lactate oxidase activity

Preparations of the apoenzyme had no residual activity and more than 90% of the original protein was recovered in the apoenzyme preparation. Fig. 1 shows the time-course for the recovery of enzyme activity when the apoenzyme was incubated with excess of FMN. The active enzyme complex obtained after incubation for 1h is referred to as holoenzyme I. The experiment shown in Fig. 1 has been repeated several times; 50% of the final enzyme activity was obtained 1–2min after mixing, but incubation for a further hour was required to obtain full activity. This suggests that the time-course for the recovery of the enzyme activity is biphasic. It should be noted that the binding of FMN to the apoenzyme, as measured by fluorescence quenching and described below, is completed within 1min. The specific activity of holoenzyme I varied with different batches from 450 to 900units/mg of protein.

Absorption spectra of the apoenzyme and holoenzyme I

The apoenzyme had a single absorption band with a maximum at 281nm. The spectrum of holoenzyme I was not identical with that of the native lactate oxidase; the absorption maximum was at 455nm instead of 458nm and the $E^{1\%}_{1cm}$ values in 0.1M-Tris–acetate buffer, pH 7.8, at 280nm and 450nm were 12.67 and 1.38 respectively. The corresponding values obtained for the native lactate oxidase in the same buffer were 18.52 and 1.73.

When stoicheiometric quantities of the apoenzyme and FMN were mixed in a split-compartment cuvette the absorption maxima shifted immediately after mixing from 374 and 445nm to 380 and 455nm (Fig. 2). This was followed by the appearance of shoulders

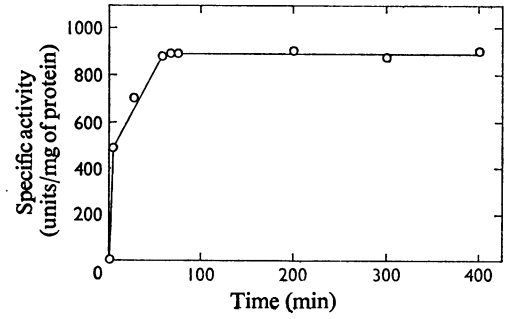


Fig. 1. *Recovery of lactate oxidase activity*

Apoenzyme (0.20mg) and commercial FMN (0.167mmol) were incubated in 0.1M-Tris–acetate, pH 7.8, at 25°C in the dark (total volume, 0.3ml). At the times indicated samples were assayed for enzyme activity.

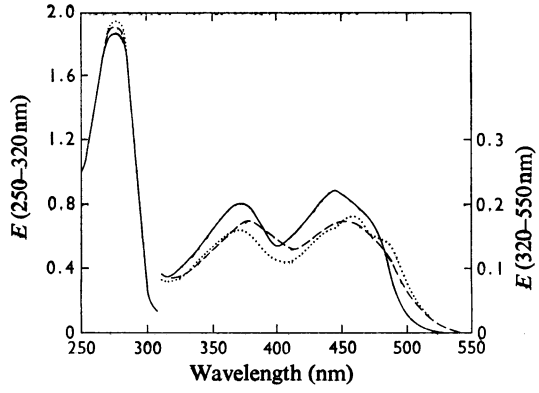


Fig. 2. *Changes in the spectrum during the initial stages of enzyme reconstitution*

Apoenzyme (2.9mg) in 1.0ml of 0.1M-Tris–acetate buffer, pH 7.8, and 36nmol of pure FMN (prepared by heat denaturation of native lactate oxidase), also in 1.0ml of the above buffer, were placed in the separate compartments of a split-compartment cuvette (path-length 1cm) (temperature 4°C). Absorption spectra were recorded before mixing (——), 3min after mixing (– – – –) and 2h after mixing (· · · ·). The specific activity of holoenzyme I at the completion of the experiment was 680units/mg of protein.

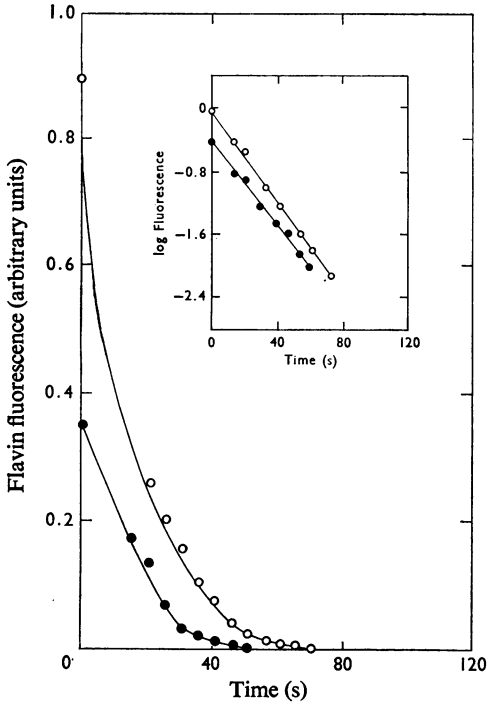


Fig. 3. *Fluorescence quenching on binding of FMN to apoenzyme*

Apoprotein (14.6μM with respect to flavin-binding sites) was mixed with pure FMN (○) or commercial FMN (●) to give final concentrations of 2.11μM and 0.432μM respectively (total volume, 1.3ml, in 0.1M-Tris–acetate, pH7.8, at 7°C). Fluorescence, in arbitrary units, was corrected for residual fluorescence. The inset shows a first-order analysis of the experimental curves.

on the 455nm band around 420 and 480nm. The time-course for these changes in the absorption spectrum correlated with the time-course for the overall increase in specific activity observed in the formation of holoenzyme I (Fig. 1).

Kinetics of quenching of FMN fluorescence

Native lactate oxidase exhibits very little flavin fluorescence. When excess of apoenzyme was added to FMN the fluorescence quenching was completed in about 1 min (Fig. 3). The reaction followed pseudo-first-order kinetics with rate constants of $6.1\times10^{-2}s^{-1}$ (commercial FMN) and $6.3\times10^{-2}s^{-1}$ (FMN from heat-denaturation of native enzyme).

Effect of FMN and apoenzyme concentration on the initial rate of flavin binding

If the binding of FMN to the apoprotein occurs by the simple model:

Table 1. *Stability of the apoenzyme*

Samples of the apoenzyme (770μg) in Tris–acetate buffer, pH7.8, were incubated at 4°C for 1h in the presence of the compounds listed below. FMN (0.48μmol) in the same buffer was then added and the solutions were incubated at 25°C for a further 2h (total volume, 0.16ml).

Additions	Concentration (mM)	Specific activity (units/mg of protein)
None	—	302
L-Lactate	10	184
EDTA	10	200
Dithioerythritol	10	498
Dithioerythritol*	10	475

* Dithioerythritol was added to this sample after the 1h incubation but before the addition of FMN.

$$A+F \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} H$$

where A is the apoenzyme, F the flavin and H the reconstituted enzyme (holoenzyme I) then rate of quenching $=-d(F)/dt=k_{+1}[A][F]-k_{-1}[H]$. Initially, $[H]=0$ and the change in [A] or [F] is negligible. If the reaction follows true second-order kinetics, the plots of the initial rate of binding versus [A] or [F] will be linear and pass through the origin, and therefore the value of k_{+1} can be calculated from the slope of this plot if the initial concentration of FMN or apoprotein is known (Hinkson, 1968).

The initial rates of binding for varying concentrations of FMN were consistent with this model and yielded a second-order rate constant of $1.01\times10^{4}M^{-1}\cdot s^{-1}$. A similar analysis for varying apoenzyme concentrations did not yield a straight line. The data, however, fitted a normal first-order plot, and the rate constant calculated from the limiting slope was $7.8\times10^{-3}s^{-1}$.

Stability of the apoenzyme

When the apoenzyme was stored at 4°C as a pellet recovered from the neutral-$(NH_4)_2SO_4$-precipitation step it retained the capacity for reconstitution with FMN for several days. Solutions of apoenzyme, however, lost this capacity within a few hours when stored at 4°C or when frozen and thawed. Table 1 shows the recovery of lactate oxidase activity after incubation of the apoenzyme with L-lactate, EDTA and dithioerythritol. There was a greater recovery of activity when dithioerythritol was added before and after the incubation.

Effect of pH on the recovery of lactate oxidase activity

Fig. 4 shows that the optimum pH for reconstituting lactate oxidase was 7.8 in Tris–acetate buffers. When

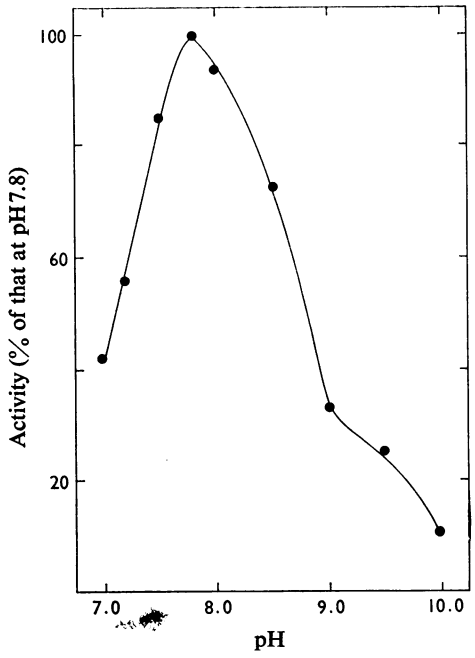


Fig. 4. *Effect of pH on the recovery of lactate oxidase activity*

Apoenzyme (199μg), 7nmol of FMN and 100μmol of buffer [Tris–acetate in the range pH7.2–10.0 and piperazine-*NN'*-bis-(2-ethanesulphonic acid) at pH7.0] were incubated at 20°C (total volume, 1.06ml). After 2h samples were assayed for lactate oxidase activity. The specific activity at pH7.8 was 638.

reconstitution was carried out in 0.1M-sodium phosphate, pH7.0, the specific activity after 3h incubation was only 23% of that obtained in Tris–acetate, pH7.8. After 48h, however, the specific activity increased to 86%.

Enzyme activity with FAD, FMN and riboflavin

Table 2 shows that enzyme activity obtained when the apoenzyme was incubated with riboflavin was 24% of that observed with FMN. Negligible activity was observed with FAD. Takemori *et al.* (1968) reported similar results with the apoenzyme from *M. phlei*.

Titration of the apoenzyme with FMN

The low specific activity of holoenzyme I (400–900) as compared with the native enzyme (1100–1350) could have resulted from a decreased binding capacity in the apoenzyme or a decrease in the rate of catalysis. The FMN-binding capacity of the apoenzyme was determined with respect to original FMN-

Table 2. *Effect of flavins on the reconstitution of lactate oxidase*

The reconstitution was carried out by incubating the apoenzyme with the flavins for 60min at 0°C in the dark. Each incubation contained 90μmol of Tris–acetate buffer, pH7.8, 160μg of apoprotein, 50μmol of flavin, and where indicated 10μmol of L-cysteine (total volume, 1.0ml).

	Enzyme activity (units)
FMN	846
FMN+cysteine	605
FAD	<50
FAD+cysteine	<50
Riboflavin	220
Riboflavin+cysteine	200

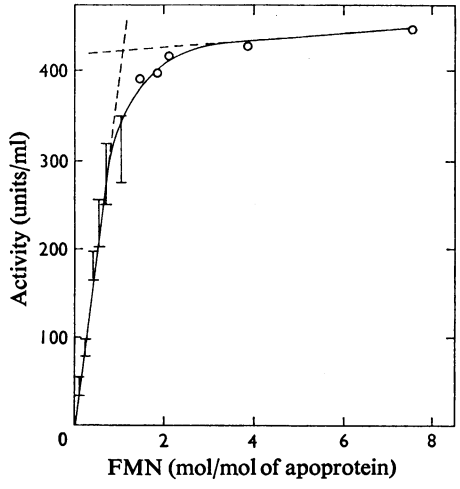


Fig. 5. *Titration of apoenzyme with FMN*

Apoenzyme (487μg) and various concentrations of FMN were incubated for 2h in the dark (total volume, 0.53ml, in 0.1M-Tris–acetate, pH7.8, at 4°C). Samples of each incubation mixture were assayed for lactate oxidase activity. The open circles represent results from single assays and the bars indicate the experimental variation from several assays.

binding sites in the native enzyme. Fig. 5 shows that when the apoenzyme was titrated with FMN 1mol of FMN was bound/44000g of protein. It was concluded that all the original active sites retained full binding capacity, since the end point of the titration agrees with the minimum molecular weight.

Dissociation constants of holoenzyme I

The dissociation constant ($K_{diss.}$) of the FMN–apoprotein complex (holoenzyme I) determined from

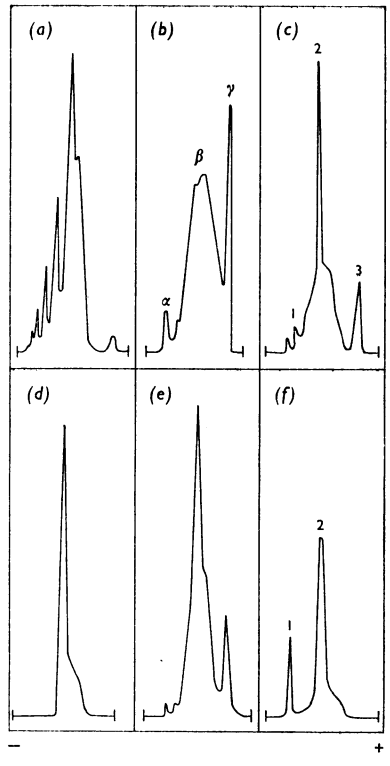


Fig. 6. *Densitometric tracings of polyacrylamide gels from various stages of the preparation*

Conditions were as described in the Materials and Methods section. Total protein loads are given in parentheses. The direction of electrophoresis was from left to right. (*a*) Apoenzyme (100μg). (*b*) Holoenzyme I (100μg). (*c*) Holoenzyme II (100μg). (*d*) Native enzyme (100μg). (*e*) Native enzyme (100μg) and holoenzyme II (100μg). (*f*) Holoenzyme II after Sephadex G-200 chromatography and concentration (see the text) (50μg).

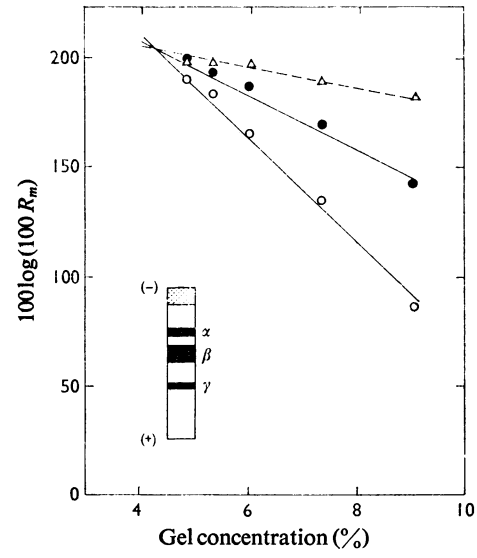


Fig. 7. *Effect of gel concentration on the relative mobilities of the protein bands in holoenzyme I*

Holoenzyme I (protein loads, 60μg) was analysed by the method of Hendrick & Smith (1968). The slopes of the lines of best fit were: α, −22.5; β, −11.08; γ, −2.96. The inset is a diagram of a polyacrylamide gel showing the three bands in holoenzyme I. ○, α band; ●, β band; △, γ band.

the titration experiment (Fig. 5) was 1.75×10^{-7}M at pH7.8.

The dissociation constant obtained from equilibrium-dialysis experiments was 1.70×10^{-6}M and from several fluorescence-quenching experiments was 5.3×10^{-7}M. Although some variation was encountered in these determinations it is apparent that the FMN is tightly bound in holoenzyme I.

Polyacrylamide disc-gel electrophoresis of the apoenzyme and holoenzyme I

Analysis of the apoenzyme by polyacrylamide disc-gel electrophoresis revealed several bands. The R_m values for these bands were 0.83, 0.51, 0.35, 0.24, 0.17 and 0.12. The densitometric tracings show that the intensity of the bands decreases in a geometric progression (Fig. 6*a*) as the rate of migration decreases.

Gels of holoenzyme I contained three distinct protein bands (designated α, β and γ). Two of these, (α and β; Fig. 6*b*) reacted positively to the enzyme-activity stain and had R_m values of 0.22 and 0.46 respectively. The β band accounted for 80% of the total protein. The R_m of the inactive γ band was 0.8.

The technique of Hendrick & Smith (1968) was used to determine the size and charge relationship between the species present in holoenzyme I. Since the plots of log R_m versus percentage gel concentration for the three protein bands, α, β and γ, gave three non-parallel straight lines intercepting at a gel concentration of 4.1% (Fig. 7), it was concluded that the three protein species differ in both molecular weight and charge. A linear standard curve of slope versus molecular weight was obtained with the following standards: ovalbumin, bovine serum albumin, fumarase, catalase and apoferritin. The molecular weights used were from tables compiled by Smith (1968). The molecular weights obtained from this standard curve were 360000 (α), 175000 (β) and approx. 40000 (γ). The molecular weight of

Table 3. *Preparation of holoenzyme II*

Apoenzyme and FMN (0.6μmol) in 2.0ml of 0.1M-Tris–acetate buffer, pH7.8, were incubated at 20°C for 3h in the dark to give holoenzyme I. This preparation was dialysed for 1 week against 0.1M-sodium phosphate buffer, pH7.0, at 4°C. The buffer was changed several times during the dialysis. Holoenzyme II was then fractionated by Sephadex G-200 and all the active fractions were pooled and concentrated as described in the text.

	Total protein (mg)	Total activity (enzyme units)	Specific activity (units/mg of protein)
Original enzyme	11.60	14300	1230
Holoenzyme I	11.40	7440	651
Holoenzyme II	11.00	10300	935
Holoenzyme II after Sephadex G-200 chromatography and concentration	8.19	8600	1050

native lactate oxidase determined by the same technique was 360000. Attempted fractionation of holoenzyme I by chromatography on Sephadex G-200 was unsuccessful.

Sedimentation-velocity study of holoenzyme I

A single symmetrical peak was observed when holoenzyme I was analysed in the ultracentrifuge (rotor speed 50740rev./min; temperature 20°C). The $s_{20,w}$ of holoenzyme I (2.0mg/ml) was 13.2S in 0.1M-Tris–acetate buffer, pH7.8. No attempts were made to study the apoenzyme in the ultracentrifuge because of the instability of this material.

Preparation of holoenzyme II

The specific activity of holoenzyme I did not alter significantly when solutions of the enzyme in 0.1M-Tris–acetate buffer, pH7.8, were stored at 4°C for 1 week. When, however, solutions of holoenzyme I were dialysed against 0.1M-sodium phosphate buffer, pH7.0, for 1 week at 4°C, the specific activity of the enzyme increased to a value close to that of native lactate oxidase. This preparation is referred to as holoenzyme II. The conditions for this change have not been investigated further.

Table 3 summarizes a typical preparation of holoenzyme II. A gel-electrophoretic analysis of holoenzyme II is shown in Fig. 6(*c*). Protein bands 1 and 2 had a positive activity strain. Band 3 was inactive in the enzyme stain, and the R_m value of this band (0.80) suggested that this was the γ species detected earlier in preparations of holoenzyme I. A sample of holoenzyme II (8.2mg) was applied to and eluted from a 15mm×56mm Sephadex G-200 column in 0.1M-sodium phosphate buffer, pH7.0. Only one peak was detected, in which the absorbance profiles at 280 and 450nm and the enzyme activity profile were coincident. The active fractions were pooled and concentrated by pressure dialysis in 0.1M-sodium phosphate buffer, pH7.0. A gel-electrophoretic analysis is shown in Fig. 6(*f*). It was noted that this preparation now contained an increased proportion of band 1. The inactive band 3 was removed by the Sephadex G-200 chromatography.

Properties of holoenzyme II

When analysed by the technique of Hendrick & Smith (1968), the molecular weights of bands 1 and 2 were 520000 and 360000. The plot of $\log R_m$ versus gel concentration gave two non-parallel straight lines intercepting at 3.8% gel concentration. It should be noted that the molecular weights of band 2 and band α in holoenzyme I were both 360000. The R_m values in the 7% gels, however, were 0.46 for band 2 and 0.22 for band α. Native enzyme also migrated with an R_m value of 0.46 in the 7% gels (Figs. 6*d* and 6*e*).

Only one symmetrical schlieren peak was observed in the sedimentation-velocity analysis. The $s_{20,w}$ of 12.33S for a 4.70mg/ml solution was slightly lower than the expected 13.6 S for the native enzyme (Sullivan, 1968).

Apparent K_m and $V_{max.}$ values of holoenzyme II obtained with L-lactate and air-saturated solutions in 0.1M-sodium citrate–citric acid buffer at 25°C were 4.4×10^{-3}M and 1690μmol/min per μmol of flavin. The results obtained with native lactate oxidase under these conditions were 4.2×10^{-3}M and 2090μmol/min per μmol of flavin. Holoenzyme II had an absorption spectrum identical with that of native enzyme (Sullivan, 1968).

The extinction coefficient at 450nm of holoenzyme II, determined as described previously (Sullivan, 1968), was 12.2×10^3litre·mol^{-1}·cm^{-1}, which is the same as that obtained for the native enzyme. The average minimum molecular weight of holoenzyme II from five analyses was 44000±2000g of protein per mol of FMN.

Discussion

A simple method has been devised for the preparation of the apoenzyme of lactate oxidase: it is based on the acid-$(NH_4)_2SO_4$ treatment used by Warburg & Christian (1938) to resolve D-amino acid oxidase. The apoenzyme was found to be quite stable when stored as a pellet at 0°C, but in solution it rapidly loses its capacity for reconstitution.

Three stages have been observed in the overall reconstitution of lactate oxidase, which is summarized in Scheme 1.

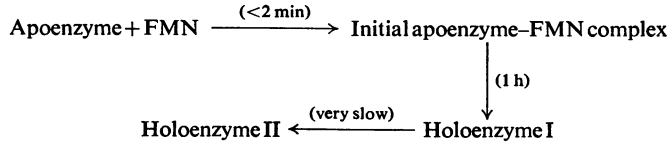
Fluorescence-quenching studies showed that the apoenzyme and FMN reacted rapidly (Fig. 3). Analyses revealed second-order kinetics with respect to FMN but first-order kinetics with respect to apoenzyme. The apoenzyme is in a large number of states of aggregation and the kinetics of fluorescence quenching could become first order with respect to apoenzyme if the rate-limiting dissociation of a polymeric species preceded flavin binding. Swoboda (1969) reported a similar kinetic analysis for the binding of FAD to the apoprotein of glucose oxidase and suggested that first-order kinetics with respect to apoenzyme could be explained if apoenzyme and flavin combined rapidly forming a fluorescent product which was subsequently converted into a non-fluorescent product in a second step. This interpretation could also be applied to lactate oxidase.

The initial FMN–apoenzyme complex possessed enzyme activity, and this increased at a slower rate for a further 60min (Fig. 1). The product formed (holoenzyme I) was quite stable under the conditions used for preparation. Holoenzyme I is clearly different from the native enzyme as judged by specific activity, adsorption spectrum, extinction coefficients and gel electrophoresis. The lower specific activity of this preparation, 40–70% of that of the native enzyme, was not the result of a loss in capacity for reconstitution. The end point obtained from a titration of FMN with the apoenzyme was 1 mol of FMN/44000g of protein. This value agrees with the minimum molecular weight of the native enzyme.

Holoenzyme I underwent modification when it was dialysed against 0.1M-sodium phosphate buffer, pH7.0. The preparation obtained, holoenzyme II, had a specific activity, flavin content, absorption spectrum, apparent $V_{max.}$ and apparent K_m similar to those of the native enzyme.

Gel electrophoresis provided some information on the composition of the apoenzyme, holoenzyme I, holoenzyme II and the native enzyme. When the native enzyme was treated with acid $(NH_4)_2SO_4$ the flavin and protein readily dissociated. The apoenzyme preparation gave a large number of protein bands in the gel-electrophoresis system. Although the apoenzyme was not analysed in detail the relative mobilities of the bands and the ordered distribution of protein suggested that the bands represent different states of aggregation of the monomer unit (i.e. monomer, dimer, tetramer etc.). In contrast with the apoenzyme, holoenzyme I consisted of only three distinct bands, α, β and γ, with molecular weights of 360000, 175000 and 40000. It seems likely that the protein bands of the apoenzyme are in equilibrium and that flavin binding stabilizes the tetramer and octamer. In the ultracentrifuge, however, holoenzyme I revealed only one species, which had a sedimentation coefficient similar to that of native lactate oxidase. This result and the results obtained by gel electrophoresis are difficult to reconcile. Native enzyme has a molecular weight of 348000, whereas the major band in the gels of holoenzyme I (the β band) had a molecular weight of 175000. It is possible that the α and β species represent an equilibrium system of the octamer and tetramer and that conditions used in the ultracentrifuge favoured the octamer, whereas the gel-electrophoresis conditions favoured dissociation. Both the α and β bands possessed enzyme activity, but the γ band was inactive. The molecular weight of this band (40000) suggests that it is an inactive monomer.

Band 2 of holoenzyme II and the native enzyme had identical R_m values in polyacrylamide gels (Figs. 6c and 6e). An ultracentrifugal analysis and gel electrophoresis indicated that the molecular weight of holoenzyme II was the same as that of the native enzyme. These results indicated that the conditions used to produce holoenzyme II enhanced the formation of an octamer (band 2). Band 2 is different from the octamer in holoenzyme I (band α), as the



Scheme 1.

R_m values in 7% gels were 0.46 and 0.22 respectively.

Holoenzyme II differs from the native enzyme in one property: it contains an active species of molecular weight 520000 which increases in proportion when the protein concentration is increased by pressure dialysis. The studies described in this paper have outlined the steps involved in the reconstitution of lactate oxidase. Although it is apparent that the polymeric state of the enzyme alters significantly during the preparation of the apoenzyme and during reconstitution, more detailed studies would be required to characterize this system.

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